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[1]Passed away on October 20, 2007
[2]Passed away on June 14, 2008

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Iatrogenic bile duct injuries: Etiology, diagnosis and management

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Abstract

Iatrogenic bile duct injuries (IBDI) remain an important problem in gastrointestinal surgery. They are most frequently caused by laparoscopic cholecystectomy which is one of the commonest surgical procedures in the world. The early and proper diagnosis of IBDI is very important for surgeons and gastroenterologists, because unrecognized IBDI lead to serious complications such as biliary cirrhosis, hepatic failure and death. Laboratory and radiological investigations play an important role in the diagnosis of biliary injuries. There are many classifications of IBDI. The most popular and simple classification of IBDI is the Bismuth scale. Endoscopic techniques are recommended for initial treatment of IBDI. When endoscopic treatment is not effective, surgical management is considered. Different surgical reconstructions are performed in patients with IBDI. According to the literature, Roux-en-Y hepaticojejunostomy is the most frequent surgical reconstruction and recommended by most authors. In the opinion of some authors, a more physiological and equally effective type of reconstruction is end-to-end ductal anastomosis. Long term results are the most important in the assessment of the effectiveness of IBDI treatment. There are a few classifications for the long term results in patients treated for IBDI; the Terblanche scale, based on clinical biliary symptoms, is regarded as the most useful classification. Proper diagnosis and treatment of IBDI may avoid many serious complications and improve quality of life.

INTRODUCTION

Iatrogenic bile duct injuries (IBDI) remain an important problem in gastrointestinal surgery. They are most frequently caused by laparoscopic cholecystectomy, which is one of the commonest surgical procedures in the world[1]. The early and accurate diagnosis of IBDI is very important for surgeons and gastroenterologists, because unrecognized IBDI lead to serious complications such as biliary cirrhosis, hepatic failure and death[2,3]. The choice of the appropriate treatment for IBDI is very important, because it may avoid these serious complications and improve quality of life in patients. Therefore, the question regarding the type of treatment for patients with IBDI is still a matter of debate. Initially, endoscopic treatment is recommended in patients with IBDI. When endoscopic techniques are not effective, different surgical reconstructions are performed[4,5]. The goal of surgical treatment is reconstruction to allow good bile flow to the alimentary tract. In order to achieve this goal, many techniques are used. There are some contradictory opinions on different surgical reconstructions in the literature.

HISTORICAL PERSPECTIVES OF RECONSTRUCTIVE BILIARY SURGERY

Anatomic knowledge of the liver and bile ducts can be traced to Babylon in 2000 BC[6]. Gallstone disease has been found in one mummy from Amen of the 21st Dynasty. Historic notes from Mesopotamia, Greece, Egypt and Roma also show an occurrence of bile duct

disease in ancient history[6]. The first surgical procedures performed on bile ducts were not complicated. In 1618, Fabricus removed gallstones. In 1867, Bobbs performed cholecystostomy. Cholecystostomies were also performed by Sims (1878), Kocher (1878) and Tait (1879)[6-8]. The first planned cholecystectomy in the world was performed by Langenbuch in 1882[6-9]. The first choledochotomy was performed by Couvoissier in 1890. Widespread use of surgical procedures on bile ducts was associated with occurrence of IBDI. The first iatrogenic bile duct injury was described by Sprengel in 1891. He also reported the first choledochoduodenostomy (ChD) for calculi (1891)[7,10]. In 1892, Doyenn reported the first choledochocholedochostomy for the same condition[7]. Cholecystoenterostomy to the colon was the first biliary-alimentary anastomosis and it was performed by Winiwater in 1881[7]. The first surgical reconstruction ("end-to-side" ChD) of IBDI was performed by Mayo in 1905[7]. The first Roux-en-Y hepaticojejunostomy (HJ) was described by Monprofit in 1908. Dahl noted Roux-en-Y HJ for surgical treatment of IBDI in 1909[7]. In 1969, Smith created a mucosal graft anastomosis in the repair of the damaged proximal bile duct[7]. In 1954, Hepp and Couinaud described the hilar plate and long extrahepatic course of the left hepatic duct. The left hepatic duct, after dissection of the hilar plate, was used in the repair of high strictures[7]. In 1948, Longmire and Sanford described a technique of finding of a branch of the left hepatic duct for anastomosis in the high biliary strictures. This technique was based on partial resection of the left hepatic lobe. In 1957, this technique was modified by Soulpaut and Couinaud. They described finding much larger ductal structures in the left lobe by following the round ligament to the origin of the 3rd segment duct[7]. In 1994, Hepp and Blumgart described a technique of hilar and intrahepatic biliary-enteric anastomosis[11].

ETIOLOGY AND PATHOGENESIS OF IBDI

Etiology of IBDI

IBDI present about 95% of all benign biliary strictures[12,13]. Benign biliary strictures encompass a wide spectrum involving not only IBDI, but also biliary disorders caused by other pathogenetic factors. The main causes of benign biliary strictures are presented in Table 1.

There are two main groups of surgical procedures leading to IBDI. The first group involves surgical procedures performed on the biliary tract such as open and laparoscopic cholecystectomy, choledochotomy and previous operations on bile ducts. The second group involves operations performed on other organs of the epigastrium such as gastric resection (most frequently the Billroth II partial gastric resection), hepatic resection and liver transplantation, pancreatic resections, biliary-enteric anastomoses, portacaval shunts, lymphadenectomy and other procedures within the hepato-duodenal ligament[12,13]. IBDI occur most frequently during cholecystectomy. Recently, the number of patients with IBDI has increased two-fold, which has been associated with widespread use of laparoscopic cholecystectomy[1]. The incidence of IBDI following open and laparoscopic cholecystectomy according to different authors in the literature is presented in Table 2.

Table 1 Main causes of benign biliary strictures

Congenital strictures	Biliary atresia and congenital cysts
Bile duct injuries	Iatrogenic: postoperative, following endoscopic and percutaneous procedures
	Following blunt or penetrating trauma of the abdomen
Inflammatory strictures	Cholelithiasis and choledocholithiasis
	Mirizzi's syndrome
	Chronic pancreatitis
	Chronic ulcer or diverticulum of the duodenum
	Abscess or inflammation of the liver or subhepatic region
	Parasitic, viral infection
	Toxic drugs
	Recurrent pyogenic cholangitis
	Primary sclerosing cholangitis
	Radiation-induced strictures
	Papillary stenosis

Table 2 Incidence of IBDI following cholecystectomy (%)

Author	IBDI incidence following OC	IBDI incidence following LC
McMahon *et al*[14], 1995	0.2	0.81
Strasberg *et al*[15], 1995	0.7	0.5
Shea *et al*[16], 1996	0.19-0.29	0.36-0.47
Targarona *et al*[17], 1998	0.6	0.95
Lillemoe *et al*[18], 2000	0.3	0.4-0.6
Gazzaniga *et al*[19], 2001	0.0-0.5	0.07-0.95
Savar *et al*[20], 2004	0.18	0.21
Moore *et al*[21], 2004	0.2	0.4
Misra *et al*[22], 2004	0.1-0.3	0.4-0.6
Gentileschi *et al*[23], 2004	0.0-0.7	0.1-1.1
Kaman *et al*[24], 2006	0.3	0.6

IBDI: Iatrogenic bile duct injuries; OC: Open cholecystectomy; LC: Laparoscopic cholecystectomy.

Pathogenesis of IBDI

There are several factors associated with an increased risk of IBDI. Coexisting acute or chronic inflammation around the gallbladder and hepato-duodenal ligament can increase the difficulty of the surgical procedure and increase the risk of IBDI. Other factors such as patient obesity, fat within the hepato-duodenal ligament, poor exposure and bleeding in the surgical area also increase the risk of IBDI. Poor prognostic factors are also male gender and long duration of symptoms before cholecystectomy.

Anatomical anomalies of the bile ducts and hepatic arteries significantly increase the risk of IBDI. The most frequent cause of IBDI is misidentification of the bile duct as the cystic duct in cases of anomalies of cystic duct insertion into the common hepatic duct. About 70%-80% of all IBDI are a consequence of

misidentification of biliary anatomy before clipping, ligating and dividing structures[12,13,25,26]. Excessive dissection along the common bile duct margins during open cholecystectomy can lead to biliary stricture because of damage to the three o'clock and nine o'clock axial arteries and their branches to the pericholedochal plexus. According to the literature, distal IBDI are accompanied by damage of axial arteries (10%-15%) and proximal IBDI are usually associated with damage to the hepatic artery and its branches (40%-60%)[26-30].

Clinical presentation of IBDI

The common clinical symptoms are jaundice, fever, chills, and epigastric pain. The clinical presentation depends on the type of injury and is divided into two groups. In patients with bile leaks, bile is present in the closed-suction drain located in the subhepatic region. If the subhepatic region is not drained, subhepatic bile collection (biloma) or abscess develops. In these patients fever, abdominal pain and other signs of sepsis occur. Generally, jaundice is not observed in these patients because cholestasis does not appear. In the second group of patients with biliary strictures, jaundice caused by cholestasis is the commonest clinical symptom[12,13].

Diagnostics of IBDI

Laboratory and radiological investigations are used in diagnosis of IBDI. Among laboratory examinations, indicators of cholestasis and liver function play an important role: serum bilirubin, alkaline phosphatase, γ-glutamyl transpeptidase, alanine and aspartate aminotransferases. In patients with IBDI without complications, the liver is not damaged. Therefore, cholestasis indicators are increased but aminotransferases are not increased in these patients. Pathological levels of aminotransferases are present in cases of secondary biliary cirrhosis as a serious complication of unrecognized or improperly treated biliary injuries. In patients with secondary biliary cirrhosis, hypoalbuminemia and coagulation defects (prolonged prothrombin time) are observed. They are the most frequently used parameters of synthetic capacity of the liver. Imaging diagnostics in IBDI involve ultrasonography of the abdominal cavity, cholangiography, endoscopic retrograde cholangiopancreatography (ERCP), computed tomography, and magnetic resonance-cholangiography. Ultrasonography of the abdominal cavity allows imaging of intrahepatic and extrahepatic bile ducts with measurement of the diameter of the common bile duct or common hepatic duct. It also shows biloma or intraabdominal abscesses in patients with bile leaks. Computed tomography is useful for more specific investigation in doubtful cases in patients with bile leaks. Percutaneous transhepatic cholangiography is useful in assessment of the bile tract proximal to the location of the damage. ERCP is a very useful method of investigation in imaging of damaged bile ducts and it allows the repair of small bile duct injuries by insertion of a biliary prosthesis. Magnetic resonance cholangiography is a sensitive (85%-100%) and non-invasive imaging modality for the biliary tract. Currently, it is the "gold standard" in preoperative diagnosis of IBDI in patients qualifying for surgical reconstruction[12,13,31,32].

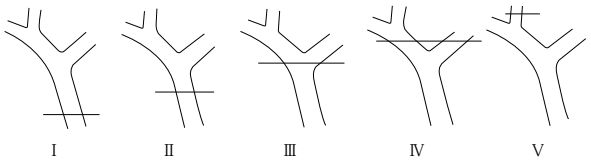

Figure 1 Bismuth classification of IBDI. Ⅰ: Common bile duct and low common hepatic duct (CHD) > 2 cm from hepatic duct confluence; Ⅱ: Proximal CHD < 2 cm from the confluence; Ⅲ: Hilar injury with no residual CHD-confluence intact; Ⅳ: Destruction of confluence: right and left hepatic ducts separated; Ⅴ: Involvement of aberrant right sectoral hepatic duct alone or with concomitant injury of CHD.

Almost 85% of IBDI are not recognized during the primary iatrogenic surgical procedure[33]. According to the literature, only 15%-30% of IBDI are recognized during the initial operation[34]. According to other data, 70% of IBDI are diagnosed within 6 mo and 80% within 12 mo after the initial operation[31].

Classification of IBDI

A number of classifications have been proposed by different authors. In our opinion, the Bismuth scale is the most useful and simple classification. It is based on the location of the injury in the biliary tract[35]. This classification is very helpful in prognosis after repair, but does not involve the wide spectrum of possible biliary injuries. The Bismuth classification is described in Figure 1. Another classification is the Strasberg scale which, in contrast to the Bismuth scale, allows differentiation between small (bile leakage from the cystic duct) and serious injuries performed during laparoscopic cholecystectomy, but it does not play an important role in the choice of surgical treatment[15,34,36]. The Mattox classification of IBDI takes into consideration the type of injuring factor (contusion, laceration, perforation, transsection, diversion or interruption of the bile duct or the gallbladder)[37]. There are several classifications in the literature for IBDI induced during laparoscopic cholecystectomy (Stewart *et al*[38], Schmidt *et al*[27], Bektas *et al*[39]).

MANAGEMENT OF IBDI

Endoscopic and radiological treatment of IBDI

Non-invasive, percutaneous radiological end endoscopic techniques are recommended as initial treatment of IBDI. When these techniques are not effective, surgical management is considered. According to the literature, the effectiveness of a radiological approach with transhepatic stenting of the damaged biliary tract is 40%-85%. The common complications of the radiological procedures are as follows: hemorrhage (hemobilia, bleeding from hepatic parenchyma or adjacent vessels), bile leakage and cholangitis. The other complications such as pneumothorax resulting from pleural violation, biliopleural fistula and perforation of adjacent abdominal

structures including the gallbladder and large bowel, are described less frequently. Percutaneous dilatation is less effective (52%) than surgical treatment (89%). According to the literature, the radiological approach is also associated with a higher number of complications (35%) than surgical management (25%). Most frequently, it is recommended in very difficult cases of very high, hilar biliary strictures or in the treatment of very small diameter bile ducts[12,13,31,40].

Endoscopic dilatation associated with insertion of biliary prosthesis during ERCP investigation is the most frequently used non-surgical method in the treatment of IBDI. According to the literature, the success of endoscopic (72%) and surgical (83%) management of IBDI is comparable. Frequency of complications in both treatment methods is also comparable (35% *vs* 26%). The common complications of endoscopic techniques regarding placement of biliary prostheses include cholangitis, pancreatitis, prosthesis occlusion, migration, dislodgement and perforation of the bile duct. Endoscopic treatment is recommended as initial treatment of benign biliary strictures, in patients with biliary fistula or when surgical treatment is not warranted[12,13,41].

Surgical treatment of IBDI

The goal of surgical treatment is to reconstruct the bile duct to allow proper bile flow to the alimentary tract. In order to achieve this goal, many techniques are used. There are contradictory reports on the effectiveness of bile duct reconstruction methods in the literature. The following operations have been reported for surgical treatment of IBDI: Roux-en-Y HJ, end-to-end ductal biliary anastomosis (EE), ChD, Lahey HJ, jejunal interposition hepaticoduodenostomy, Blumgart (Hepp) anastomosis, Heinecke-Mikulicz biliary plastic reconstruction and Smith mucosal graft[11,18,42-46].

Various surgical techniques including immediate surgical repair: In the case of recognition of IBDI during laparoscopic cholecystectomy, immediate cholangiography and conversion to an open procedure in order to define the extent of the injury are required. The injury should be repaired by an experienced hepatobiliary surgeon. If this is impossible, a patient should be transferred to a hepatobiliary surgery referral center after adequate drainage of the subhepatic region. Bile ducts of diameter less than 2-3 mm without communication with a main biliary tract, should be ligated in order to avoid postoperative bile leak leading to development of biloma and abscess in the subhepatic region. Bile ducts of diameter more than 3-4 mm should be repaired, not ligated, because they drain a wider hepatic area. Interruption of common hepatic duct or common bile duct continuity can be repaired by immediate tension-free EE with or without a T tube, using absorbable sutures. Security of the immediately repaired bile duct with a T tube is controversial. According to the literature, in liver transplantation, EE over a T tube is associated with a significantly higher stricture rate than choledochocholedochostomy without a T tube (25% *vs* 11%). If the bile duct loss is too long and immediate and EE is not possible without tension, Roux-en-Y HJ is recommended[5,12,13,25,31,47].

Surgical reconstructions: A number of reconstructions are used in surgical treatment of IBDI. There are a few conditions for proper healing of each biliary anastomosis. The anastomosed edges should be healthy, without inflammation, ischemia or fibrosis. The anastomosis should be tension-free and properly vascularized. It should be performed in a single layer with absorbable sutures[25,38].

Currently, Roux-en-Y HJ is the most frequently performed surgical reconstruction of IBDI. In this surgical technique, a proximal common hepatic duct is identified and prepared and the distal common bile duct is sutured. End-to-side or end-to-end HJ is performed in a single layer using interrupted absorbable polydioxanone (PDS 4-0 or 5-0) sutures[48,49]. Most authors prefer HJ because of the lower number of postoperative anastomosis strictures. According to Terblanche *et al*[45], HJ is effective in 90% of cases. However, after this reconstruction, bile flow into the alimentary tract is not physiological, because the duodenum and upper part of the jejunum are excluded from bile passage. Physiological conditions within the proximal gastrointestinal tract are changed as a result of duodenal exclusion from bile passage. An altered bile pathway is a cause of disturbances in the release of gastrointestinal hormones[48-50]. There is a hypothesis that in patients with HJ, the bile bypass induces gastric hypersecretion leading to a pH change secondary to altered bile synthesis and release of gastrin. A higher number of duodenal ulcers is observed in patients with HJ, which may be associated with a loss of the neutralizing effect of the bile, including bicarbonates and secondary gastric hypersecretion[51]. Laboratory investigations revealed increased gastrin and glucagon-like immunoreactivity plasma levels and decreased triglycerides, gastric inhibitory polypeptide and insulin plasma levels in patients with HJ[51]. An altered pathway of bile flow is also a cause of disturbance in fat metabolism in patients undergoing HJ[51,52]. Moreover, the total surface of absorption in these patients is also decreased as a result of exclusion of the duodenum and upper jejunum from the passage of food. This hypothesis was supported by a study performed in our center. We compared early and long term results of two surgical reconstructions of IBDI: Roux-en-Y HJ and EE. The study showed a significantly lower weight gain in patients undergoing HJ in comparison to patients following physiological EE[49]. The other disadvantage of HJ is a lack of ability to control endoscopic examination and endoscopic dilatation of the strictured biliary anastomosis. In order to resolve this problem, a longer jejunal loop (jejunostomy) is prepared and sutured to the abdominal subcutaneous tissue in the right subcostal region. Jejunostomy can be open or closed with the possibility of opening in a case of biliary

anastomosis stricture, which should be endoscopically dilated. Jejunostomy is associated with bile loss of about 40 mL/d[53].

EE is a physiological biliary reconstruction[49,54]. In this type of reconstruction, extensive mobilization of the duodenum with the pancreatic head through the Kocher maneuver, excision of the bile duct stricture, and refreshment of the proximal and distal stumps should be performed. Anastomosis is performed in a single layer with interrupted absorbable PDS 4-0 or 5-0 sutures[49]. This reconstruction is not recommended by most authors because of the higher number of anastomosis strictures in comparison with HJ. We recommend EE first, because in some patients, extensive mobilization of the duodenum with the pancreatic head by the Kocher maneuver allows tension-free anastomosis after the extensive bile duct length loss. Excision of the bile duct stricture, dissection and refreshing of the proximal and distal stumps as far as the tissues are healthy and without inflammation, and the use of non-traumatic, monofilament-interrupted 5-0 sutures allows the achievement of good long term results. Use of an internal Y tube conducting the right and left hepatic ducts into the duodenum through the EE and the papilla of Vater also allows the proper healing of this anastomosis. In our department, this reconstruction was performed when the bile duct loss was from 0.5 to 4 cm. It allowed the achievement of very good long term results with effectiveness comparable to HJ. Establishing a physiological bile pathway allows proper digestion and absorption, which causes a greater weight gain in patients following EE, as noted in our study[49]. Another essential advantage of EE is the possibility of control of the endoscopic examination in these patients. Fewer early complications are observed after EE than HJ, which is associated with opening of the alimentary tract and a higher number of anastomoses (biliary-enteric and entero-enteric)[49].

Other biliary reconstruction methods are used less frequently. ChD is actually a rarely performed operation recommended by some authors only in cases of injury within the distal portion of the common bile duct. It guarantees physiological bile flow into the duodenum and anastomosis endoscopic control, as well as being technically easier. It is recommended in some cases of distal strictures, when use of the jejunal loop, as a result of numerous adhesions, is impossible. It should be performed on the large common bile duct (> 15 mm diameter) because the postoperative strictures are more frequent within the narrow duct. ChD should be created between the duodenum and the distal common bile duct in order to decrease the risk of so-called sump syndrome noted in 0.14%-3.3% of cases in the literature. Following ChD, recurrent ascending cholangitis because of bile reflux is noted in 0%-4% of patients[11,31]. A higher rate of bile duct cancer in patients with ChD in comparison to HJ was noted by Tocchi *et al*[55] during a 30-year observation period (7.6% *vs* 1.9%).

Jejunal interposition hepaticoduodenostomy, using 25-35 cm of the jejunal loop, is performed in some surgical centers. This reconstruction includes three types of anastomosis (biliary-enteric, enteric-duodenal and entero-enteric). Biliary-enteric anastomosis is performed in a single layer with interrupted absorbable 5-0 sutures and enteric-duodenal anastomosis in a single layer with interrupted or continuous absorbable 4-0 sutures. The advantage of this reconstruction is physiological bile flow into the duodenum, which prevents duodenal ulcers caused by changes in the neurohormonal axis within the upper alimentary tract[10,56].

The repair of hilar IBDI requires special surgical techniques. In the past, the so-called "mucosal graft technique" described by Smith in the 1960s was performed[57,58]. This reconstruction involves creating a mucosal dome of jejunum (by removing a seromuscular patch) near the end of the Roux-en-Y loop through which a straight rubber tube is passed *via* hepatic ducts and through the liver parenchyma. This technique is based on the hypothesis that the jejunal mucosa grafts to the biliary epithelium, and a mucosa-to-mucosa anastomosis is created. Short-term results were good, but in the long term a high number of anastomosis strictures was observed. Therefore, currently, not Smith but the Blumgart-Hepp technique is used in reconstruction of hilar IBDI. In this technique, the dorsal surface of the left hepatic duct is placed parallel to the quadrate hepatic lobe; dissection and opening of the left hepatic duct longitudinally allows creation of a wide anastomosis of 1-3 cm in diameter[11,25,57-60].

Biliary drainage: There are several methods of biliary drainage securing the anastomosis: external T tube, external Y tube, Rodney Smith drainage and internal Y tube. External T drainage involves using a typical Kehr tube with insertion of its short branches into the bile duct and passage of its long branch through the abdominal wall to the outside. Y drainage involves insertion of short branches of the Kehr tube into both right and left hepatic ducts, splinting of the anastomosis and passage of its long branch through the jejunal loop and abdominal wall to the outside (external Y drainage) or into the duodenum by the papilla of Vater (internal Y drainage). An external T or Y tube is removed percutaneously and an internal Y tube is removed endoscopically. Most frequently, external T drainage is used in biliary-enteric anastomosis and internal Y drainage in EE. In Rodney Smith drainage, two straight rubber tubes splinting the biliary-enteric anastomosis are passed *via* the hepatic ducts, through the liver parenchyma and through the abdominal wall to the outside. This drainage type is used in high intrahilar biliary-enteric anastomosis. In the past, it was used in the Smith "mucosal graft technique"[54,58-60].

The use and duration of biliary drainage is still controversial. The advantage of biliary drainage is limitation of the inflammation and fibrosis occurring after the surgical procedure. In the opinion of some authors, the presence of the biliary tube prevents anastomosis stricture[61]. The disadvantage of biliary drainage is a higher risk of postoperative complications[62]. Mercado *et al*[63]

Table 3 Terblanche clinical classification for assessment of long-term results of surgical bile duct repair

Grade	Result	
I	Excellent	No biliary symptoms with normal liver function
II	Good	Transitory symptoms, currently no symptoms and normal liver function
III	Fair	Clearly related symptoms requiring medical therapy and/or deteriorating liver function
IV	Poor	Recurrent stricture requiring correction, or related death

recommend using transanastomotic stents when there is a thin bile duct less than 4 mm in diameter, and when there is inflammation within the ductal anastomosed edges which makes proper healing of the anastomosis questionable. The duration of drainage is also controversial. According to most authors, the optimal length of time for biliary drainage is about 3 mo. Investigations showed that longer durations of biliary drainage do not provide any advantage[18,64].

RESULTS OF SURGICAL TREATMENT OF IBDI

Short-term results and early complications

According to most authors, the early postoperative morbidity rate is 20%-30% and mortality rate 0%-2%[31,42,44]. The most frequent early complication is wound infection, which is described in 8%-17.7%[32,48,60,65]. Other complications reported in the literature are the following: bile collection, intra-abdominal abscess, biliary-enteric anastomosis dehiscence, biliary fistula, cholangitis, peritonitis, eventration, pneumonia, circulatory insufficiency, intra-abdominal bleeding, sepsis, infection of the urinary tract, pneumothorax, acute pancreatitis, thrombosis and embolic complications, diarrhea, ileus and multi-organ insufficiency[18,32,42,66].

Long term results

Assessment of long term results is the most important in surgical treatment of IBDI. Proof of successful surgical treatment is the absence of biliary anastomosis stricture. In referral centers, a successful outcome after surgical repair of IBDI is observed in 70%-90% of patients[3,5,45]. Two-thirds (65%) of recurrent biliary strictures develop within 2-3 years after the reconstruction, 80% within 5 years, and 90% within 7 years. Recurrent strictures 10 years after the surgical procedure are also described in the literature[3,31,67]. A satisfactory length of follow-up, which is necessary in order to assess the long term results of the repair procedure, is 2-5 years[18,31,64]. Some authors recommend 10 or 20 years of observation[43,45].

There are a number of classifications in order to assess the long term outcomes of bile duct surgical repairs. In our opinion, the Terblanche clinical grading (1990) is the most useful classification. It is based on clinical biliary symptomatology and is presented in Table 3[45]. Other less frequently used classifications by Nielubowicz *et al*[68] (1973), Lygidakis *et al*[69] (1986), Muñoz *et al*[70] (1990) and McDonald *et al*[66] (1995) are described in the literature.

CONCLUSION

Surgical procedures performed within the biliary tract are very common. The incidence of IBDI has increased recently, and has been associated with increased use of laparoscopic cholecystectomy worldwide. It is essential to be careful in the proper visualization of the surgical area and the identification of structures before ligation or transsection in order to decrease the risk of bile duct injuries during surgery. When biliary injury develops, early recognition and appropriate treatment are most important. Early and correct treatment allows avoidance of serious complications in patients with IBDI. Following bile duct repair, patients require long term and careful postoperative observation because of the possibility of biliary anastomosis stricture.

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TOPIC HIGHLIGHT

Kazuhiro Hanazaki, MD, Professor and Chairman, *Series Editor*

What is artificial endocrine pancreas? Mechanism and history

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Abstract

The artificial endocrine pancreas is a feedback control instrument that regulates insulin delivery on a minute-by-minute basis according to measured blood glucose levels. Only one type of bedside-type artificial endocrine pancreas is now available in Japan: STG-22 (Nikkiso Co. Ltd., Japan). In the insulin infusion algorithm, insulin is infused on the basis of its proportional and derivative actions, to blood glucose concentrations with a constant time delay. The bedside-type artificial endocrine pancreas has been proven to be useful not only as a therapeutic tool for diabetes mellitus, but also as an elegant research tool for investigating the pathophysiology of the disease, by using the euglycemic hyperinsulinemic glucose clamp technique. The wearable type of closed-loop system has been developed recently. The breakthrough is the establishment of a needle-type glucose sensor. The development of closed-loop glycemic control systems that enable long-term physiological regulation has focused on implantable devices. Much effort has been expended to realize these devices.

Key words: Diabetes mellitus; Artificial pancreas; Blood glucose; Insulin; Infusion

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INTRODUCTION

The strategy used in the therapy of diabetes mellitus can be divided into three periods. First, doctors attempted to prevent acute metabolic derangements and prolong the lifespan of patients through use of insulin (discovered in 1921 by Banting and Best) and through the introduction of oral hypoglycemic agents. Second, in association with prolongation of lifespan, an increase in chronic complications, especially microangiopathy, was observed. To counter these, the techniques of hemodialysis or kidney transplantation were established. The application of photocoagulation for preventing the blindness caused by diabetic retinopathy became popular. We are now in the third stage, in which we are attempting to prevent the onset of these chronic complications. Many retrospective studies and recently performed prospective studies have revealed that strict glycemic regulation is essential to prevent the onset of microangiopathy[1-3].

HISTORY OF ARTIFICIAL ENDOCRINE PANCREAS

As early as 1959, Professor E Perry McCullagh, an endocrinologist at The Cleveland Clinic, demonstrated the concept of an implantable artificial endocrine pancreas. The closed-loop regulatory system, which consisted of a glucose monitoring device, transmitter, and insulin syringe, was looked upon as the future treatment device for diabetes mellitus.

The development of an artificial endocrine pancreas to substitute for the diseased pancreatic β cell function

has been attempted widely. Albisser *et al*[4] in Toronto in 1974 and Shichiri *et al*[5] in Osaka in 1975 succeeded with the clinical use of an artificial endocrine pancreas that consisted of an autoanalyzer for blood glucose determination, a minicomputer system, and a pump driving system. Next, the size of the whole system was reduced, which created a bedside-type artificial endocrine pancreas; Biostator (Miles Laboratory Inc., Elkhart, IN, USA) was developed (no longer available)[6] and another type was developed by the Osaka University group[7]. These devices have been used clinically on a short-term basis, and have a good reputation as elegant research tools to study the pathophysiology of diabetes mellitus.

BEDSIDE-TYPE ARTIFICIAL ENDOCRINE PANCREAS

Principle of the system

The artificial endocrine pancreas is a device that is composed of a sensor, computer and set of pumps. These components are connected in such a way as to form a closed loop for the subjects. The system is shown in Figure 1. By means of an indwelling dual-lumen catheter, venous blood is drawn into an analyzer that is modified for continuous blood glucose measurement. The computer receives the electrical signals generated by the glucose analyzer and interprets these in accordance with its internal algorithms that are programmed with specific parameters. In turn, the computer instructs one pump to delivery insulin, the amount varying according to the level of the blood glucose and its rate of change. Similarly, glucose or glucagon may be administered by another pump in a counter-regulatory manner when hypoglycemia tends to occur.

Glucose sensor

In our first artificial endocrine pancreas system, continuous glucose measurement was conducted with a Technicon AutoAnalyzer Ⅱ using a modification of the glucose oxidase method[5-7]. However, to minimize the blood sampling volume and to make the whole system smaller, a glucose sensor for continuous glucose monitoring of the whole blood was developed by combining glucose oxidase membrane with an electrode that measures hydrogen peroxide, one of the reaction products, polarographically. A key component of a low-noise blood glucose sensor with long-term stability is its membrane, therefore, hydrophilic Cuprophan 100 pmol/L with a pore size of 3 nm, was applied to cover the immobilized glucose oxidase (Figure 2).

Intravenous insulin infusion algorithm

To develop an insulin infusion algorithm, mathematical models and a computer algorithm are required. By applying the mechanical control theory, Albisser *et al*[4] have proposed a set of relationships to translate information about blood glucose levels into rates of delivery of insulin

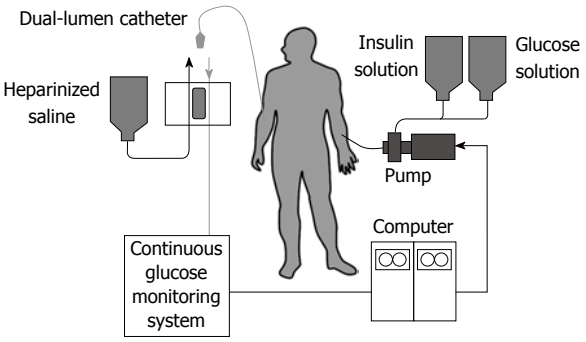

Figure 1 Schematic diagram of the bedside-type artificial endocrine pancreas.

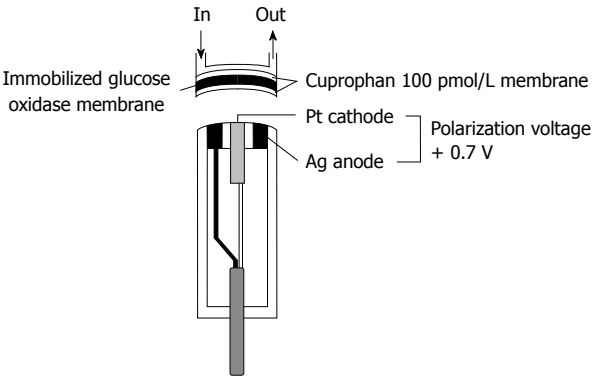

Figure 2 Structure of a glucose sensor of the bedside-type artificial endocrine pancreas. The immobilized glucose oxidase was covered with hydrophilic Cuprophan 100 pmol/L.

and glucagon (or glucose). In this algorithm, the rate of insulin infusion is regulated by the proportional (static) and derivative (dynamic) control mechanisms, which provides for single-phase insulin release, as well as a biphasic response to measured blood glucose concentrations. However, glucose infusion is necessary to prevent severe hypoglycemia caused by excessive insulin infusion in response to a hyperglycemic state.

It is well known that, in rat islets perfused with glucose, a biphasic response of insulin secretion is observed. With the aid of a control theory, we assume that insulin secretion responds not only to the glucose concentration itself, but also to the rate of change in glucose concentration. In other words, against the stepwise input of glucose concentration, an initial rapid insulin secretion is achieved by the derivative action and a second milder increase in secretion is achieved by the proportional action as an output. This relationship has been simulated successfully by using the transfer function with the first-order delay in both proportional and derivative action as shown in Figure 3[8]. By applying this principle, an insulin infusion algorithm has been developed.

The characteristics of our insulin infusion algorithm are as follows: (1) the amount of insulin infusion is small enough to keep or mimic the physiological plasma insulin concentration; and (2) glucagon infusion is

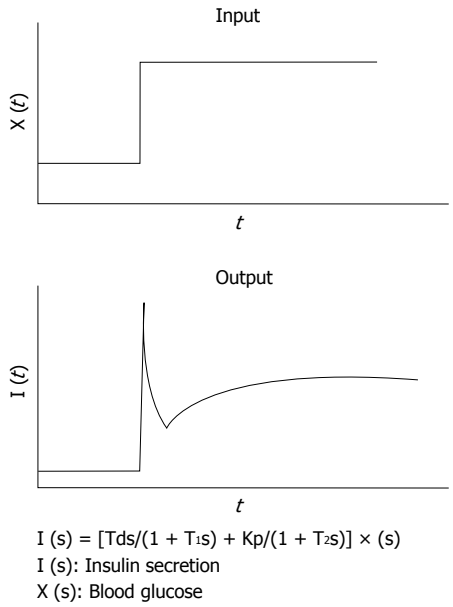

Figure 3 Mathematical model for insulin infusion algorithm. The stepwise input of glucose concentration [X (t)] and biphasic response of insulin secretion [I (t)] are depicted. The relationship between input and output is expressed in the transfer function. I (s), X (s) and dX (s) are the Laplace transformed form of I (t), X (t) and dX (t)/dt, respectively. T_1 and T_2 are the first order delay in response.

not necessarily needed because the negative derivative action of blood glucose concentration reduces the insulin infusion rate when blood glucose is falling. The details of the computer algorithm have been described previously[6,8-10].

Self-adaptive control algorithm for compensating insulin sensitivity changes

Even though the insulin secretory dynamics of healthy subjects are accomplished in patients with diabetes whose insulin sensitivity is low or super-normal, it is necessary to change manually the parameters for deciding insulin infusion rates for the adaptive control of blood glucose. Therefore, a computer algorithm for self-adaptive control has been established. Firstly, under the blood glucose regulation with artificial endocrine pancreas, the real rate of change in blood glucose concentration in each subject is calculated, and the difference between this and the projected rate of change in blood glucose concentration is assumed to be the index of insulin sensitivity. Secondly, according to the calculated insulin sensitivity, the computer automatically changes the parameters that regulate the insulin infusion rate[11].

Glucose infusion algorithm

A counter-regulatory system might be useful and safe for the prevention of hypoglycemia caused by increased endogenous insulin secretion, and the change in insulin sensitivity that is observed frequently during insulin treatment with the artificial endocrine pancreas in patients with diabetes. In the glucose infusion algorithm, glucose is infused on the basis of proportional and derivative actions of blood glucose concentration with a time delay constant between blood withdrawal and initiation of glucose infusion[10,12].

Clinical application of bedside-type artificial endocrine pancreas

Only one type of bedside-type artificial endocrine pancreas is now available in Japan: STG-22 (Nikkiso Co. Ltd., Japan; Figure 4). Using this system, perfect blood glucose regulation with physiological plasma insulin profiles can be obtained in patients with diabetes. At present, clinical applications of the bedside-type artificial endocrine pancreas on a short-term basis include: blood glucose control in diabetic coma or diabetic ketoacidosis[13], during surgery[14-16], delivery[17], during hemodialysis in diabetic nephropathy[18], the prediction of the insulin requirement[19], and blood glucose control in a hypoglycemic state such as in the case of insulinoma[20].

In addition, by using this system, the euglycemic hyperinsulinemic glucose clamp study for the determination of insulin sensitivity in patients with diabetes is now applied widely[21,22]. The euglycemic hyperinsulinemic clamp study was performed using an artificial pancreas according to the method of DeFronzo *et al*[23]. In brief, insulin is infused in a continuous fashion at a rate of 1.12-1.50 mU/kg per minute, after the priming insulin infusion during the first 10 min of the clamp at the same doses. Blood glucose levels were determined every 5 min during the 2-h clamp study, and euglycemia (5.0 mmol/L) was maintained by infusion of variable amounts of 10%-20% glucose solution. The total-body glucose disposal rate was evaluated as the mean of the glucose infusion rate during the last 30 min of the clamp. The insulin resistance index by the clamp was calculated by dividing the mean glucose infusion rate by the steady-state plasma insulin levels during the last 30 min of the clamp. The Artificial Organs Registry in Japan shows that the clinical applications of this bedside-type artificial endocrine pancreas have been increasing over time. The cumulative number of cases, including clinical and experimental applications, reached 14418 from 1983 to 2002. The number of applications was 465 in 2002 (29 for blood glucose control, 341 for laboratory and clinical research, and 95 for animal experiments) (Figure 5).

TREND IN THE DEVELOPMENT OF ARTIFICIAL ENDOCRINE PANCREAS

Wearable artificial endocrine pancreas

The ultimate goal of the development of the artificial endocrine pancreas is to achieve long-term strict glycemic regulation. In 1982, we succeeded in miniaturizing a glucose monitoring system to a needle-type, which consisted of a platinum anode and a silver cathode (0.4 mm in diameter and 2 cm in length). The electrode loaded with 0.6 V polarographic voltage measures hydrogen

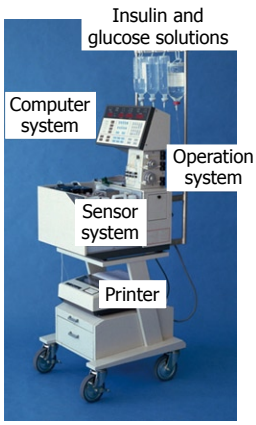

Figure 4 Bedside-type artificial endocrine pancreas (STG-22, Nikkiso Co. Ltd. Japan).

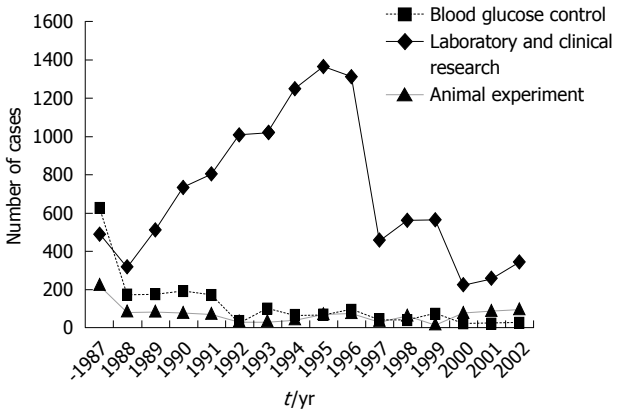

Figure 5 Number of cases to whom the bedside-type artificial endocrine pancreas had been applied in Japan. Artificial Organs Registry Report in Japan, 2002.

peroxide produced. It has been demonstrated that this sensor possesses excellent sensor characteristics suitable for application *in vivo*. We then developed a wearable type artificial endocrine pancreas, which consisted of a needle-type glucose sensor, microcomputer system, insulin and glucagon infusion pump systems, and a battery. The total system was packed into a small unit (12 cm × 15 cm × 6 cm in size and 400 g in weight)[24-26]. However, the major obstacle in extending the term of glycemic control is the lack of a stable and reliable glucose sensor.

To obtain stable and reliable measurement of subcutaneous tissue glucose concentrations for at least 4 d, we have developed two types of glucose sensors: a miniaturized extracorporeal glucose monitoring system based on a microdialysis sampling method[27]; and a ferrocene-mediated needle-type glucose sensor covered with biocompatible membrane[28]. We have also reported that subcutaneous tissue glucose concentration was monitored continuously using these systems for 4 d. Furthermore, we have developed a newly designed wearable type artificial endocrine pancreas (12.6 cm × 2.9 cm × 9.6 cm in size and 250 g in weight) (KAP-003; Nikkiso, Tokyo, Japan), which consists of a glucose sensing system, microcomputer system, syringe pump for insulin infusion, and a battery[29] (Figure 6).

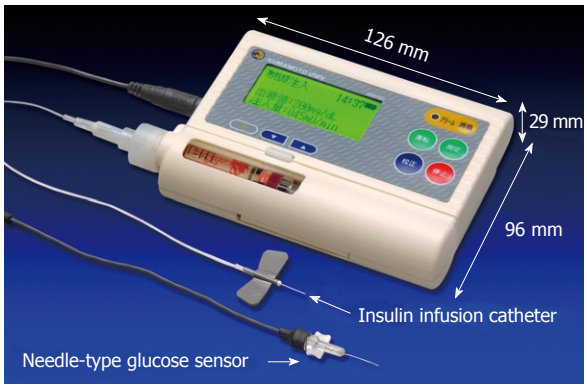

Figure 6 Newly designed wearable artificial endocrine pancreas. The total system is packed into a small unit (12.6 cm × 2.9 cm × 9.6 cm) weighing 250 g (KAP-003, Nikkiso). A glucose sensor is placed in the subcutaneous tissue and measures glucose concentration continuously. The blood glucose concentration (BG, in mg/dL) and the insulin infusion rate (IIR, in mU/min) appear every minute on a large LCD display.

Implantable artificial endocrine pancreas

The trend in the development of an artificial endocrine pancreas is now from a wearable to an implantable one. However, many problems remain to be solved for each part of the devices. Technology derived from the development of various implantable artificial organs will be beneficial for the accomplishment of the long-term clinical application of the devices.

Our study has suggested that although closed-loop portal and peripheral venous insulin delivery systems are equally effective in terms of blood glucose control and insulin requirements, portal insulin delivery is superior to peripheral delivery in maintaining more appropriate hepatic glucose handling and physiological insulin profiles. These results indicate that the portal vein is the most suitable insulin delivery route for the implantable artificial endocrine pancreas[30,31].

With regard to the metabolic efficacy and insulin requirement, intraportal insulin therapy is expected to be more effective than intraperitoneal insulin therapy. However, because the technique of placing an insulin catheter into the portal vein in humans is associated with severe invasion and high risks, such as infection and catheter thrombosis, there have been few reports of applying intraportal insulin therapy to patients with diabetes. Recently, there have been several reports of new methods that overcome these problems[32,33]. Thus, with technical improvement, it should be possible to safely infuse insulin intraportally.

The application of a one-chip microcomputer could make the processor system smaller. The technology derived in the process of developing the clinical applications of implantable insulin infusion pumps has contributed to the completion of the effector implantation. Implantation of the entire system requires a small and powerful long-life battery. A transcutaneous energy transmission system is one of the candidates for this. As a stable and reliable long-life implantable glucose sensor is not yet available, it is not practical to implant

all these parts of these apparatus intracorporeally. It would be adequate to implant the computer and infusion pumps and retain the glucose sensor extracorporeally to regulate insulin infusion rates by telemetric means. In the near future, an implantable artificial endocrine pancreas with a telecommunications system will be available for the treatment of diabetes mellitus.

CONCLUSION

Successful glycemic control in patients with diabetes using the artificial pancreas emphasizes the importance of continuous glycemic monitoring for strict glycemic control. However, the major obstacle for extending the term of glycemic control in patients with diabetes is the development of an implantable, high-precision glucose sensor for tissue glucose determination. A needle-type glucose sensor, which is a miniature hydrogen peroxide electrode covered by membrane with biological activity, can be implanted easily and is exchangeable. The sensor has the *in vitro* and *in vivo* characteristics suitable for continuous tissue glucose monitoring. A wearable artificial endocrine pancreas, which incorporates a needle-type glucose sensor, has been devised and has regulated glycemia physiologically in patients with diabetes for > 6 d. Further improvements in sensor design, especially in membranes with biocompatibility, might reduce the host reactions to the sensor implanted in tissue and thus extend its biological life.

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TOPIC HIGHLIGHT

Kazuhiro Hanazaki, MD, Professor and Chairman, *Series Editor*

Perioperative intensive insulin therapy using artificial endocrine pancreas in patients undergoing pancreatectomy

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Abstract

Perioperative glycemic control is important for reducing postoperative infectious complications. However, clinical trials have shown that efforts to maintain normoglycemia in intensive care unit patients result in deviation of glucose levels from the optimal range, and frequent attacks of hypoglycemia. Tight glycemic control is even more challenging in those undergoing pancreatic resection. Removal of lesions and surrounding normal pancreatic tissue often cause hormone deficiencies that lead to the destruction of glucose homeostasis, which is termed pancreatogenic diabetes. Pancreatogenic diabetes is characterized by the occurrence of hyperglycemia and iatrogenic severe hypoglycemia, which adversely effects patient recovery. Postoperatively, a variety of factors including surgical stress, inflammatory cytokines, sympathomimetic drug therapy, and aggressive nutritional support can also affect glycemic control. This review discusses the endocrine aspects of pancreatic resection and highlights postoperative glycemic control using a closed-loop system or artificial pancreas. In previous experiments, we have demonstrated the reliability of the artificial pancreas in dogs with total pancreatectomy, and its postoperative clinical use has been shown to be effective and safe, without the occurrence of hypoglycemic episodes, even in patients after total pancreatectomy. Considering the increasing requirement for tight perioperative glycemic control and the recognized risk of hypoglycemia, we propose the use of an artificial endocrine pancreas that is able to monitor continuously blood glucose concentrations with proven accuracy, and administer automatically substances to return blood glucose concentration to the optimal narrow range.

Key words: Blood glucose; Diabetes mellitus; Hyperglycemia; Pancreas; Artificial; Pancreatectomy

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INTRODUCTION

Under normal conditions, blood glucose homeostasis is regulated by hepatic and/or pancreatic metabolism[1-3]. The role of the hepatocyte in producing glucose in the fasting and stressed state, or for postprandial glucose uptake is critical for metabolic homeostasis[1,2]. These functions depend largely on three circulating glucoregulatory hormones that are secreted by the pancreas: insulin, glucagon, and pancreatic polypeptide (PP)[1-3]. After pancreatectomy, insufficiency or deficiency of these hormones causes glucose intolerance, a form of secondary diabetes mellitus termed pancreatogenic diabetes[4-7]. The appropriate method for glycemic control in pancreatogenic diabetes after pancreatectomy has yet to be established because of the instability of blood glucose levels, especially in patients after total pancreatectomy. In this Topic Highlight, we review the problem of pancreatogenic diabetes in patients who have undergone pancreatectomy. In addition, as a solution

to pancreatogenic diabetes, we demonstrate the safety and usefulness of tight blood glucose control using an artificial endocrine pancreas, following proximal, distal and total pancreatectomy.

PANCREATOGENIC DIABETES

Pancreatogenic diabetes is characterized by diabetes with frequent episodes of iatrogenic hypoglycemia and hyperglycemia[8], which is referred to as brittle diabetes (Figure 1). This is true particularly for patients after total or subtotal pancreatectomy. The condition is difficult to treat because of a paradoxical combination of enhanced peripheral insulin sensitivity and decreased hepatic insulin sensitivity, in addition to decreased glucagon secretion[8-12]. Patients become hyperglycemic because of unsuppressed hepatic glucose production, when insulin replacement is insufficient. In contrast, patients become hypoglycemic when insulin replacement is barely excessive, as a result of the enhanced peripheral insulin sensitivity and a deficiency of pancreatic glucagon secretion[10].

Shortly after surgical resection of the pancreas, 8%-23% of patients develop pancreatogenic diabetes, which increases to 40%-50% during follow-up[5-7]. With the increasing incidence of pancreatectomy[13,14], pancreatogenic diabetes as a sequela to surgical resection is an urgent medical issue.

ACTIONS OF THE GLUCOREGULATORY HORMONES

Insulin

Insulin-secreting β cells are distributed evenly throughout the pancreas. Insulin decreases serum glucose concentration through suppressing hepatic glyconeogenesis, glycogenolysis and facilitating hepatic glycogen synthesis[1,2]. Insulin receptors are expressed on nearly every cell throughout the body and insulin decreases blood glucose concentration through facilitating glucose uptake into insulin-receptor-bearing cells[10]. In addition to the glucoregulatory effect, insulin also has an anti-inflammatory effect[15,16].

Glucagon

Glucagon-secreting α cells are located predominantly in the body and tail of the pancreas. Together with the β cells, the function of α cells is essential for maintaining the remarkable consistency of glucose levels under various states of supply and demand[17]. During fasting, glucagon is responsible for maintaining adequate glucose production in hepatocytes by stimulating glycogenolysis and gluconeogenesis. Thus, glucagon works as a counter-regulatory mechanism for hypoglycemia. Surgical resection that causes reduction of α cells may be associated with severe hypoglycemic episodes, especially after total or distal pancreatectomy.

PP

PP-secreting cells are located mainly in the ventral pancreatic head and uncinate process[18-20]. Studies on a canine model of chronic pancreatitis with accompanying PP deficiency have demonstrated hepatic resistance to insulin and inappropriate hepatic glucose production, despite physiological levels of insulin[21]. Kono *et al*[22] have shown that with co-infusion of PP in total pancreatectomized dogs, smaller amounts of insulin infusion are required to normalize glucose levels. Earlier studies have also demonstrated that PP reverses abnormal glucose production after pancreatectomy[11]. Therefore, PP deficiency may serve as a potentially reversible pathophysiological factor that contributes to altered glucose metabolism[8] following proximal or total pancreatectomy.

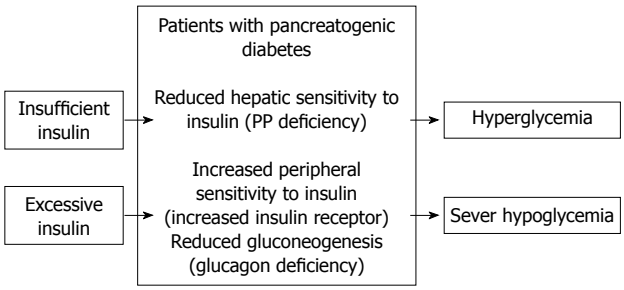

Figure 1 Pathophysiology of pancreatogenic diabetes according to published reports[10-14].

GLUCOSE CONTROL AFTER PANCREATECTOMY

Diabetes mellitus is a well-established risk factor for postoperative infectious complications[23-25], and much effort has focused on preoperative glycemic control. However, after van den Berghe *et al*[26] reported that tight glycemic control had a beneficial impact on the mortality rate of patients admitted to the intensive care unit (ICU), increased attention has been paid to postoperative glycemic control. Hyperglycemia itself, even in non-diabetic patients, has also been reported to be associated with an adverse outcome in surgical patients[27].

Despite this increasing attention to postoperative glycemic control, tight control of blood glucose levels is still not practiced widely, because of the difficulty of keeping glucose levels within the aimed-for range, and frequent occurrences of hypoglycemia[28,29]. Therefore, we recommend strongly the use of an artificial endocrine pancreas equipped with the ability to monitor accurately and control automatically blood glucose levels efficiently, safely and in a hands-free manner[22,30-34].

EXPERIMENTAL STUDY

The initial aim of this experimental study was to investigate the effect of PP infusion on insulin requirements after total pancreatectomy in dogs[22]. However, the study also demonstrated clearly the usefulness and safety of the artificial pancreas in controlling blood glucose after total pancreatectomy[22]. We have used STG-22 (Nikkiso Co. Ltd., Tokyo, Japan) which is a bedside-type artificial

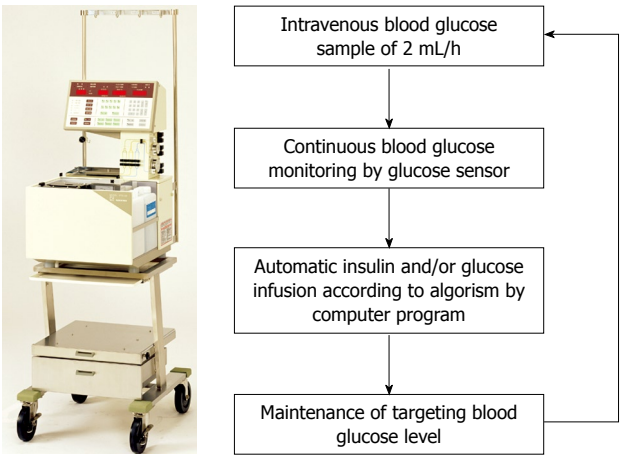

Figure 2 STG-22, a bedside-type artificial endocrine pancreas system with closed-loop.

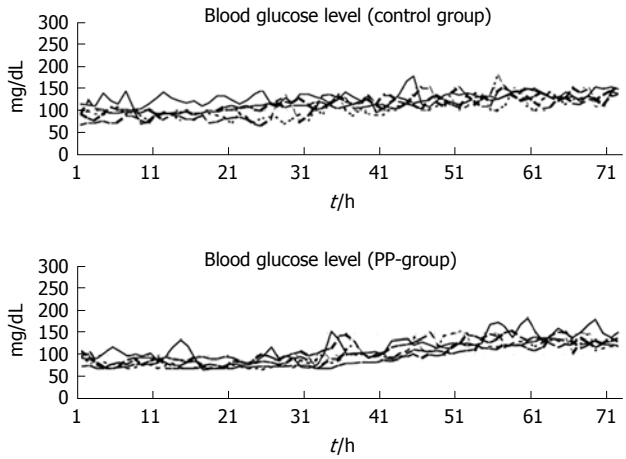

Figure 3 Continuous blood glucose levels over 72 h in 10 dogs after total pancreatectomy[22].

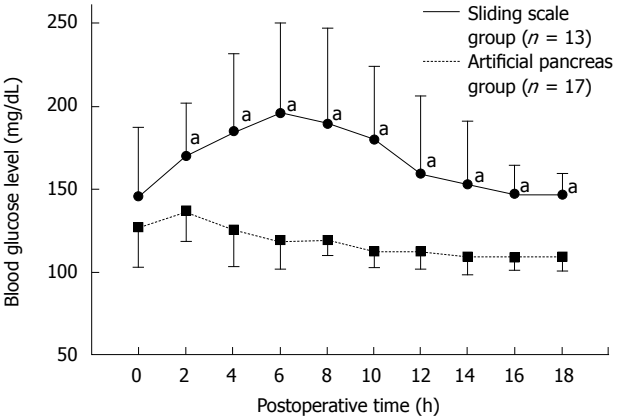

Figure 4 Postoperative blood glucose levels in the sliding scale (*n* = 13) and artificial pancreas (*n* = 17) groups during the first 18 h following pancreatectomy[38]. Significant statistical difference ($^aP < 0.05$).

endocrine pancreas[34]. The Nikkiso Company developed the STG-22 unit in 1984 as an artificial endocrine pancreas, which was only a single device with a closed-loop system (Figure 2). Peripheral venous blood for glucose monitoring was sampled continuously at 2 mL/h. STG-22 is capable of measuring continuously the blood glucose level with its glucose sensor, and automatically infuses insulin and/or glucose to adjust the blood glucose level in accordance with a target glucose value, which is the so called closed-loop system[34]. Ten dogs underwent total pancreatectomy. Following surgery, five dogs were supported solely by the artificial pancreas for 72 h, while the other five were supported by the artificial pancreas plus an infusion of bovine PP at 2 pmol/kg per minute. Mean blood glucose levels and insulin requirements were compared between the two groups. In all 10 dogs, the blood glucose concentration was controlled tightly at a mean level of 110 ± 4 mg/dL, and there was no difference in mean blood glucose level between the two groups (Figure 3). The insulin requirement in the group treated with PP was 90.0 ± 20.8 mU/kg for the first day and 562.7 ± 126.5 mU/kg for the second day. This requirement was significantly less than that of the group without PP: 445.0 ± 151.9 mU/kg for the first day and 1007.7 ± 144.9 mU/kg for the second day. During the operation of the artificial pancreas, there were no serious complications, such as catheter thrombosis, migration, infection, or episodes of hypo/hyperglycemia.

CLINICAL STUDY

In addition to endocrine insufficiency after pancreatic surgery, other factors such as surgical stress, inflammatory proteins[35], sympathomimetic drug therapy[3], and aggressive nutritional support can also make glycemic control difficult. To date, only one study has focused on tight postoperative glycemic control in patients undergoing pancreatic resection[36]. Thirty patients with pancreatic neoplasia were divided prospectively into two groups: sliding scale (n = 13) and artificial pancreas (n = 17) groups. Blood glucose concentrations were monitored continuously by an artificial pancreas and glycemic control was achieved using the sliding scale method or the artificial pancreas itself.

In the sliding scale group, postoperative blood glucose levels rose steadily, and reached a maximum value of approximately 200 mg/dL between 4 and 6 h after pancreatectomy. In the artificial pancreas group, blood glucose levels decreased gradually, and reached the target range (80-110 mg/dL) by 6 h postoperatively (Figure 4). Total insulin administered per patient during the first 18 h after surgery was significantly higher in the artificial pancreas group (107 ± 109 IU) compared to the sliding scale group (8 ± 6 IU; $P < 0.01$). No hypoglycemia was recorded in either group. This study demonstrated the usefulness of the artificial pancreas for the first time in a clinical setting, using a number of cases and a prospective design.

TOTAL PANCREATECTOMY AND POSTOPERATIVE GLYCEMIC CONTROL

Total pancreatectomy results in an extreme form of pancreatogenic diabetes. The blood glucose concentration

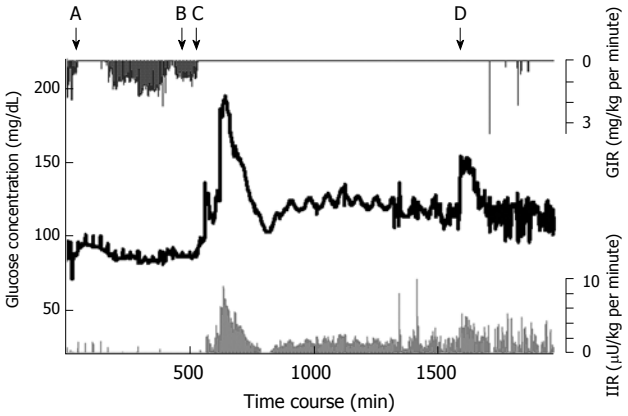

Figure 5 Blood glucose concentration during and after total pancreatectomy in a middle-aged female patient. Blood glucose is shown by the black line. The insulin infusion rate (light grey line) increased according to the increase in glucose concentration. A: Beginning of surgery; B: Wound closure; C: Beginning of parenteral nutrition in ICU; D: Beginning of combined enteral and parenteral nutrition; GIR: Glucose infusion rate; IIR: Insulin infusion rate.

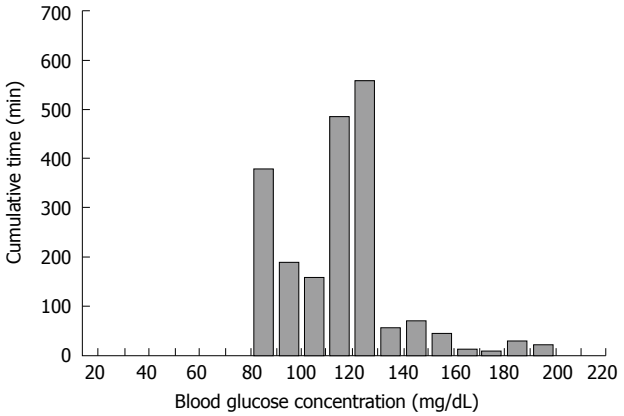

Figure 6 Histogram depicting the range and cumulative time for the blood glucose concentration of a middle-aged female who underwent total pancreatectomy.

goes up and down within a short duration, and iatrogenic hypoglycemia frequently affects prognosis. The patient described here was a middle-aged woman who underwent total pancreatectomy, and during the acute postoperative phase, received catecholamines to maintain blood pressure, and nutritional support of 25 kcal/kg per day soon after transfer from the operating theatre to the ICU.

Her blood glucose concentration profile is shown in Figure 5. An artificial pancreas (STG-22) was operated for 1994 min, automatic and manual calibration was performed seven times, and each calibration took approximately 8 min. Failure of blood withdrawal occurred seven times, which was a total of 20 min (data during calibration and failure of blood withdrawal was eliminated from the data analysis). Blood sample failures were resolved easily by trained ICU nurses.

The glucose concentration was stable during surgery and immediately after completion of total pancreatectomy (A to B in Figure 5). The glucose concentration increased with the beginning of parenteral nutrition in the ICU, and immediate administration of insulin suppressed the severe hyperglycemia. With the reduction in glucose concentration, the insulin infusion rate decreased to avoid hypoglycemia (the glucose surge after C in Figure 5). Her glucose concentration increased again with the beginning of enteral nutrition (Figure 4D), followed by increased insulin infusion. Total insulin administered during the operation of STG-22 was 96 IU. Her blood glucose concentration was 80-139 mg/dL for 91.3% of the time (Figure 6). No episode of hypoglycemia, defined as a blood glucose concentration < 40 mg/dL, occurred. The artificial pancreas was shown to be safe and efficient in the patient after total pancreatectomy.

FUTURE DIRECTIONS

Recently, the Normoglycemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulation (NICE-SUGAR) study has reported that glycemic control for critically ill patients in the ICU results in beneficial effects on survival when the blood glucose concentration is targeted at $\leqslant$ 180 mg/dL[37]. However, when limited to the surgical ICU setting, tight glycemic control also provides benefits for postoperative patients, according to a recent meta-analysis which included the results of the NICE-SUGAR study[38]. In light of current knowledge, a prospective randomized control trial using the artificial endocrine pancreas is needed urgently to compare different ranges of blood glucose concentrations after major surgery. Such a study would ascertain the optimal range for blood glucose concentrations without hypoglycemia, which presumably weakens the potency of tight glycemic control.

In the future, along with efforts to reduce postoperative complications after pancreatectomy by improving surgical methods, drainage and patient selection, glycemic control should receive more attention, especially during the early postoperative period. Undoubtedly, it is very important to find out the optimal range for blood glucose levels and the optimal caloric intake shortly after pancreatectomy, and develop more sophisticated mechanical devices to control blood glucose.

CONCLUSION

Pancreatectomy often results in pancreatogenic diabetes for which insulin treatment is known to be difficult. To reduce the postoperative infectious complications related to hyperglycemia and iatrogenic hypoglycemia caused by conventional blood glucose control, perioperative use of an artificial pancreas is recommended as an ideal method for tight glycemic control after pancreatic surgery. The operation of an artificial endocrine pancreas is still somewhat resource-intensive and expensive, but if mortality and morbidity are reduced, it is worthwhile.

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TOPIC HIGHLIGHT

Kazuhiro Hanazaki, MD, Professor and Chairman, *Series Editor*

Perioperative insulin therapy using a closed-loop artificial endocrine pancreas after hepatic resection

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Abstract

Postoperative hyperglycemia is common in critically ill patients, even in those without a prior history of diabetes mellitus. It is well known that hyperglycemia induced by surgical stress often results in dysregulation of liver metabolism and immune function, impairing postoperative recovery. Current evidence suggests that maintaining normoglycemia postoperatively improves surgical outcome and reduces the mortality and morbidity of critically ill patients. On the basis of these observations, several large randomized controlled studies were designed to evaluate the benefit of postoperative tight glycemic control with intensive insulin therapy. However, intensive insulin therapy carries the risk of hypoglycemia, which is linked to serious neurological events. Recently, we demonstrated that perioperative tight glycemic control in surgical patients could be achieved safely using a closed-loop glycemic control system and that this decreased both the incidence of infection at the site of the surgical incision, without the appearance of hypoglycemia, and actual hospital costs. Here, we review the benefits and requirements of perioperative intensive insulin therapy using a closed-loop artificial endocrine pancreas system in hepatectomized patients. This novel intensive insulin therapy is safe and effectively improves surgical outcome after hepatic resection.

Key words: Artificial pancreas; Hepatic resection; Hyperglycemia; Intensive insulin therapy; Surgical site infection

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INTRODUCTION

Anatomically, the liver is situated downstream from the pancreas. It is a primary site for the metabolism of pancreatic hormones, such as insulin and glucagon, which have a central role in the regulation of peripheral blood glucose levels[1]. In addition, the liver is positioned downstream from the gut, from which it absorbs a large amount of ingested glucose, and is involved in glycogenolysis and gluconeogenesis. Accordingly, reduced liver function following hepatic resection using the Pringle procedure[2] may result in metabolic disturbances of the pancreatic hormones and glucose intolerance.

Hyperglycemia has a deleterious effect on cells that passively take up glucose independent of insulin, including hepatocytes, alveolar cells, endothelial cells, neurons, and immune cells. Postoperative hyperglycemia is common in critically ill patients, even in those without a prior history of diabetes mellitus[3-5]. It is well known that hyperglycemia induced by surgical stress often results in dysregulation of liver metabolism and immune function, leading to impaired postoperative recovery[6,7]. Thus, the prevention of glucose toxicity in the mitochondrial compartment is important[8].

In this Topic Highlight, we review the benefits and requirements of tight glycemic control in hepatic surgery, with a focus on postoperative infection control. We suggest that perioperative intensive insulin therapy using a closed-loop artificial endocrine pancreas system is safe and effectively improves surgical outcome after hepatic resection.

CURRENT STATE OF TIGHT GLYCEMIC CONTROL IN CRITICALLY ILL PATIENTS

In large randomized trials in which the use of tight blood glucose control (80-110 mg/dL) with intensive insulin therapy was compared with standard blood glucose control (< 200 mg/dL) in surgical intensive care unit (ICU) patients, strict control of postoperative blood glucose levels was shown to significantly reduce patient mortality and morbidity[9,10]. In addition, postoperative hyperglycemia has been shown to be associated with an increased risk of surgical site infection (SSI)[11,12]. Current evidence suggests that maintaining normoglycemia postoperatively improves surgical outcome and reduces mortality and morbidity in critically ill patients[8,11,12]. These observations led to several short-lived multicenter randomized controlled studies designed to evaluate the benefit of tight glycemic control with intensive insulin therapy[13,14]. The main reason for the early cessation of these clinical trials was the high incidence of hypoglycemia (10%-17%) induced by the intensive insulin therapy[15,16], which could not be prevented because of technical limitations at that time[17-19]. However, the subsequent development of accurate continuous blood glucose monitoring devices and closed-loop systems for computer-assisted blood glucose control in the ICU will likely reduce the incidence of hypoglycemia in these situations[8].

For the reasons described above, achieving tight glycemic control with intensive insulin therapy has come under increased scrutiny in the management of patients in the surgical ICU. Recently, we demonstrated in two retrospective studies and in one randomized clinical trial that perioperative tight glycemic control using a closed-loop glycemic control system for patients undergoing liver resection was safe and effective in decreasing the incidence of SSI without increasing the risk of hypoglycemia.

INSULIN THERAPY IN THE SURGICAL ICU

In all three studies, perioperative blood glucose concentrations were monitored continuously using the STG-22 system, developed by Nikkiso Co. (Tokyo, Japan). Patients were divided into two groups: one in which glucose levels were controlled by manual injection of insulin according to the commonly used sliding-scale method (SS group)[20,21], and a second group in which programmed infusions of insulin were administered as determined by the control algorithm of a closed-loop artificial endocrine system (AP group).

Conventional insulin therapy using the sliding-scale method

Blood glucose levels in patients in the SS group were monitored continuously by the artificial pancreas and patients were checked routinely by nursing staff every 2 h. Blood glucose levels in these patients were controlled by subcutaneous injections of regular human insulin, with the dose determined by the sliding-scale method and the target blood glucose level to avoid hypoglycemia set at 150-200 mg/dL[20,21].

Novel insulin therapy using a closed-loop artificial endocrine pancreas system

The STG-22 unit was developed in 1984 by Nikkiso Co. as a closed-loop artificial endocrine pancreas system. The STG-22 system is a reliable and accurate device that measures blood glucose concentrations continuously and is comparable to the ABL 800FLEX machine (Radiometer Medical ApS, Brønshøj, Denmark) recommended by the National Committee for Clinical Laboratory Standards[22,23]. The STG-22 closed-loop glycemic control system is composed of a glucose sensor for the detection and/or monitoring of glucose and pumps for the infusion of appropriate amounts of insulin or glucose[24,25]. The insulin and glucose pumps are regulated by a computer on the basis of target blood glucose values that are defined prior to initiation of the system. The STG-22 maintains stable blood glucose concentrations by automatic infusion of regular insulin or glucose into the circulation[24,25]. In the ICU, peripheral blood was sampled continuously at 2 mL/h over the first 18 h postoperatively to monitor glucose levels. In addition, the STG-22 was used to evaluate the patients' insulin requirements.

Statistical analysis

Continuous variables are presented as the mean ± SD. Dichotomous variables are presented as both absolute numbers and percentages. Data were analyzed using Student's *t*-test (two-tailed), with dichotomous variables analyzed by the χ^2 test (two-tailed) or Fisher's exact test (two-tailed). $P < 0.05$ was considered significant. All analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

RETROSPECTIVE STUDIES

The benefits of using a closed-loop glycemic control system in patients after hepatectomy were investigated in two retrospective studies. The aim of the first study was to evaluate the usefulness of the closed-loop system in providing continuous monitoring and strict control of postoperative blood glucose levels in patients after hepatic resection[25]. The aim of the second study was to identify, using multivariate analysis, risk factors and predictors of SSI, as well as how to prevent the development of SSI, in a consecutive series of patients undergoing hepatic resection for liver disease in a single institution.

In the first study[25], postoperative blood glucose levels increased initially in the SS group, reaching a plateau of approximately 250 mg/dL between 4 and 7 h after hepatectomy. Thereafter, blood glucose levels decreased, returning to normal within 16 h after surgery. In the AP group, blood glucose decreased gradually, reaching target levels (90-110 mg/dL) within 12 h after surgery. Total insulin administered per patient during the first 16 h after surgery was significantly higher in the AP group compared with the SS group (183 ± 188 IU *vs* 8 ± 7 IU, respectively, $P < 0.001$). These data suggest that the sliding-scale method is not as effective as the closed-loop artificial endocrine system in preventing hyperglycemia resulting from disturbed glucose metabolism following liver resection.

In the second study[26], the association between SSI and various clinical parameters was investigated in 152 patients following hepatic resection. The incidence of SSI in these patients was 14.5%. Multivariate analysis identified four independent parameters that were correlated with the occurrence of SSI, namely (1) body mass index > 23.6 kg/m^2, (2) estimated blood loss volume > 810 mL, (3) the presence of postoperative bile leak organ/space SSI; and (4) use of the sliding-scale method for postoperative glucose control. No SSI was observed after liver resection in patients in whom postoperative blood glucose levels were controlled by an artificial pancreas. The results of this second retrospective study demonstrated that a lack of postoperative glycemic control is associated with a significantly higher incidence of postoperative infectious complications and a longer period of hospitalization.

PROSPECTIVE RANDOMIZED CLINICAL TRIAL

A prospective randomized trial was conducted in patients undergoing hepatic resection to evaluate the postoperative condition of the patients and the effects of a closed-loop artificial pancreas on tight glycemic control during intensive insulin therapy after hepatectomy[27]. Patients were randomly assigned to receive intensive insulin therapy using a closed-loop glycemic control system (i.e. an artificial pancreas; target blood glucose 80-110 mg/dL; AP group) or conventional insulin therapy using the sliding-scale method (target blood glucose 150-200 mg/dL; SS group). Perioperative blood glucose levels were monitored continuously in both groups using a closed-loop system. Neither group experienced hypoglycemia (blood glucose < 40 mg/dL). Although perioperative blood glucose levels in the AP group were close to 100 mg/dL, those in the SS group were > 150 mg/dL, which is the same as in our first retrospective study[25] (Figure 1). The incidence of SSI was significantly lower in the AP than in the SS group (2.3% *vs* 18.2%, respectively, $P = 0.030$), the duration of hospitalization was significantly shorter for patients in the AP group compared with the SS group (14.3 ± 5.9 d *vs* 18.7 ± 11.7 d, respectively, $P = 0.049$). The impact on hospital costs is one of the most frequently discussed consequences of SSI within a clinical setting. A few studies have presented data on the incidence and costs of infection for the hospital and post-discharge periods. As there were no incidences of post-discharge SSI in the current study, the costs-of-illness during hospitalization were incorporated into the overall costs. Total hospital costs were significantly lower for patients in the AP group than in the SS group (16 407 ± 5284 $ *vs* 21 879 ± 15 784 $, respectively, $P = 0.047$). There is evidence suggesting that SSI prolongs the length of hospitalization for patients undergoing cardiac surgery, caesarean section, orthopedic surgery and general surgery, and increases the total costs of a patient's treatment[28]. These results support the notion that intensive insulin therapy using a closed-loop glycemic control system after hepatic resection results in the maintenance of near normoglycemia, contributing to a reduction in both the incidence of SSI and total hospital costs per patient due to a decreased duration of hospitalization. Thus, the closed-loop system promises more effective and safer intensive insulin therapy in hepatectomized patients. An important question that has arisen from this study is how to choose candidates for intensive insulin therapy using a closed-loop system. This important issue must be addressed in future studies.

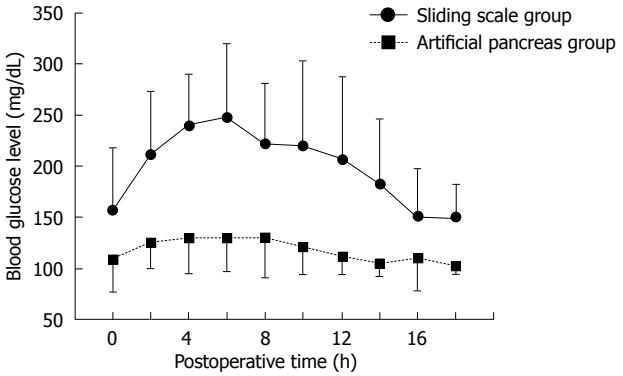

Figure 1 Postoperative blood glucose levels in hepatectomized patients in whom blood glucose was controlled using either the sliding-scale method or a closed-loop artificial endocrine pancreas system (Okabayashi *et al*[27] *Diabetes Care* 2009).

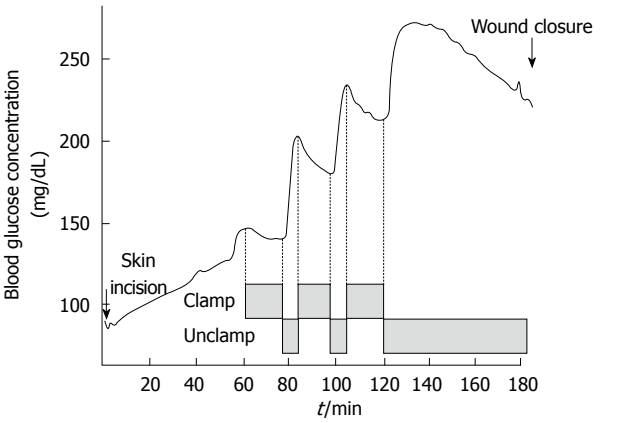

Figure 2 Typical trend of blood glucose concentrations during the Pringle maneuver for hepatic resection (Maeda *et al*[32] *Am J Surg* 2009).

PROBLEMS ASSOCIATED WITH INTENSIVE INSULIN THERAPY IN LIVER SURGERY

The possible risks of targeting normoglycemia include intraoperative hyperglycemia, hypoglycemia, and the need for perioperative parenteral nutrition.

The Pringle maneuver was introduced in liver surgery to reduce hepatic hemorrhage[2] and is now widely used in hepatic resections to control intraoperative bleeding[29-31]. This inflow-occlusion technique involves total compression of the hepatoduodenal ligament, generally by clamping it for 15 min during hepatic parenchymal resections, followed by 5 min of unclamping[31,32]. The clamping-unclamping procedure is repeated until the hepatic resection is complete. Glucose concentrations just before the first Pringle maneuver were significantly higher than baseline, but decreased gradually with the first clamping of the hepatoduodenal ligament. After unclamping of the hepatoduodenal ligament, there was an immediate and marked increase in glucose levels. The decrease and subsequent increase in glucose levels are seen with following rounds of clamping and unclamping of the hepatoduodenal ligament. After the surgery is completed, glucose concentrations gradually decline (Figure 2)[32]. In future studies, we will determine whether the rapid fluctuations in blood glucose levels during the Pringle maneuver should be controlled.

The most feared risk associated with intensive insulin therapy is postoperative hypoglycemia, which may cause convulsions, coma, and brain damage, as well as cardiac arrhythmias[15]. In ICU studies, the risk of severe hypoglycemia (glucose < 40 mg/dL) has been shown to increase from 5.0% to 18.7% with intensive insulin therapy[10,15,16]. Van den Berghe[8] suggested that clinical outcome by intensive insulin therapy (targeting blood glucose level of 80-110 mg/dL) was more effective for reducing hospital mortality and morbidity in critically ill adult patients compared with moderate intensive insulin therapy (targeting blood glucose level of 110-150 mg/dL). However, a large international randomized trial in 2009 showed that a blood glucose target of less than 180 mg/dL resulted in lower mortality than a target of 81-108 mg/dL[16]. Furthermore, contrary to this report[8], a recent meta-analysis[15] did not support the benefits of intensive insulin therapy that it was not associated with significantly reduced hospital mortality but was associated with an increased risk of hypoglycemia. These randomized trials had several issues as follows: (1) trials were not blinded, (2) unusually high mortality in the usual care group, (3) parenteral nutrition (different administration of energy at the ICU), and (4) a markedly increased risk of hypoglycemia. To address this issue on the focus of a more effective blood glucose control method under impartial conditions without hypoglycemia, we are constructing a prospective randomized comparison study between intensive insulin therapy and moderate intensive insulin therapy using a closed-loop artificial endocrine pancreas. There were no occurrences of hypoglycemia during intensive insulin therapy using a closed-loop glycemic control system in our series of studies. In our experience of more than 200 patients in the surgical and medical ICU, we have not seen hypoglycemia develop in any patient when intensive insulin therapy is administered using a closed-loop glycemic control system (data not shown).

Enteral feeding was started as soon as possible after patients were hemodynamically stable. However, if the energy intake target could not be achieved, parenteral feeding was initiated to compensate for the deficit. In our series, all patients were given parenteral nutrition following surgery[33], with the total caloric requirement calculated according to the Harris-Benedict equation[34]. Based on the results of our studies, maintaining an adequate calorie level, and controlling postoperative glucose levels with insulin therapy contributed to a reduction in the incidence of SSI. Moreover, the duration of hospitalization after liver surgery was reduced in patients who received perioperative tight glycemic control with intensive insulin therapy, and it is likely that this is related to a reduction in postoperative complications due to infection (SSI). In our studies, tight glycemic control was maintained for 18 h in patients in the surgical ICU after liver resection, and excellent glucose control was achieved without hypoglycemia using the closed-loop system.

Another outcome of our studies was that a brief period of glycemic control impacted on the incidence of SSI. Bacterial growth curves for *Escherichia coli*, *Streptococcus*, *Proteus*, *Staphylococcus* and *Pseudomonas*, among others, indicate that under optimum conditions the greatest growth occurs between 2 and 18 h[35]. We strongly believe that by instigating perioperative tight glycemic control for a brief period (at least 18 h) after surgery, postoperative infectious morbidity is decreased.

FUTURE DIRECTIONS

Clearly, we support a recent report that suggests that the development of accurate, continuous blood glucose

monitoring devices (preferably closed-loop systems) for computer-assisted blood glucose control in the ICU will help prevent hypoglycemia[17]. However, using currently available technology, tight glucose control (i.e. targeting blood glucose levels of 80-110 mg/dL) using a closed-loop system in the surgical ICU was not achieved in 100% of cases, with the reported range being 60%-100%. Thus, further studies are needed to determine a better algorithm with which more accurate tight glucose control can be achieved. Regardless, we believe that by using the closed-loop glycemic control system during intensive insulin therapy in the ICU, the incidence of hypoglycemia and/or problems of nutritional support are reduced, as is the burden on nursing staff that is normally associated with the requisite frequent monitoring of blood glucose levels.

CONCLUSION

Intensive insulin therapy using a closed-loop artificial endocrine pancreas system during hepatic resection not only maintained near normoglycemia, but also contributed to a reduction in the rate of SSI and a decrease in total hospital costs due to shortened hospitalization. The closed-loop glycemic control system promises to revolutionize intensive insulin therapy for patients with disturbed glucose metabolism after liver resection.

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TOPIC HIGHLIGHT

Kazuhiro Hanazaki, MD, Professor and Chairman, *Series Editor*

Relationship between perioperative glycemic control and postoperative infections

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Abstract

Perioperative hyperglycemia in critically ill surgery patients increases the risk of postoperative infection (POI), which is a common, and often costly, surgical complication. Hyperglycemia is associated with abnormalities in leukocyte function, including granulocyte adherence, impaired phagocytosis, delayed chemotaxis, and depressed bactericidal capacity. These leukocyte deficiencies are the cause of infection and improve with tight glycemic control, which leads to fewer POIs in critically ill surgical patients. Tight glycemic control, such as intensive insulin therapy, has a risk of hypoglycemia. In addition, the optimal targeted blood glucose range to reduce POI remains unknown. Since 2006, we have investigated tight perioperative blood glucose control using a closed-loop artificial endocrine pancreas system, to reduce POI and to avoid hypoglycemia. In this Topic Highlight, we review the relationship between perioperative glycemic control and POI, including the use of the artificial pancreas.

Key words: Glycemic control; Surgical site infection; Artificial pancreas; Insulin therapy; Glucose toxicity

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INTRODUCTION

Recent evidence suggests that perioperative hyperglycemia is the main risk factor for the development of postoperative infection (POI)[1,2]. Intensive glucose control[3-7] leads to fewer POIs in critically ill surgical patients[1]. However, tight glycemic control[8-12] such as intensive insulin therapy (IIT)[3-7] has a risk of hypoglycemia. In addition, the optimal target range for blood glucose to reduce POI remains unknown[1,2,13-15]. We recently demonstrated that tight perioperative glycemic control can be achieved by using an artificial endocrine pancreas[16,17] for surgical patients, and that it was a safe and effective treatment for decreasing the incidence of POI, without increasing the risk of hypoglycemia[18-22].

In this Topic Highlight, we review the relationship between perioperative glycemic control and POI, including the use of an artificial pancreas.

RELATIONSHIP BETWEEN PERIOPERATIVE HYPERGLYCEMIA AND POI

Perioperative hyperglycemia in critically ill surgery patients increases the risk of POI, which is a common, and often costly, surgical complication[1,2,18-22]. It is well known that diabetic patients are at higher risk of postoperative complications, including POI, than non-diabetic surgery patients. Indeed, hyperglycemia correlates positively with POI in diabetic surgical patients[23] and with a poor prognosis following stroke or head injury[24,25]. POI is exacerbated by perioperative hyperglycemia in critically ill surgery patients[1].

Strict glycemic control decreases the risk of infection

and improves other outcomes for trauma, cardiac, and critically ill non-diabetic surgery patients. Ramos *et al*[1] associated postoperative hyperglycemia with an increased risk of 30 d postoperative infectious complications and a longer hospital stay, independent of diabetic status. On the basis of these findings, they recommended evaluation of the possible benefits of postoperative glycemic control in general surgical patients[1].

Hyperglycemia is also associated with a sustained decrease in polymorphonuclear leukocyte function[26]. Abnormalities in leukocyte function[27] have been identified that are caused by the hyperglycemic state. These include abnormalities in granulocyte adherence[28], impaired phagocytosis[29], delayed chemotaxis[30], and depressed bactericidal capacity[29,31]. The degree of hyperglycemia that has been shown to impair phagocytic function is as low as 200 mg/dL[32]. These leukocyte deficiencies appear to improve with tight glycemic control[33]. Together with the demonstrated role of perioperative hyperglycemia in POI, it is clear that tight glycemic control should improve the clinical outcome for all surgical patients.

Insulin displays a potent and acute anti-inflammatory effect by inhibiting the tissue factor, plasminogen activator inhibitor-1[34], and the intranuclear nuclear factor κB (NF κB)[35]. Insulin plays a vital role, not only in blood glucose control, but also as an anti-inflammatory and anti-oxidant agent. Insulin suppresses the proinflammatory effects of NF κB, activator protein 1, early growth response 1, and high mobility group box 1[34-36], as well as inhibiting nicotinamide hypoxanthine dinucleotide oxidase action to reduce reactive oxygen species production[34,35].

IIT has been reported to reduce infection rates after neurosurgery; therefore, this approach might be an appropriate blood glucose control method to prevent POI[37]. However, clinical evidence is still needed to confirm the efficacy of IIT for strict blood glucose control in preventing POI. Although improved control of blood glucose fluxes have high potential to improve survival and decrease morbidity in surgical patients, the association between perioperative blood glucose range and the incidence of POI remains unclear in the majority of surgical patients due to lack of evidence. Further studies, including prospective randomized controlled trials, are necessary to clarify this issue.

THE OPTIMAL TARGET RANGE FOR BLOOD GLUCOSE TO PREVENT POI IN SURGICAL PATIENTS

Good long-term glycemic control is strongly associated with significantly fewer POIs in diverse surgical populations[38]. Zerr *et al*[39] reported that elevated blood glucose levels of more than 200 mg/dL in diabetic patients are associated with a higher incidence of deep sternal wound infection. Use of perioperative glycemic control in the range of 150-200 mg/dL significantly reduced POI in diabetic patients undergoing open heart surgery[40].

Van den Berghe *et al*[3] demonstrated that tight glycemic control (IIT) to maintain blood glucose levels in the target range of 80-110 mg/dL improved morbidity and mortality in the surgical intensive care unit (ICU). However, a recent large study by NICE-SUGAR Study Investigators[15] completely denied the effectiveness of IIT shown by Van den Berghe *et al*[3]. Contrary to Van den Berghe's first trial[3], IIT increased mortality among adults in the ICU and a blood glucose target less than 180 mg/dL (most frequent blood glucose value of 142 mg/dL) resulted in lower mortality than did a target of 81-108 mg/dL (most frequent blood glucose value of 107 mg/dL)[15]. Recent meta-analysis[41] including NICE-SUGAR[15], concluded that IIT significantly increased the risk of hypoglycemia and conferred no overall mortality benefit among critically ill patients; however, this therapy might be beneficial to patients admitted to a surgical ICU. Some patients might benefit from IIT, although the characteristics of such patients remain to be clearly defined, as does the effect of different blood glucose algorithms, the method of measuring blood glucose, and the influence of nutritional strategies[41]. Unfortunately, however, the optimal targeted blood glucose range to prevent POI remains unclear, especially in the intraoperative targeted blood glucose zone. IIT is also a risk factor for hypoglycemia ($\leqslant$ 40 mg/dL)[3,4,12-15], which is linked to serious neurological events. Hypoglycemia is the major potential harm of tight glucose control. Tight glycemic control without hypoglycemia will be required in the future.

TIGHT PERIOPERATIVE GLYCEMIC CONTROL USING A CLOSED-LOOP ARTIFICIAL ENDOCRINE PANCREAS SYSTEM

Since 2006, we have been developing a closed-loop artificial pancreas system[16-20] (STG-22, Nikkiso Co. Ltd., Tokyo) to achieve perioperative glycemic control and prevent POI in general surgery, without the risk of hypoglycemia associated with tight glycemic control.

Firstly, we confirmed that the STG-22[21,22] could reliably and accurately measure blood glucose concentration similarly to the ABL 800FLEX machine (Radiometer Medical Aps, Br0nsh0j, Denmark) recommended by the National Committee for Clinical Laboratory[21,22]. This closed-loop glycemic control system maintained stable blood concentrations by the automatic infusion of regular insulin and/or glucose into the circulation[18].

Secondly, we performed two prospective randomized clinical trials and found that tight perioperative glycemic control using a closed-loop artificial pancreas system (STG-22, Nikkiso Co. Ltd., Tokyo) decreased surgical site infection (SSI) in patients who underwent pancreatectomy[19] or hepatectomy[20]. In our reports, perioperative blood glucose levels were continuously monitored using an artificial endocrine pancreas[16-20] and glucose levels were controlled using either the sliding scale method[42] (SS group: targeted blood glucose zone

of 150-200 mg/dL) or the artificial pancreas (AP group: targeted blood glucose zone of 80-110 mg/dL). In our study of patients who had undergone a pancreatectomy, the AP group, maintained near-normal glycemia, whereas the blood glucose levels of the SS group plateaued at approximately 200 mg/dL between 4 and 6 h after pancreatectomy[19]. In addition, the incidence of SSI in the AP group (0%) was significantly lower than that of the SS group (about 30%). In our study of patients who had undergone a hepatectomy[20], the AP group again maintained near-normal glycemic control and a reduction in the incidence of SSI. As a consequence, the length of stay and cost of hospitalization was reduced. It is worth noting that there were no incidents of hypoglycemia using a closed-loop artificial pancreas system, despite maintaining blood glucose levels similar to those obtained with IIT. Our results support the conclusions of previous reports[1,38-40] that glycemic control could be a simple intervention to decrease the risk of infectious complications resulting from hyperglycemia after surgery.

Thirdly, to date, more than 200 general surgeries (hepatic resection, pancreatic resection, esophageal resection, and emergency operations) have been performed under tight perioperative glycemic control using an artificial pancreas (STG-22) with no incidence of hypoglycemia[20]. To avoid hypoglycemia in tight glycemic control and improve POI in surgery, the closed-loop artificial endocrine pancreas system is an effective and safe means. Furthermore, novel perioperative glycemic control using an artificial pancreas markedly improved the labor burden on nursing staff, and hence reduced concerns about hypoglycemia[43].

Finally, because an ideal comparative study of tight glycemic control should be carried out under conditions without risk of hypoglycemia, blood glucose control using a closed-loop artificial pancreas system might be beneficial for the detection of the optimal target range for blood glucose in surgical patients.

CONCLUSION

Perioperative hyperglycemia increases the risk of POI. Therefore, perioperative glycemic control in patients undergoing surgery is beneficial to reduce POI. As a closed-loop artificial pancreas system is able to perform tight glycemic control without increasing the risk of hypoglycemia, it might be a safe and useful blood glucose control system in critically ill surgical patients. At present, however, the perioperative optimal target range for blood glucose to reduce POI remains uncertain. Further studies are needed to address this issue.

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TOPIC HIGHLIGHT

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Intraoperative glycemic control procedures and the use of an artificial pancreas

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Abstract

Strict intraoperative glycemic control can significantly decrease the incidence of postoperative infection; however, anesthesiologists must carefully control blood glucose levels as well as properly manage the respiratory and cardiovascular systems. However, standard blood glucose measurement systems and insulin dosing algorithms, which are necessary for achieving strict glycemic control, have not yet been developed. An artificial pancreas (STG-22™; Nikkiso Co., Tokyo, Japan) is considered a highly accurate blood glucose monitoring system capable of closed-loop control of blood glucose. The device has, however, many problems to be addressed since it is a large and expensive system with little versatility, and it requires a large amount of blood to be collected. Therefore, the development of less invasive and inexpensive systems with future technological progress is greatly anticipated.

Key words: Strict glycemic control; Artificial pancreas; Anesthesiologist; Sliding scale

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INTRODUCTION

Management of the respiratory and cardiovascular systems is the most important task of an anesthesiologist. Traditionally, the purpose of intraoperative glycemic control is to prevent three major problems: hypoglycemia, ketoacidosis, and hyperglycemia-associated osmotic diuresis (dehydration). So far, particular attention has been given only to the case of abnormal hyperglycemia. On the other hand, postoperative glycemic control appears to influence a patient's prognosis, and as a result, this has drawn attention to intraoperative glycemic control[1,2]. In this report, the recent concept of glycemic control, problems of glycemic control procedures and effects of intraoperative glycemic control on a patient's prognosis are reviewed based on recent publications.

NEW CONCEPT OF GLYCEMIC CONTROL

The concept of glycemic control in the care of critically ill patients largely changed after van den Berghe *et al*[3] published a report in 2001. They comparatively investigated two groups of patients admitted to a surgical intensive care unit. In the first group, insulin control was instituted when the blood glucose level exceeded 100 mg/dL in order to maintain the blood glucose level in the target range from 80 to 110 mg/dL (strict glycemic control group). In the second group, insulin control was instituted if the blood glucose level exceeded 215 mg/dL in order to maintain the blood glucose level in the target range from 180 to 200 mg/dL (conventional glycemic control group). The results of insulin control were remarkable. The mortality rate of patients during the stay in the intensive care unit was 4.6% for the strict glycemic control group *vs* 8.0% for the conventional treatment group. The mortality rate among patients who remained in the intensive care unit for five or more days was 10.6% for the strict glycemic control group *vs* 20.2% for the conventional treatment group. The in-hospital mortality rate was reduced by 3.7% by strict

glycemic control. However, the test protocol has been questioned since there is a large discrepancy between the actual mortality rate of 26.3% in the examined group and the mortality rate predicted by APACHE Ⅱ score (median score 7). To address this criticism, a study was conducted in a medical intensive care unit and results were reported in 2006[4]. This study, where long-term prognosis following a similar glycemic control was monitored for one year or longer, showed the benefits of strict glycemic control in patients who remained in the intensive care unit for three or more days, but not in those who stayed for a shorter period. The mortality rate of the strict glycemic control group was 41.5% compared with the 50.9% of the conventional treatment group. However, the underlying detailed mechanism was not clarified and various basic and clinical studies to clarify this mechanism are presently ongoing.

DOES INTRAOPERATIVE GLYCEMIC CONTROL INFLUENCE PATIENT PROGNOSIS?

To date, there have been no randomized controlled trials to assess the effects of intraoperative glycemic control on patient prognosis. However, it is likely that intraoperative hyperglycemia decreases immunocompetence and increases the incidence of wound infection[5-10]. Various types of anesthetic agents also alter blood glucose level[11]. Furthermore, it has been reported that the incidence of wound infection is markedly high among hyperglycemic patients whose blood glucose level was reported to be 149 mg/dL or above intraoperatively[12,13]. Moreover, every 18 mg/dL increase in blood glucose level from 110 mg/dL is reported to increase the risk of wound infection by 17%[14]. Based on these results, intraoperative glycemic control that maintains blood glucose at a level not higher than 150 mg/dL is considered necessary. On the other hand, intraoperative glycemic control reportedly increases the risks of hypoglycemia due to unstable actions of insulin owing to hypothermia and impaired tissue perfusion[15,16]. Therefore, the establishment of concrete glycemic control procedures is eagerly anticipated, and the development of a continuous intraoperative blood glucose monitoring system is considered very important.

CURRENT STATUS OF GLYCEMIC CONTROL PROCEDURES

As mentioned above, concrete glycemic control procedures for achieving strict glycemic control have not yet been established. The detailed procedures described by van den Berghe *et al*[3] in 2001 were not very clear. According to another line of thought, full-time staff can be designated to carry out frequent blood glucose measurements in order to achieve glycemic control. While this validates this line of thought, it can not be executed in general practice. To perform accurate and safe glycemic control, the following elements are considered important: (1) frequency of blood glucose measurements, (2) accuracy of blood glucose measurement results, (3) accuracy of the insulin dosing algorithm, and (4) blood collection volume.

FREQUENCY OF BLOOD GLUCOSE MEASUREMENT

As for the frequency of blood glucose measurement, continuous monitoring is preferable to intermittent measurements. Two types of system capable of continuous blood glucose monitoring are currently used. One measures glucose concentration in the intracellular substance obtained by directly inserting an electrode subcutaneously, and the other collects venous blood continuously for blood glucose monitoring[17,18]. The former system is based on the near proportional relation between glucose concentration in the intracellular substance and blood glucose level. However, the measurement stability of this type of system is in question because rapid changes in blood glucose level are not readily reflected in the intracellular substance. On the other hand, an artificial pancreas (STG-22^{TM}) was developed by Nikkiso (Tokyo, Japan) and this device is considered the only system in the world representing the latter type of system, which collects venous blood continuously and performs blood glucose monitoring. Furthermore, this system carries out direct measurements of blood glucose and can therefore show rapid changes in blood glucose level (Figures 1 and 2). Although STG-22^{TM} is expected to serve as a standard blood glucose monitoring system, it cannot be used for all patients because of its high cost. Generally, intermittent blood glucose measuring systems are highly versatile and can be used in any facility. However, many difficulties are encountered in their use for strict glycemic control, mostly attributed to increased workloads from frequent measurements and large measurement errors[19]. It is considered that the achievement of strict glycemic control is currently supported by the efforts and dedication of medical staff as well as through proper education[18].

PROBLEMS ENCOUNTERED REGARDING THE VOLUME OF BLOOD COLLECTED

To achieve successful strict glycemic control, blood glucose measurement must be performed at least every hour. Since around 0.5 mL of blood is required for a single blood glucose measurement, a total of 12 mL of blood in point-of-care blood glucose testing must be collected daily. When blood glucose control is unstable, more frequent measurements are needed and approximately 20 mL of blood must be collected daily solely for blood glucose measurement. This becomes a considerable burden for patients with anemia and for

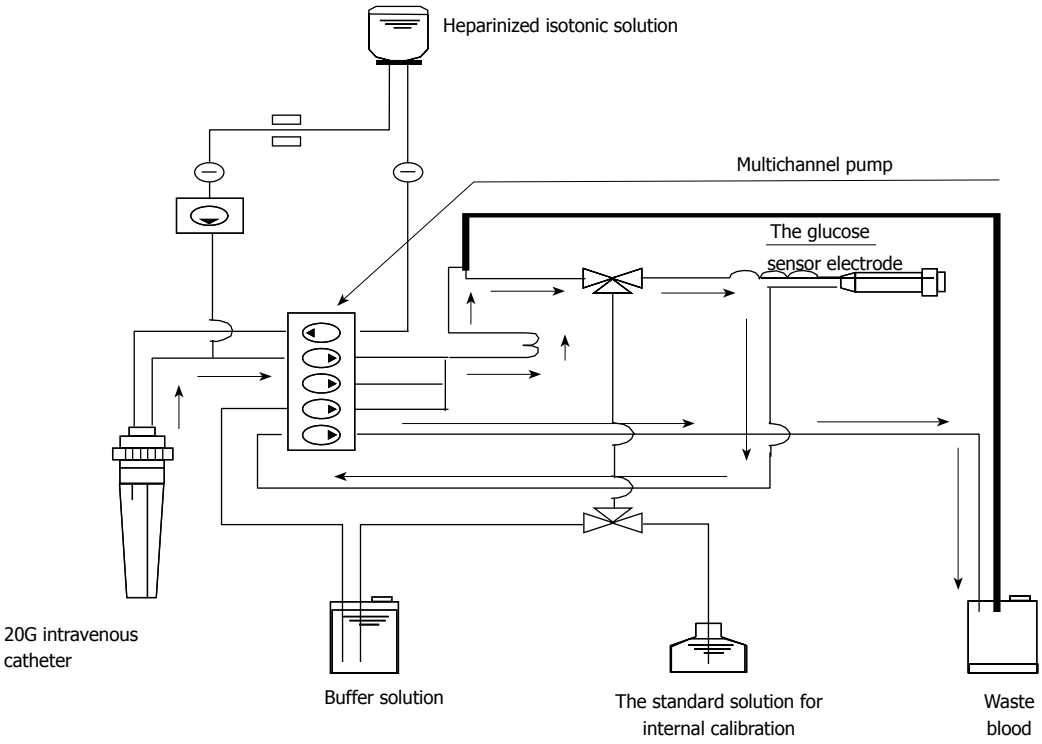

Figure 1 The whole circuit of the STG-22™. Arrows indicate the direction of blood sampling.

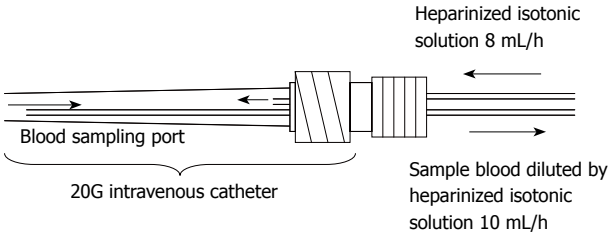

Figure 2 This figure shows the dual lumen catheter technique.

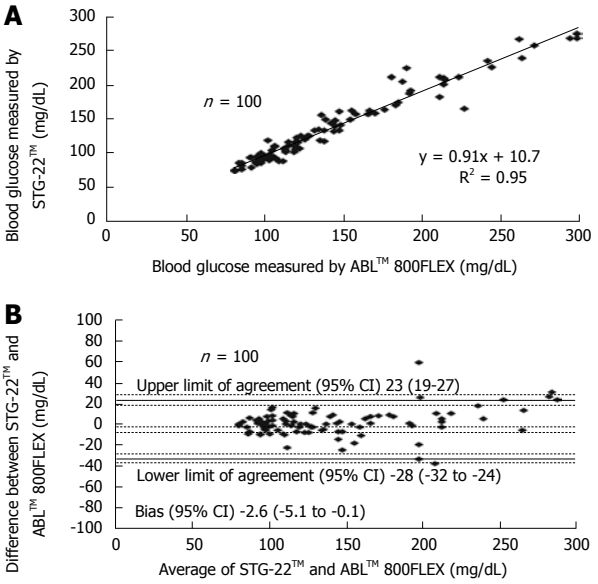

Figure 3 Scatter plot of individual blood glucose levels measured by both STG-22™ and ABL™ 800FLEX during surgery[20].

pediatric patients, and thus the development of blood glucose measurement technologies requiring no blood collection is greatly anticipated.

ACCURACY OF BLOOD GLUCOSE MEASUREMENT RESULTS

Measurement errors become an issue during strict glycemic control for maintaining blood glucose at a level between 80 and 110 mg/dL. According to the standards established by the International Organization for Standardization, the errors defined for glucose concentrations of 4.1 mmol (74 mg/dL) or above must not be greater than 20%, and those for glucose concentrations of 4.1 mmol (74 mg/dL) or below must not be greater than ± 0.8 mmol (14 mg/dL), and these values are not guaranteed by the accuracy of portable blood glucose measurement systems. STG-22™, which is currently being used for a clinical study by our group, is a highly accurate and reliable system. Its measurement errors are below 21% during intraoperative measurement[20] (Figure 3) and not more than 15% during postoperative measurements[21] (Figures 4-6). These findings suggest that STG-22™ can sufficiently perform closed loop control of blood glucose based on measurement results.

ACCURACY OF INSULIN DOSING ALGORITHMS

Sliding scale dosing of insulin, described previously as a special feature, is not described here. A program that calculates insulin dosage based on the blood glucose level, rate of its change and target blood glucose level

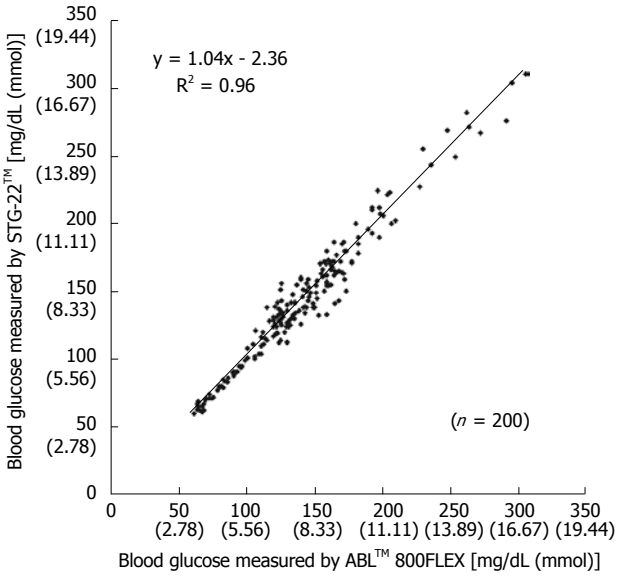

Figure 4 Scatter plot of individual blood glucose levels measured by both STG-22™ and ABL™ 800FLEX in post-surgical patients[21].

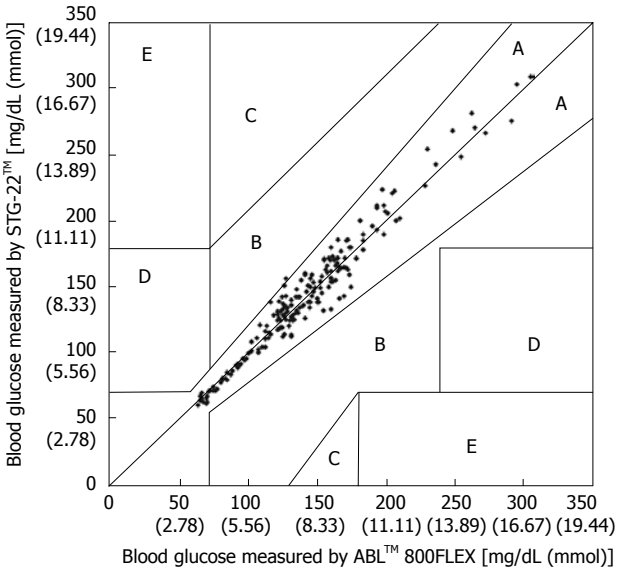

Figure 5 Error grid analysis for evaluation of blood glucose measured by STG-22™ compared with those measured by ABL™ 800FLEX. Zone A, B: Accurate or acceptable; Zone C: Unnecessary corrections that could lead to a poor outcome; Zone D: Dangerous failure to detect and treat; Zone E: 'Erroneous treatment'[21].

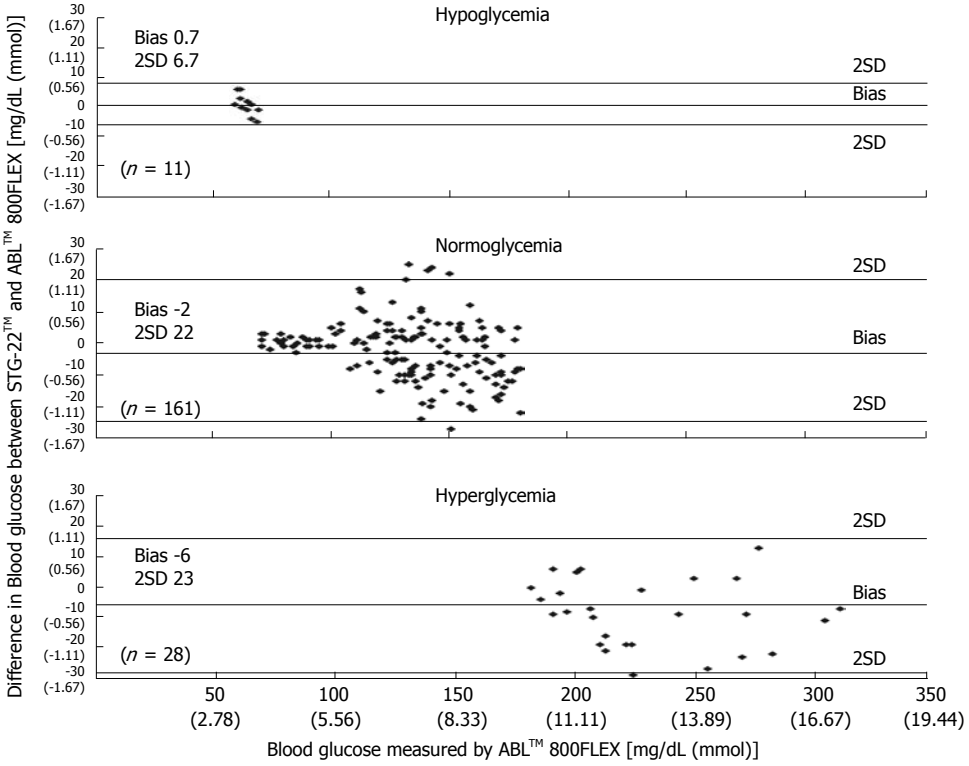

Figure 6 Bland-Altman plot of blood glucose measurements from STG-22™ and ABL™ 800FLEX[21].

is expected to be used widely in clinical applications, since such a program has been reported to decrease the incidence of hypoglycemia by approximately 0.3%[22]. On the other hand, STG-22™ is the only system equipped with a closed-loop algorithm that enables continuous measurement of blood glucose levels and provides updated results. This system utilizes Shichiri's algorithm, which calculates insulin dosage on the basis of the fact that glucose-stimulated insulin secretion is based on the proportional-derivative action[23]. The results of our previous studies have proven that use of STG-22™ achieves good postoperative glycemic control among patients who received surgical treatments[24,25]. Here, we aim to further evaluate the applicability of this system to intraoperative glycemic control.

NEW BLOOD GLUCOSE MEASUREMENT TECHNOLOGY

A continuous noninvasive blood glucose monitoring system is an ideal instrument for strict glycemic control. Pasic *et al*[26] previously developed a blood glucose measurement system by combining microdialysis and an optic fiber, and they succeeded in performing continuous *in vitro* measurement for 3 d. Maruo *et al*[27] have also developed a near-infrared based blood glucose monitoring system, and its application to patients in the intensive care unit showed a good outcome. However, these systems present disadvantages in terms of convenience and accuracy, making strict glycemic control difficult. Further improvements to these systems are needed.

NEW INSULIN DOSING TECHNOLOGIES

Recently, an insulin inhalant has been approved in the US and Europe. In terms of drug assimilation, the lung possesses a large surface area for drug absorption. Moreover, the rate of solute absorption is fast in the alveolar epithelium, and mucosal clearance in the alveoli is slower than that in the bronchi. Therefore, the lung is a very suitable route for administering high-molecular-weight insulin. Because of the reasons mentioned above, the lung can therefore possibly be used as a new route for administering drugs when peripheral circulation is impaired. In support of this, Barnett *et al*[28] have reported that long-term inhaled insulin therapy has no adverse effects on pulmonary function and thus its wide application is expected in the future.

CONCLUSION

A highly accurate continuous blood glucose monitoring system and closed-loop control of blood glucose are essential for anesthesiologists to achieve strict intraoperative glycemic control. STG-22™ is the only system that performs both functions; however, it requires 50 mL of blood per day for continuous blood glucose monitoring, and the collection of such a volume is invasive and remains laborious. With technological progress, the development of blood glucose monitoring systems with improved convenience, accuracy and minimal invasiveness are anticipated in the future.

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TOPIC HIGHLIGHT

Kazuhiro Hanazaki, MD, Professor and Chairman, *Series Editor*

Blood glucose control in patients with severe sepsis and septic shock

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Abstract

The main pathophysiological feature of sepsis is the uncontrollable activation of both pro- and anti-inflammatory responses arising from the overwhelming production of mediators such as pro- and anti-inflammatory cytokines. Such an uncontrollable inflammatory response would cause many kinds of metabolic derangements. One such metabolic derangement is hyperglycemia. Accordingly, control of hyperglycemia in sepsis is considered to be a very effective therapeutic approach. However, despite the initial enthusiasm, recent studies reported that tight glycemic control with intensive insulin therapy failed to show a beneficial effect on mortality of patients with severe sepsis and septic shock. One of the main reasons for this disappointing result is the incidence of harmful hypoglycemia during intensive insulin therapy. Therefore, avoidance of hypoglycemia during intensive insulin therapy may be a key issue in effective tight glycemic control. It is generally accepted that glycemic control aimed at a blood glucose level of 80-100 mg/dL, as initially proposed by van den Berghe, seems to be too tight and that such a level of tight glycemic control puts septic patients at increased risk of hypoglycemia. Therefore, now many researchers suggest less strict glycemic control with a target blood glucose level of 140-180 mg/dL. Also specific targeting of glycemic control in diabetic patients should be considered. Since there is a significant correlation between success rate of glycemic control and the degree of hypercytokinemia in septic patients, some countermeasures to hypercytokinemia may be an important aspect of successful glycemic control. Thus, in future, use of an artificial pancreas to avoid hypoglycemia during insulin therapy, special consideration of septic diabetic patients, and control of hypercytokinemia should be considered for more effective glycemic control in patients with severe sepsis and septic shock.

Key words: Blood glucose; Diabetes mellitus; Insulin; Hypercytokinemia; Inflammation mediators

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INTRODUCTION

There are many pathophysiological changes during severe sepsis and septic shock, and one of the most striking is metabolic derangement. Among the metabolic changes, hyperglycemia is the most important[1,2]. Accordingly therapeutic approaches to hyperglycemia in the management of severe sepsis and septic shock have had much attention. Intensive insulin therapy became popular in the intensive care unit (ICU) after Van den Berghe's research reporting its effectiveness on glycemic control[3,4]. However, a recent large-scale randomized trial indicated that such glycemic control is not effective in reducing ICU mortality and that glycemic control with intensive insulin therapy increases the risk of hypoglycemia, and complications arising from hypoglycemia[5]. Therefore, in this paper we will discuss the effectiveness of intensive insulin therapy in the ICU and the future perspectives on tight glycemic control from the viewpoint of the correlation between inflammatory hypercytokinemia and hyperglycemia.

METABOLIC CHANGE IN SEPSIS

Recent advances in molecular biology have contributed to the tremendous progress in understanding the pathophysiology of sepsis. Now it is widely accepted that the main features of sepsis are the uncontrollable activation of not only pro-inflammatory, but also anti-inflammatory responses, because of overwhelming production of pro-inflammatory and anti-inflammatory mediators[6-8]. Such overwhelming production of mediators causes many pathological changes in vital organs and systems including metabolic changes[9]. One such metabolic change is hyperglycemia arising from muscle glycolysis and lipolysis, and subsequent gluconeogenesis and glycolysis in the liver[9-12]. The other feature of metabolic change in sepsis is hyperlactatemia due to glycolysis in muscle caused by counterregulatory hormones and cytokines, sometimes referred to as the "lactate shuttle"[9,13]. Hyperglycemia in critical illness, such as severe sepsis, is not only a marker of severity of illness and the predictor of poor outcome[1,2], but also has many kinds of adverse effects on vital organs. One such adverse effect on the innate immune system impairs the ability of the host to combat infection, resulting in reduced neutrophil activity such as chemotaxis, formation of reactive oxygen species, and phagocytosis of bacteria despite accelerated diapedesis of leukocytes into peripheral tissue, as well as specific alterations in cytokine patterns, with increased concentrations of the early proinflammatory cytokines, tumor necrosis factor-α and interleukin (IL)-6, and a reduction of endothelial nitric oxide formation[14]. Recently it has also been reported that the variability of the glucose level in blood is independently associated with hospital mortality in septic patients[15,16] and that severity of sepsis has a strong effect on glycemic variability in blood[13].

Without question, treatment of severe sepsis and septic shock starts with control of the infection source. Antibiotics, drainage of abscesses, and operations to control the source, when indicated, are essential in the initial treatment[17]. Furthermore, hemodynamic stabilization is also very important for the initial treatment of such patients[18].

On the other hand, an epoch-making paper by Van den Berghe in 2001 reported that tight glycemic control with intensive insulin therapy in the surgical ICU, to control the blood glucose level between 80 and 110 mg/dL, resulted in improvement in survival and a shortened length of hospital stay[3]. The same authors reported later that intensive insulin therapy reduced morbidity but not mortality in the medical ICU[4]. These studies[3,4] led the influential guidelines for the management of severe sepsis and septic shock to recommend tight glycemic control as one of the most important therapeutic approaches[19]. Since the publication of the guidelines, tight glycemic control in ICU patients has become popular and it is now one of the standard clinical practices in the ICU. This recommendation remained the same in the revised version of the guidelines published in 2008[20].

CLINICAL EFFECT OF GLYCEMIC CONTROL IN SEPTIC PATIENTS

Since the publication of the papers by Van den Berge[3,4], indicating that tight glycemic control between 80 and 110 mg/dL with intensive insulin therapy reduces morbidity and mortality among critically ill patients in the surgical ICU, and that the intensive insulin therapy significantly reduces morbidity but not mortality among all patients in the medical ICU, many secondary clinical trials on tight glycemic control have been carried out. A meta-analysis and systematic reviews on tight glycemic control were also published. In a review, the Van den Berghe group reconfirmed that maintaining strict normoglycemia with the use of intensive insulin improves the outcome of critically ill patients[21]. They also published a paper in which they concluded that intensive insulin therapy reduced mortality of all medical/surgical ICU patients, except those with a prior history of diabetes, that intensive insulin therapy did not cause harm, and that a blood glucose target < 110 mg/dL was most effective, but also carried the highest risk of hypoglycemia[22].

On the other hand, Brunkhorst and the SepNet group from Germany published a paper which found that use of intensive insulin therapy placed critically ill patients with sepsis at increased risk for serious adverse events related to hypoglycemia, without showing any benefit, and they stopped the trial for safety reasons[23]. Treggiari and colleagues also showed that a policy of intensive insulin therapy in a group of ICU patients was not associated with a decrease in hospital mortality, and they concluded that further study was needed prior to widespread implementation of intensive insulin therapy in critically ill patients[24]. The meta-analysis by Wiener and colleagues published in JAMA concluded that, in critically ill adult patients, tight glucose control is not associated with significantly reduced hospital mortality, but that it is associated with an increase risk of hypoglycemia[25]. Finally, the long-awaited result of the NICE-SUGAR (Normoglycemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulator) trial was published recently. This concluded that intensive glucose control increased mortality among adults in the ICU and that a blood glucose target of 180 mg/dL or less resulted in lower mortality than did a target of 81-108 mg/dL[5]. Furthermore it was also pointed out that intensive insulin therapy increased the labor requirement of nursing staff in the ICU[26]. Now the statement by Merz and Finfer, that each ICU should define a blood glucose range which can be achieved without causing a significant increase in severe hypoglycemia, and which fits within the constraints of their nursing and economic resources sounds fair and acceptable[26]. They also concluded that the upper limit of glucose control should currently be 140-180 mg/dL[26]. Preiser also suggested that a blood glucose range of 80-110 mg/dL may not be normal or desirable and that lowering of blood glucose by intensive insulin therapy can induce a shortage in the provision of glucose, the predominant source of energy useable by the

myocardium during ischemia, and neuroglycopenia in cases of cerebral injury[27].

MECHANISM OF BENEFICIAL OR HARMFUL EFFECTS OF TIGHT GLYCEMIC CONTROL

There are many papers which illustrate the mechanism of the beneficial effect of tight glycemic control. Wade tell us that hyperglycemia can alter cytokine production and phagocytosis both by means of hyperosmotic stress and by mechanisms other than hyperosmolality[28]. Egi from Bellomo's group suggested that variability of glucose concentration is a significant independent predictor of ICU and hospital mortality, and that decreasing the variability of blood glucose concentration might be an important aspect of glucose management[29]. The question whether intensive insulin therapy *per se* or a lowered glucose level by intensive insulin therapy is the main mechanism of the beneficial effect of tight glycemic control with intensive insulin therapy has not yet been answered.

On the other hand, Jeschke *et al*[30] reported that insulin therapy improves the systemic inflammatory reaction to severe trauma. Vanhorebeek from Van den Berghe's group reported that protection of hepatocyte mitochondrial ultrastructure and function is one of the mechanisms of the beneficial effect of strict blood glucose control with insulin in critically ill patients[31]. Another researcher from Van den Berghe's group also reported that intensive insulin therapy prevented critical polyneuropathy/myopathy and the necessity for treatment with prolonged mechanical ventilation[32,33]. Dugo and colleagues showed, in an experimental study, that the inhibitory effect of insulin on the activity of glycogen synthase kinase-3β, contributed to the protective effect of insulin against organ injury/dysfunction caused by excessive systemic inflammation, independently of any effects on blood glucose[34]. Another possible mechanism of the beneficial effect of intensive insulin therapy or tight glycemic control is through RAGE (receptor of advanced glycation end product). Now RAGE is considered to play an important role in the pathophysiology of severe sepsis and septic shock. It is possible that tight glycemic control can reduce the production of AGE (advanced glycation end product), and that tight glycemic control can thereby reduce the inflammatory response mediated through AGE and RAGE interaction[35]. This could be another mechanism of the beneficial effect of tight glycemic control in severe sepsis.

There are many papers reporting that hypoglycemia during intensive insulin therapy is the main reason why intensive insulin therapy cannot show a beneficial effect on mortality in severe sepsis and septic shock. Waeschle *et al*[16] showed that the risk of hypoglycemia with intensive insulin therapy is very high among patients with severe sepsis and septic shock. The meta-analysis mentioned above on the benefit and risks of tight glucose control in critically ill adults by Wiener[24], indicated that intensive insulin therapy increased the risk of hypoglycemia among critically ill patients including those with severe sepsis and septic shock. Krinsley and Grover indicated that even a single episode of severe hypoglycemia was independently associated with increased risk of mortality and therefore that safe implementation of tight glycemic control requires appropriate monitoring to reduce the risk of this complication[36]. They proposed to move beyond tight glucose control to safe effective glucose control avoiding hypoglycemia[37]. The mechanism by which hypoglycemia increased mortality in severe sepsis and septic shock has not yet been fully elucidated. However, brain damage because of an energy deficit in the brain through hypoglycemia is possibly one mechanism[38].

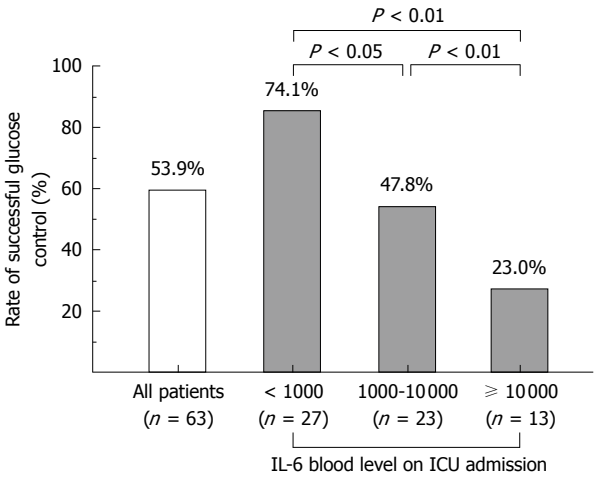

Figure 1 Correlation between IL-6 blood level on ICU admission and success rate of blood glucose control among patients with severe sepsis and septic shock. The blood glucose level is targeted to be between 110 and 150 mg/dL with intensive insulin therapy.

FUTURE PERSPECTIVES ON TIGHT GLYCEMIC CONTROL

It is now clear that to benefit from tight glycemic control, we should avoid hypoglycemia. On the other hand it has also become clear that it is not so easy to keep the blood glucose level within the targeted range even though the targeted range is not very tight such as between 150 and 180 mg/dL in patients with severe sepsis and septic shock.

In our ICU, we routinely check IL-6 blood levels in every patient every day. The reason why we specifically measure IL-6 is not that we think IL-6 is the most important proinflammatory cytokine, but rather that IL-6 is the most easily measurable cytokine because of its relatively high blood level and relatively long half-time in the blood[39]. Since blood levels of many kinds of cytokines change synergistically in sepsis, we do not need to measure blood levels of various cytokines to make a diagnosis of hypercytokinemia but we can measure only one of the most easily measurable cytokines to make the diagnosis of hypercytokinemia in

sepsis. It is reported that if a patient has an IL-6 level of more than 1000 pg/mL, this patient can be diagnosed with systemic inflammatory response syndrome or hypercytokinemia[40].

We applied tight glycemic control with a target blood glucose level of 110-150 mg/dL in patients with severe sepsis and septic shock. The overall success rate for tight glycemic control was only 53.9%. However when we subgrouped the patients according to the IL-6 blood level on ICU admission, we found that the success rate of tight glycemic control was relatively high in subgroups whose IL-6 blood level on ICU admission was lower than 1000 pg/mL. On the other hand, the success rate of tight glycemic control was very low among the patients whose initial IL-6 blood level in the ICU was higher than 10000 pg/mL as shown in Figure 1. These data indicate that hypercytokinemia correlates with hyperglycemia in sepsis and that countermeasures to hypercytokinemia in sepsis would be one of the key factors for successful glycemic control. Recently, we published a paper showing the efficacy of continuous hemodiafiltration (CHDF) with a cytokine-adsorbing hemofilter made from polymethyl methacrylate (PMMA) membrane in patients with septic shock[41]. We found that, in septic patients with severe hypercytokinemia, blood glucose control became easier once we lowered the blood level of cytokines with PMMA-CHDF.

Since one of the reasons why tight glycemic control could not show a beneficial effect on critically ill patients is hypoglycemia during intensive insulin therapy, avoidance of hypoglycemia should always be considered[36,37]. For this purpose an artificial pancreas has promising potential[42]. On the other hand, a pharmacological dose of a steroid is recommended in the guidelines for the management of severe sepsis and septic shock[18,19]. However, steroid administration may create some difficulties in glycemic control in septic patients[43]. An artificial pancreas may be most effective on such patients with severe sepsis and septic shock receiving steroid therapy.

Another important issue of tight glycemic control in critically ill patients, including those with severe sepsis and septic shock, is whether tight glycemic control would also be effective in critically ill diabetics. It is proposed that precisely defined target glucose levels, treatment intervention and the avoidance of hypoglycemic episodes during insulin therapy should be studied before the widespread application of tight glycemic control in critically ill diabetic patients[44]. Egi and colleagues reported that, unlike nondiabetic patients, diabetic patients showed no clear association between hyperglycemia during the ICU stay and mortality, and there were markedly lower odds ratios of death at all levels of hyperglycemia suggesting that hyperglycemia may have different biological and/or clinical implications in critically patients with diabetes mellitus[45].

As mentioned above, it has not yet been fully elucidated whether intensive insulin therapy or normoglycemia with intensive insulin therapy really has the beneficial effect on severely septic patients. However, if normoglycemia is the key mechanism of tight glycemic control with intensive insulin therapy, modulation of glucose use and gluconeogenesis in sepsis with adrenergic β receptor blockade is one of the future approaches in this area[46].

CONCLUSION

It is now suggested that tight glycemic control with a target blood glucose level of 90-110 mg/dL does not improve clinical outcome and that less strict glycemic control with a target blood glucose level of 140-180 mg/dL is more effective. Also specific targeting of glycemic control in diabetic patients should be considered. Since there is a significant correlation between success rate of glycemic control and the degree of hypercytokinemia in septic patients, some countermeasures to hypercytokinemia may be an important aspect of successful glycemic control. Thus, in future, use of an artificial pancreas to avoid hypoglycemia during insulin therapy, special consideration of septic diabetic patients, and control of hypercytokinemia should be considered for more effective glycemic control in patients with severe sepsis and septic shock.

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TOPIC HIGHLIGHT

Kazuhiro Hanazaki, MD, Professor and Chairman, *Series Editor*

Problems associated with glucose toxicity: Role of hyperglycemia-induced oxidative stress

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Abstract

Glucose homeostasis deficiency leads to a chronic increase in blood glucose concentration. In contrast to physiological glucose concentration, chronic super-physiological glucose concentration negatively affects a large number of organs and tissues. Glucose toxicity means a decrease in insulin secretion and an increase in insulin resistance due to chronic hyperglycemia. It is now generally accepted that glucose toxicity is involved in the worsening of diabetes by affecting the secretion of β-cells. Several mechanisms have been proposed to explain the adverse effects of hyperglycemia. It was found that persistent hyperglycemia caused the functional decline of neutrophils. Infection is thus the main problem resulting from glucose toxicity in the acute phase. In other words, continued hyperglycemia is a life-threatening risk factor, not only in the chronic but also the acute phase, and it becomes a risk factor for infection, particularly in the perioperative period.

Key words: Glucose toxicity; Diabetes; Complication; Surgery; Surgical site infection; Emergency medicine; Critical care medicine

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INTRODUCTION

Chronic hyperglycemia is a characteristic of the diabetic condition, while glucose toxicity is the main cause of diabetic complications, which are often observed only several years after the beginning of the illness[1]. Glucose toxicity, in its narrow sense, can indicate a clinical condition where control of diabetes in particular is poor, since hyperglycemia itself reduces the insulin secretion capacity of pancreatic β-cells, and the resultant increase in insulin resistance leads to further hyperglycemia. This vicious circle finally leads to the total incapacity of β-cells to secrete insulin[2,3].

On the other hand, acute hyperglycemia, similar to chronic hyperglycemia, is known to cause injury to many organs. Hyperglycemia in the acute phase causes the functional decline of neutrophils, and is a risk factor that causes infection in the perioperative period. In the first half of this review, we will present an introduction to the various mechanisms known to be involved in the control of glucose homeostasis and in the development of glucose toxicity. In the latter half, diabetic complications (chronic and acute) and implications for the fields of surgery, emergency and critical care medicine will be presented and discussed.

GLUCOSE HOMEOSTASIS

Glucose homeostasis is maintained by the highly coordinated interaction of three physiologic processes: insulin secretion, tissue glucose uptake and hepatic glucose production. In this way, the body tries to maintain a constant supply of glucose for cells by keeping glucose concentration in the blood constant. Normal glucose homeostasis represents the balance between intake (glucose absorption from the gut), tissue utilization (glycolysis, pentose phosphate pathway activity, tricarboxylic acid cycle activity, glycogen synthesis) and endogenous production (glycogenolysis and gluconeogenesis)[4]. The most important metabolic fuels are glucose and fatty acids. Glucose is preferentially used by brain and muscles,

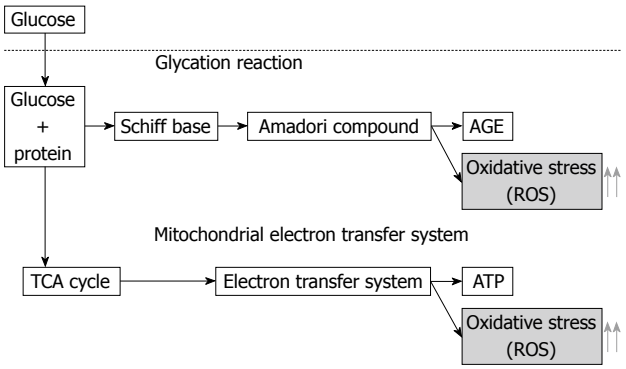

Figure 1 Increase in oxidative stress in the diabetic state. Acceleration of glycation response and the intramitochondrial electron transfer system was detected in the diabetic state, causing oxidative stress as the responses accelerated. Black arrows indicate pathway, grey arrows indicate increase or decrease. AGE: Advanced glycosylation end products; ROS: Reactive oxygen species; TCA cycle: Tricarboxylic acid cycle; ATP: Adenosine tri-phosphate.

and to ensure a continuous supply of glucose to the brain and other tissues, metabolic fuels are stored for use in time of need. Glucose homeostasis is controlled primarily by the anabolic hormone insulin and also by some insulin-like growth factors[5]. Several catabolic hormones (glucagons, catecholamines, cortisol, growth hormone, and adrenocorticotropic hormone) may antagonize the action of insulin and are known as anti-insulin or counter-regulatory hormones[6].

It is often found that critically ill patients incur hyperglycemia because of insulin resistance even if it is not complicated by diabetes[7]. When severe stress occurs, insulin resistance and an insulin secretion decrease result from the response to stress by the neuroendocrine system because secretion of anti-insulin hormones is enhanced. This leads to enhancement of glucogenesis in the liver, of lipolysis in adipose tissue, and of protein catabolism in skeletal muscle. This is known as surgical diabetes. Patients with diabetes are more susceptible to stress and easily develop exacerbation of diabetes, resulting in an increase in the incidence of complications.

MECHANISM OF GLUCOSE TOXICITY

It has been found that oxidative stress is associated with the molecular mechanism of the decreased insulin biosynthesis and secretion, which is the main etiology of glucose toxicity. Because pancreatic islet cells show extremely weak manifestation of antioxidative enzymes[8,9], it is thought that the pancreas may be more susceptible to oxidative stress than other tissues and organs.

Pathway of oxidative stress production

Metabolic reactions continuously produce reactive oxygen species (ROS), such as superoxides (O_2^-), hydroxyl radicals (OH^-), peroxyl radicals (ROO^-) or nitric oxide. ROS are involved in a diversity of biological phenomena, such as inflammation, carcinogenesis, aging, and atherosclerosis. However, several antioxidant enzymes help to maintain low levels of ROS. Oxidative stress corresponds

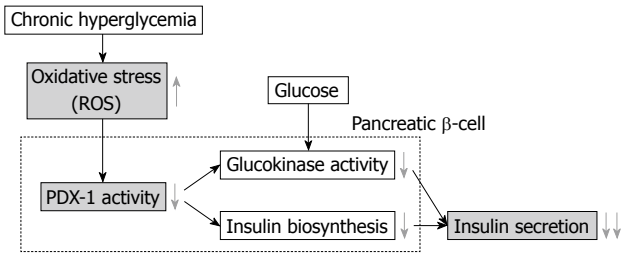

Figure 2 The mechanism of insulin secretion reduction due to glucose toxicity. DNA binding capacity of PDX-1 decreases as a result of oxidative stress caused by hyperglycemia, while insulin biosynthesis and secretion also decrease. Black arrows indicate pathway, grey arrows indicate increase or decrease. PDX-1: Pancreatic duodenal homeobox-1.

to the overproduction of ROS that can damage cellular components, such as lipids, proteins or DNA[10]. There are strong indications that oxidative stress may be a key event in diabetic complications[11,12]. It is reported that 8-hydroxy-2-deoxyguanosine (8-OHdG), which is an indicator of oxidative damage of DNA, increases in patients with type 2 diabetes mellitus[13], and that 8-OHdG, 4-hydroxy-2-nonenal and heme oxygenase-1, all oxidative stress markers, increase in the pancreatic islet cells of type 2 diabetes mellitus animal models[14,15]. Generation of ROS in diabetes seems to be directly linked to chronic hyperglycemia. Various pathways are thought to be involved in the increase in oxidative stress in a hyperglycemic state (Figure 1). With the first pathway, more oxidative stress is caused by hyperglycemia because a non-enzymatic glycosylation reaction (glycation) is enhanced in the hyperglycemic state. ROS are then generated by the Amadori compound, which is an intermediate metabolite, before leading to the production of metabolites, known as advanced glycosylation end products (AGE) as the result of a glycation reaction[16]. Another pathway involves the mitochondrial electron transfer system, which also becomes a source of oxidative stress. This system is located in the mitochondrial inner membrane, where Adenosine tri-phosphate is produced as an important organic energy source. In this electron transfer system, water molecules are generated by deoxidation of four of the electrons of oxygen molecules, ROS is produced as an intermediate product in this process, and some of the ROS leak out from this system. Part of the oxygen used for this process is produced as superoxide anions even under physiological conditions, and their production increases in the hyperglycemic state[17]. Furthermore, the hexosamine pathway also becomes a source of oxidative stress. It was found that glucosamine, which is an intermediate metabolite in this process, also brings about oxidative stress[18]. Because this hexosamine pathway is enhanced in the diabetic state, the oxidative stress thus generated will increase.

Reduction of insulin biosynthesis and secretion by oxidative stress

Figure 2 shows the mechanism of the reduction of insulin secretion by oxidative stress. It is known that biosynthesis

of insulin decreases when pancreatic β-cells are exposed to chronic hyperglycemia in animal models of type 2 diabetes mellitus, and a similar phenomenon was induced by oxidative stress caused in the diabetic state. In other words, promoter activity of the insulin gene and mRNA expression decrease, and insulin gene expression is thus inhibited, when the β-cell line and isolated pancreatic islet cells are exposed to oxidative stress. It was also found that the DNA binding capacity of Pancreatic duodenal homeobox-1 (PDX-1), which is a very important transcription factor for insulin genes, decreases[18,19]. Finally, as for the control of glucokinase transcription by PDX-1, it is reported the promoter activity, manifestation, and the enzyme activity of glucokinase decreases with oxidative stress[20]. In fact, the use of antioxidant drugs resulted in an improvement of insulin secretion capacity as well as an increase in insulin mRNA expression[21]. On the other hand, it was also found that oxidative stress and activation of the c-Jun N-terminal kinase pathway are involved in a decline in insulin biosynthesis and secretion due to chronic hyperglycemia[22].

Increase in insulin resistance caused by oxidative stress

Glucotoxicity not only affects the secretion of pancreatic hormones but also participates in insulin resistance of insulin-sensitive tissues, which include liver, skeletal muscle, and adipose tissue. Insulin resistance has been shown to be present before the onset of chronic hyperglycemia, although the latter may contribute to aggravation of the diabetic state by increasing insulin resistance[23].

Oxidative stress is also strongly suspected to be involved in chronic hyperglycemia-induced insulin resistance[24]. Indeed, it is known that incubation of primary adipocyte cells with chronic high glucose concentration can induce oxidative stress[25]. Moreover, it was demonstrated that oxidative stress induces insulin resistance in the 3T3-L1 adipocyte cell line by inhibiting the translocation of Glut 4 to the plasma membrane[26]. Finally, it was found that oxidative stress can induce insulin resistance in intact rat muscle[27].

GLUCOSE TOXICITY AND DIABETIC COMPLICATIONS

The prevalence of diabetes mellitus is increasing worldwide at an alarming rate due to population growth, obesity, sedentary life style and aging. Consequently, diabetic complications are also on the increase. Prevention and treatment of complications are considered to be most important for general care of diabetic patients. The basic causes of complications include tissue metabolism disorders caused by chronic hyperglycemia, which results in damage to many organs. The main diabetic complications are listed in Table 1. They are divided into chronic and acute complications based on the disease course.

Table 1 Types of diabetic complications

Chronic complications
Microvascular diseases: retinopathy, neuropathy, nephropathy
Macrovascular diseases: aortic sclerosis, stroke, myocardial infarction, angina pectoris, obstructive peripheral vascular disease, *etc*
Others: cataract, dermatopathy, hypertension, osteopenia, osteomalacia, arthropathy, soft tissue fibromatosis, *etc*
Acute complications
Diabetic coma: ketoacidotic coma, non-ketotic hyperosmolar coma, lactic acidosis
Acute infection: bacterial, mycotic, viral, *etc*

Chronic complications

When metabolic disorders due to diabetes continue for many years, vascular tissue is affected the most. Chronic complications are divided into microvascular diseases that are specific to and common in diabetes and macrovascular diseases that are not specific but frequent and thus important for a prognosis. Vascular endothelial cell function in the blood vessels of diabetic patients is impaired, and many basic research endeavors have demonstrated that vasodilative reaction is also impaired[28,29]. Hyperglycemia-induced oxidative stress is also involved in the development of both macrovascular and microvascular diabetic complications[30].

Chronic hyperglycemia can induce microvascular complications such as retinopathy, neuropathy or nephropathy[31]. The retina is highly sensitive to oxidative stress since it has higher oxygen uptake and glucose oxidation than any other tissue[32]. Studies of diabetic rat retina and retinal cells incubated with a high-concentration of glucose have shown that the concentration of superoxides is elevated[33]. It has been demonstrated in animal models that oxidative stress is not only involved in the development of retinopathy but also in the persistence of the pathology after normalization of glucose concentration, probably as the result of persistent ROS[34]. Oxidative stress is also strongly suspected to be involved in the development of diabetic neuropathy[35]. Several studies have shown the capacity of antioxidant enzymes to prevent or reverse the toxic effect of chronic hyperglycemia in the nerves[36]. Moreover, oxidative stress may contribute to the pathogenesis of diabetic nephropathy since the presence of high concentrations of mitochondrial oxidative stress markers has been demonstrated in the urine and kidneys of diabetic rats[37].

Chronic hyperglycemia can also induce macrovascular complications. Cardiovascular complications are the most prevalent cause of death in diabetic patients. Moreover, it has been clearly shown that chronic hyperglycemia during diabetic and pre-diabetic states is linked to an increased risk for the development of cardiovascular diseases[38]. Long-term incubation of macrovessels with high-concentration glucose was found to strongly increase the risk of cardiovascular, cerebrovascular and peripheral arterial diseases[39]. Activation of protein kinase C by hyperglycemia is thought to play a central role in vascular complications since it leads to: (1) modification

of contractile protein function, (2) an increase in the activity of nitric oxide synthase, and (3) activation of the angiotensin-converting enzyme (ACE). Activation of ACE has been linked with apoptosis and necrosis of cardiomyocytes and endothelial cells[40]. The importance of ACE in cardiovascular disease development was confirmed by studies showing the inhibition of ACE can protect against cardiovascular diseases[41]. Finally, protein glycation is another factor probably involved in the development of cardiovascular diseases[42].

Acute complications

Hyperglycemia can exacerbate a number of perioperative problems, including cardiac, neurologic, and infectious complications. In general, most outcomes tend to improve with treatment of hyperglycemia[43]. Diabetic coma is a form of consciousness disturbance that is characteristic of diabetes in acute complications. Diabetic coma has three categories, ketoacidotic coma due to hyperglycemia, non-ketotic hyperosmolar coma without ketoacidosis, and, rarely, lactic acidosis.

Infection in acute complications is a clinical condition that is not specific to but can easily become complicated in diabetic states. Diabetic patients have reduced immune function and enhanced bactericidal activity, so that special attention is required since the infection focus expands much faster than in non-diabetic patients. This becomes a problem particularly in the fields of surgery, emergency and critical care medicine.

Association between hyperglycemia and infection

It has been confirmed that perioperative appropriate glycemic control promotes wound healing. Perioperative infectious complications, including surgical site infection, represent serious postoperative complications. It is well known that, compared with non-diabetic patients, diabetic patients suffer from an increased incidence of perioperative infections, especially surgical site infection. It was reported that patients with preoperative elevation of HbA1c levels show a significantly higher incidence of surgical site infection than patients with normal HbA1c levels[44,45].

Recent basic researches have found that the functional decline of neutrophils is caused by a hyperglycemic state[46], and that the mechanism of this decline includes increased adhesive capacity and diminished chemotaxis, phagocytic activity and bactericidal capacity[47,48]. Neutrophilic function is reduced in proportion to an increase in the blood glucose level, and 200 mg/dL is assumed to be the threshold of neutrophil dysfunction. Furnary *et al*[49] reported that the incidence of deep sternal wound infection decreased from 2.0% to 0.8% in a patient group whose blood glucose level was kept below 200 mg/dL by insulin administration, and there are other reports of reduced infectious risk due to strict glycemic control[50]. Maintenance of a perioperative hyperglycemic state, since the stress response is a risk factor of postoperative infection, is extremely important for postoperative infection prophylaxis.

CONCLUSION

Several recent clinical studies have demonstrated the efficacy of strict glycemic control for reducing the mortality rate of post-operative or emergency patients[51-53]. It was thought that perioperative strict glycemic control was essential to relieve a perioperative inflammatory response and improve patient outcome. However, despite frequent blood glucose testing, it has been shown that intensive insulin therapy is sometimes difficult to perform when using sliding-scale manual insulin injection. Consequently, hypoglycemic events could not be avoided during intensive insulin therapy with intermittent blood glucose sampling. It has therefore been suggested that continuous blood glucose monitoring would be beneficial for maintaining target blood glucose levels[54,55]. We also believe that it is necessary to establish more accurate glycemic control methods because, in view of the global increase in diabetes, it is expected that glycemic control in surgical and emergency settings will become increasingly important.

Recently the usefulness of a closed-loop system (artificial endocrine pancreas), which provides continuous monitoring and strict control of blood glucose, was reported. STG-22™ (Nikkiso, Tokyo, Japan) is a novel artificial endocrine pancreas with a closed-loop glycemic control system that provides continuous blood glucose monitoring through a glucose sensor electrode and subsequent automatic insulin and glucose infusion to maintain appropriate blood glucose levels[54-57]. The usefulness of a closed-loop system providing continuous monitoring and strict control of post-operative blood glucose in patients after hepatic resection was also reported[58]. Accurate and continuous blood glucose monitoring and close glycemic control may be possible with an artificial endocrine pancreas. Establishment of a new perioperative blood glucose control method with the aid of an artificial endocrine pancreas is urgently needed.

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ORIGINAL ARTICLES

Molecular determinants of the profibrogenic effects of endothelin-1 in pancreatic stellate cells

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Abstract

AIM: To gain molecular insights into the expression and functions of endothelin-1 (ET-1) in pancreatic stellate cells (PSC).

METHODS: PSCs were isolated from rat pancreas tissue, cultured, and stimulated with ET-1 or other extracellular mediators. Cell proliferation was assessed by measuring the incorporation of 5-bromo-2′-deoxyuridine into DNA and cell migration was studied in a transwell chamber assay. Gene expression at the level of mRNA was quantified by real-time Polymerase chain reaction. Expression and phosphorylation of proteins were monitored by immunoblotting, applying an infrared imaging technology. ET-1 levels in cell culture supernatants were determined by an enzyme immunometric assay. To study DNA binding of individual transcription factors, electrophoretic mobility shift assays were performed.

RESULTS: Among several mediators tested, transforming growth factor-β1 and tumour necrosis factor-α displayed the strongest stimulatory effects on ET-1 secretion. The cytokines induced binding of Smad3 and NF-κB, respectively, to oligonucleotides derived from the ET-1 promoter, implicating both transcription factors in the induction of ET-1 gene expression. In accordance with previous studies, ET-1 was found to stimulate migration but not proliferation of PSC. Stimulation of ET-1 receptors led to the activation of two distinct mitogen-activated protein kinases, p38 and extracellular signal-regulated kinases (ERK)1/2, as well as the transcription factor activator protein-1. At the mRNA level, enhanced expression of the PSC activation marker, α-smooth muscle actin and two proinflammatory cytokines, interleukin (IL)-1β and IL-6, was observed.

CONCLUSION: This study provides novel lines of evidence for profibrogenic and proinflammatory actions of ET-1 in the pancreas, encouraging further studies with ET-1 inhibitors in chronic pancreatitis.

Key words: Chronic pancreatitis; Endothelin-1; Fibrosis; Pancreatic stellate cells

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INTRODUCTION

Pancreatic fibrosis represents a key feature of chronic pancreatitis and pancreatic cancer. Fibrosis not only accompanies the tumour, but plays an active role in its progression[1,2]. The cellular and molecular basis of pancreatic fibrogenesis has therefore attracted significant interest in pancreatologists in recent years. In 1998, pancreatic stellate cells (PSC) were identified as the main source of extracellular matrix (ECM) proteins in the diseased pancreas[3,4]. In response to profibrogenic stimuli, PSC undergo phenotypic changes termed activation, which include proliferative activity, expression of myofibroblastic markers [such as α-smooth muscle actin (α-SMA)], and enhanced synthesis of ECM components. The activation process is also triggered when PSC are isolated from healthy pancreas and when cultured in cell culture dishes. Numerous extracellular mediators and intracellular pathways of PSC activation have been described since 1998, which are summarized in recent reviews[2,5]. Thus, platelet-derived growth factor

(PDGF) has been identified as a strong PSC mitogen, while transforming growth factor (TGF)-β1 is considered the most relevant stimulator of ECM synthesis[6,7]. In addition, proinflammatory cytokines, including tumour necrosis factor (TNF)-α, and ethanol metabolites, have been implicated in pancreatic fibrogenesis[7-9]. Despite many efforts, however, little progress has been made so far with respect to the development of therapeutic strategies to interrupt fibrogenesis in the context of chronic pancreatitis and pancreatic cancer. One reason is the lack of efficient antifibrotic drugs that are applicable in patients and not in experimental settings only.

Recent studies on fibroproliferative disorders in different organs have indicated an antifibrotic effect of endothelin (ET)-receptor antagonists[10]. Using bosentan, a clinically available dual ET-receptor antagonist targeting the two receptor types ETRA and ETRB, we recently observed inhibition of key functions of activated PSC, including proliferation and collagen synthesis[11]. When bosentan was applied to rats with chronic pancreatitis, at least a tendency towards a diminished disease progression was observed in a subgroup of animals with less severe disease. Based on the results of these experiments, we were further interested in the molecular role of the ET-1/ET receptor axis in the process of stellate cell activation.

In the present study, we have analyzed the biological and molecular effects of ET-1 in PSC and addressed the question of how ET-1 expression in PSC is regulated. The experiments were built on earlier reports by our group and others, which had suggested an autocrine loop of ET-1. Specifically, ET-1 was previously shown to activate extracellular signal-regulated kinases (ERK) 1 and 2, and to enhance PSC contraction and migration[11-13]. Our novel data deciphers ET-1 signalling in greater detail, and identifies, for the first time, target genes of ET-1 in PSC. Interestingly, these genes include not only α-SMA (which is directly linked to the activation process), but also two proinflammatory cytokines, interleukin (IL)-1β and IL-6. Furthermore, our investigations revealed TGF-β1 and TNF-α as potent inducers of ET-1 expression in PSC. Together, the data suggest that ET-1 is an important player in a network of proinflammatory and profibrogenic mediators that fosters interactions between inflammatory cells and PSC in a vicious cycle of inflammation and fibrosis.

MATERIALS AND METHODS

Materials

Iscove's modified Dulbecco's medium (IMDM) and all supplements for cell culture were obtained from Biochrom (Berlin, Germany), Nycodenz from Nycomed (Oslo, Norway), the rat-specific ET-1 enzyme immunometric assay (EIA) kit from Biotrend (Köln, Germany), and the 5-bromo-2'-deoxyuridine (BrdU) labelling and detection enzyme-linked immunosorbent assay kit as well as the polynucleotide kinase from Roche Diagnostics (Mannheim, Germany). Human connective tissue growth factor (CTGF) was delivered by EMP Genetech (Ingolstadt, Germany), and recombinant cytokines [rat interferon (IFN)-γ and PDGF, human TNF-α and TGF-β1] by R&D Systems (Minneapolis, MN, USA). TRIzol and all reagents used for reverse transcription and Taqman™ real-time polymerase chain reaction (PCR) were from Applied Biosystems (Foster City, CA, USA). PVDF membrane was supplied by Millipore (Schwalbach, Germany), protein- and phosphoprotein specific antibodies to ERK1/2 and p38 (all raised in rabbits) by New England BioLabs (Frankfurt, Germany), and Odyssey® blocking buffer, stripping buffer and secondary antibodies for immunoblotting by LI-COR (Bad Homburg, Germany). Immunoglobulins used in gel shift assays were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), DNA oligonucleotides from BioTeZ (Berlin, Germany), and [γ-^{32}P] ATP from Hartmann Analytic (Braunschweig, Germany). Collagenase, ET-1, tissue culture dishes (Corning plasticware), carboxyfluorescein succinimidyl ester (CFSE), as well as standard laboratory chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

PSC were isolated from the pancreas of male LEW.1W inbred rats by collagenase digestion of the organ and Nycodenz (120 g/L) density gradient centrifugation essentially as previously described[14]. The cells were resuspended in IMDM supplemented with 17% foetal calf serum (FCS), 10 mL/L non-essential amino acids (dilution of a 100 × stock solution), 10^5 U/L penicillin and 100 mg/L streptomycin, and cultured at 37℃ in a 5% CO_2 humidified atmosphere. All experiments were performed with cells that were passaged only once. Therefore, PSCs in primary culture were grown to subconfluency, harvested by trypsination (usually, on day seven after isolation), and recultured at equal seeding densities.

Quantification of DNA synthesis

Cell proliferation was assessed by measuring incorporation of BrdU into newly synthesized DNA. Therefore, cells growing in 96-well plates were treated under serum-free conditions with ET-1 as indicated. At the time of ET-1 application, BrdU labelling was initiated by adding labelling solution at a final concentration of 10 μmol/L (in the culture medium). Twenty-four hours later, labelling was stopped, and BrdU uptake was measured according to the manufacturer's instructions.

Cell migration assay

Serum-starved PSC were labelled with the fluorescence dye CFSE, which was dissolved in dimethyl sulfoxide and applied at a final concentration of 333 μmol/L (chosen based on the results of assay optimization). The cells were incubated with the dye for 20 min in darkness at room temperature. Migration assays were performed under serum-free conditions in 24-transwell plates using inserts with a pore size of 8 μm. Therefore, labelled cells were seeded at a density of 50 000 cells per well into the upper chamber, while ET-1 was added to the medium of the lower chamber as indicated. After an incubation period of 24 h, cells in the transwell inserts were removed by medium aspiration and mechanical

detachment from the upper side of the membrane. Cells adhering to the lower side of the filter or the bottom of the lower chamber were harvested by trypsination and transferred into 96-well plates. Fluorescence intensity was then measured using a fluorescence microplate reader (λ_{ex} = 492 nm, λ_{em} = 517 nm).

Immunoblotting

Protein extracts from equal numbers of PSCs (pretreated as indicated) were prepared and subjected to immunoblot analysis as previously described[14]. After electrophoresis, proteins were blotted onto PVDF membrane. The filters were rinsed in phosphate-buffered saline (PBS) and blocked for 1 h using Odyssey® Blocking Buffer, before primary antibodies were added to the blocking solution. One hour later, the blots were washed four times for 5 min in PBS containing 0.1% Tween 20 (PBS-T), and exposed to secondary antibody (IRDye® 800CW conjugated goat anti-rabbit IgG) for 1 h. Subsequently, the membranes were washed again (PBS-T, three times for 5 min), rinsed in PBS and scanned at a wavelength of 800 nm using an Odyssey® Infrared Imaging System. Signal intensities were quantified by means of the Odyssey® software version 3.0. Prior to reprobing with additional primary antibodies, the blots were treated with stripping buffer according to the instructions of the manufacturer.

Quantitative reverse transcriptase-PCR using real-time TaqMan™ technology

Total RNA from cells pretreated as indicated was isolated with TRIzol reagent according to the manufacturer's instructions. Next, RNA was reverse transcribed into cDNA by means of TaqMan™ Reverse Transcription Reagents and random hexamer priming. Relative quantification of target cDNA levels by real-time PCR was performed in an ABI Prism 7000 sequence detection system (Applied Biosystems) using TaqMan™ Universal PCR Master Mix and the following Assay-on-Demand™ rat gene-specific fluorescently labelled TaqMan™ MGB probes: Rn00573960_g1 (CTGF), Rn00561129_m1 (ET-1), Rn00580432_m1 (IL-1β), Rn00561420_m1 (IL-6), Rn00572010_m1 (TGF-β1), and Rn01527838_g1 [hypoxanthine phosphoribosyl transferase (HPRT), used as house-keeping control gene]. The Custom TaqMan Gene Expression Assay specific for rat α-SMA (GenBank Accession Number: X06801) has been described previously[11]. Following the guidelines of the manufacturer, PCR was performed under the following conditions: 95℃ for 10 min, 50 cycles of 15 s at 95℃, 1 min at 60℃. The reactions were performed in triplicate, and repeated six times with independent samples. Relative expression of each mRNA compared with HPRT was calculated according to the equation $\Delta Ct = Ct_{target} - Ct_{HPRT}$. The relative amount of target mRNA in control cells and cells treated as indicated was expressed as $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct_{treatment} = \Delta Ct_{ET\text{-}1} - \Delta Ct_{control}$.

ET-1 polypeptide quantification

ET-1 polypeptide concentrations in PSC culture supernatant were determined by EIA. Therefore, cells were seeded at a density of 50000 cells per well into 24-well plates. After an overnight incubation, medium was replenished and cells were exposed to different mediators as indicated. 48 h later, supernatants were collected and stored at -80℃ until they were subjected to EIA analysis. The assay was performed according to the instructions of the manufacturer. FCS of the culture medium was also analyzed and found to contain no detectable amounts of ET-1 (data not shown).

Electrophoretic mobility shift assays (EMSA)

PSC growing in six-well plates were incubated for 16 h in FCS-free culture medium, before they were treated as indicated. Nuclear extracts were prepared as previously described[14,15]. For EMSA experiments, nuclear proteins of 10^5 cells were incubated with double-stranded oligonucleotides which were end-labelled with [γ-^{32}P] ATP by polynucleotide kinase. The sequence of the activator protein (AP)-1 probe was 5'-CGCTTGA TGACTCAGCCGATC-3' (consensus binding motif underlined). To study inducible protein binding to the ET-1 promoter, the following probes derived from the rat promoter sequence[16] (accession number: S76970) were applied: 5'-GATTGTCAGACGGCGGGCGTCTGC CTCTGAAG-3' (corresponding to bases -196 to -165), and 5'-AGCCGTGATTTCCTCTAGAGC-3' (-163 to -144). The first oligonucleotide contains two consensus motifs for Smad transcription factors (underlined), which in the homologous region of the human ET-1 promoter have been identified as core sequences within a TGF-β response element[17]. The underlined bases of the second oligonucleotide correspond to a putative, although imperfect NF-κB/c-Rel binding site. Binding reaction and supershift analysis (initiated by adding 0.5 μg antibody) were performed as described[14,15]. Protein-DNA complexes were analyzed by electrophoretic separation on a 6% non-denaturating polyacrylamide gel. Dried gels were exposed to X-ray film.

Statistical analysis

Results are expressed as mean ± SE for the indicated number of separate cultures per experimental protocol. Statistical significance was analyzed using Wilcoxon's rank sum test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of ET-1 on PSC functions and gene expression

Initial studies dealt with the biological effects of ET-1 in PSC. In accordance with previous reports[12,13], ET-1 stimulated migration but had no effect on PSC proliferation (Figure 1). Next, we analyzed regulation of gene expression by ET-1, using real-time PCR. Interestingly, ET-1 significantly enhanced expression of α-SMA, suggesting direct stimulation of myofibroblastic differentiation (Figure 2). Furthermore, we focussed on cytokines and growth factors that have previously been implicated in autocrine or paracrine maintenance and

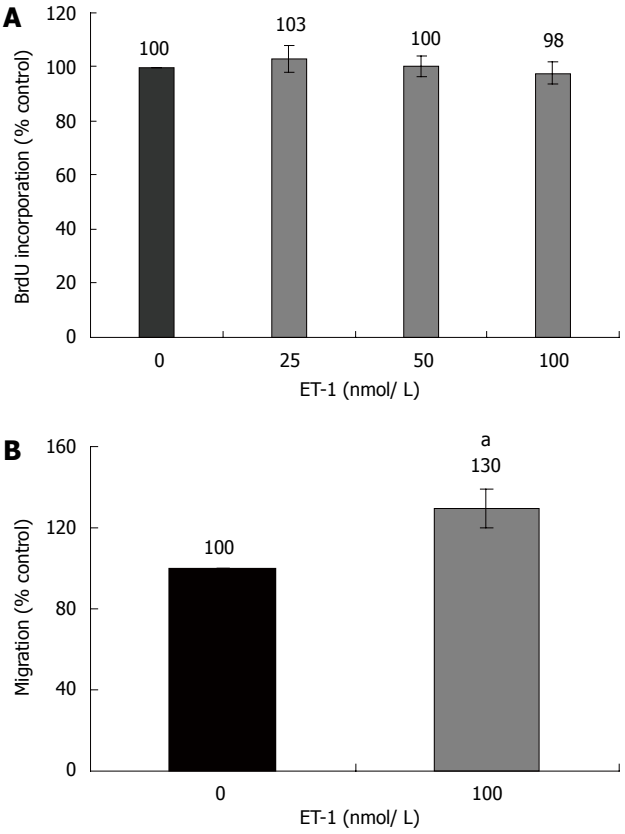

Figure 1 Effects of ET-1 on PSC proliferation and migration. A: PSCs growing in 96-well plates were treated, under FCS-free conditions, with ET-1 at the indicated concentrations for 24 h. Cell proliferation was assessed with the BrdU DNA-incorporation assay. One hundred percent BrdU incorporation corresponds to untreated PSCs; B: CFSE-labelled cells were seeded into the upper chamber of 24-transwell plates, whereas ET-1 (100 nmol/L) was added to the lower chamber as indicated. Cell migration under FCS-free conditions was analyzed as described in the "Materials and Methods" section. One hundred percent cell migration corresponds to the intensity of the fluorescence signal received from untreated PSCs. Data in (A) and (B) are presented as mean ± SE ($n \geqslant 6$ separate cultures); $^{a}P < 0.05$ *vs* control cultures.

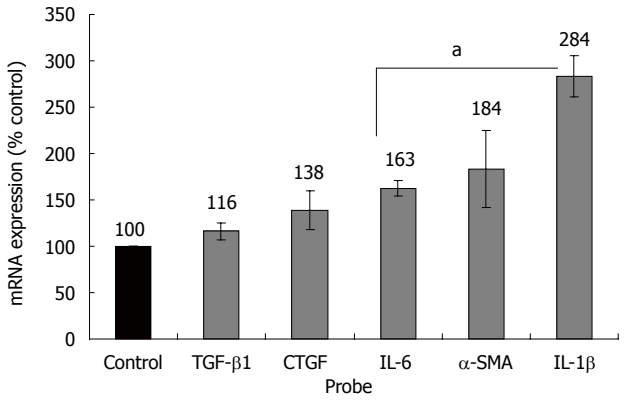

Figure 2 Effects of ET-1 on PSC gene expression. PSC growing in 6-well plates were starved of serum for 1 h before they were stimulated with ET-1 (100 nmol/L) as indicated. The mRNA expression of TGF-β1, CTGF, IL-6, α-SMA, IL-1β and the housekeeping gene HPRT was analyzed by real time PCR, and relative amounts of target mRNA were calculated. One hundred percent mRNA expression of each gene corresponds to untreated PSC. Data of 6 independent experiments (with triplicate samples) were used to calculate mean ± SE; $^{a}P < 0.05$ *vs* control cultures.

enhancement of PSC activation[6-8,18,19]. ET-1 significantly increased expression of two proinflammatory mediators, IL-1β and IL-6, but not of TGF-β1 and CTGF, two potent inducers of collagen synthesis.

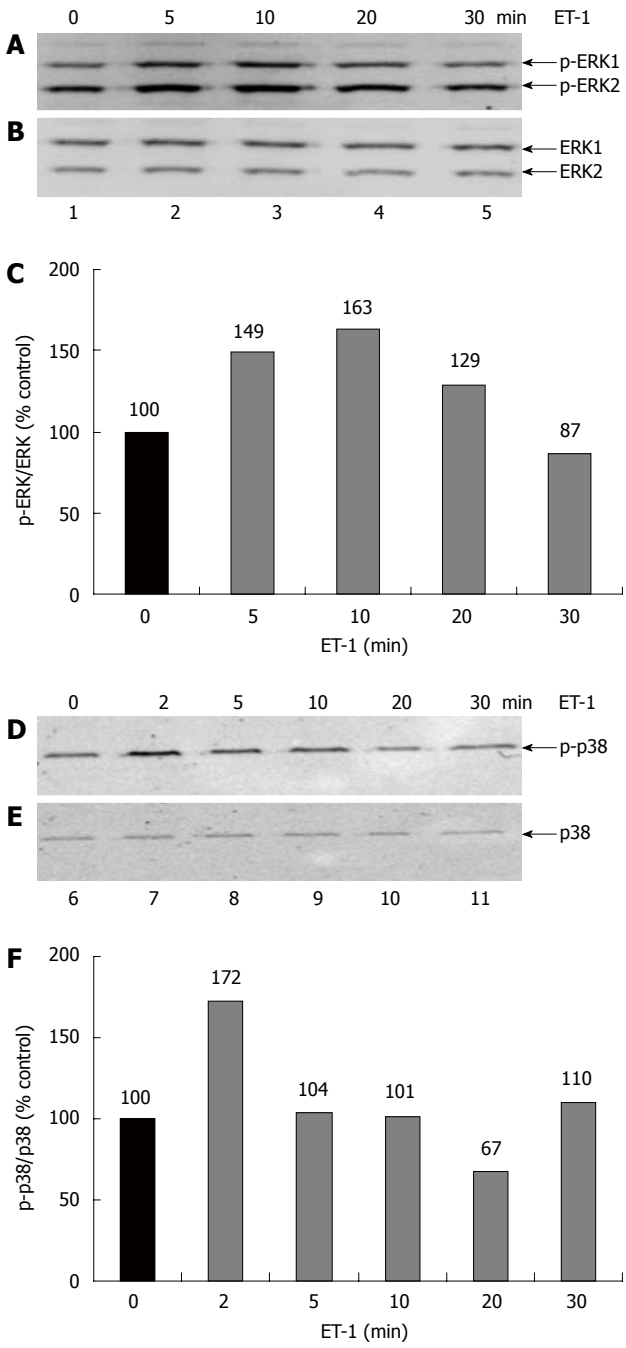

Figure 3 ET-1 induces phosphorylation of ERK1/2 and p38. PSC were starved of serum for 16 h before they were stimulated with ET-1 (100 nmol/L) for the indicated periods of time (A, D). ERK1/2 and p38 phosphorylation were analyzed by immunoblotting (B, E). Reprobing of the blots with anti-ERK1/2 and anti-p38 protein-specific antibodies revealed no systematic differences in the ERK1/2 and p38 amount among the samples (C, F). Fluorescence signal intensities of phospho (p)-ERK1/2, ERK1/2 protein, phospho (p)-p38 and p38 protein were quantified using Odyssey® software version 3.0. Subsequently, the ratios of phospho-ERK/ERK protein (C) and phospho-p38/p38 (F) protein were calculated. A ratio of one hundred percent corresponds to unstimulated control cultures. The data shown are representative of three independent experiments.

Transduction of the ET-1 signal in PSC

Extending our previous pilot study[11], we observed that ET-1 induced a rapid and transient phosphorylation of two

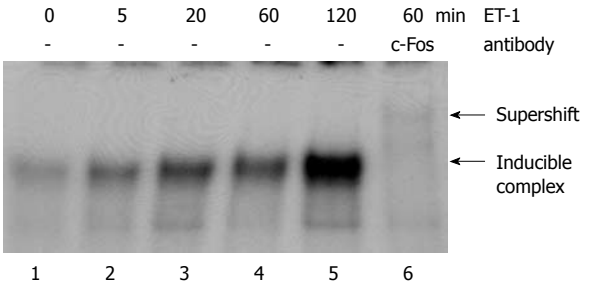

Figure 4 ET-1 activates DNA binding of AP-1. Serum-starved PSC growing in 6-well plates were incubated with ET-1 (100 nmol/L) for the indicated periods of time. Nuclear extracts were subjected to EMSA analysis using a [^{32}P]-labelled oligonucleotide with an AP-1 binding motif. Supershift analysis was performed by incubating the binding reaction with a c-Fos-specific antibody. Shifted complex and supershifted band (lane 6, faint since antibody binding may diminish binding of c-Fos to the oligonucleotide) are pointed out by arrows. Results are representative of three independent experiments.

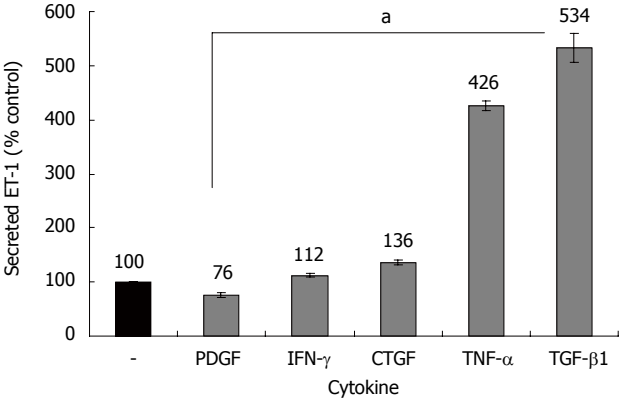

Figure 5 Regulation of ET-1 expression in PSC. PSC growing in 24-well plates were treated with PDGF (10 μg/L), IFN-γ (100 μg/L), CTGF (2 mg/L), TNF-α (10 μg/L), and TGF β1 (5 μg/L) for 48 h as indicated. Afterwards, supernatants were collected and subjected to EIA analysis of the ET-1 content. Data from six separate cultures were used to calculate mean ± SE; $^{a}P < 0.05$ *vs* control cultures.

distinct types of mitogen-activated protein (MAP) kinases, ERK1/2 and p38 (Figure 3). EMSA experiments revealed that ET-1 stimulation strongly increased DNA binding of the transcription factor complex AP-1 (Figure 4). As indicated by the results of a supershift analysis, the DNA/protein complex contained the AP-1 subunit c-Fos (Figure 4, lane 6). ET-1 stimulation of PSC was also associated with some enhancement of the DNA binding of NF-κB. The relevance of this finding, however, remained uncertain since activation of NF-κB was quite weak (data not shown).

Regulation of ET-1 gene expression

In the course of the studies, four mediators involved in PSC activation (PDGF, CTGF, TNF-α and TGF-β1)[2] as well as one antagonist, the antiproliferative and antifibrotic cytokine IFN-γ[20], were tested regarding their effects on ET-1 synthesis in PSC (Figure 5). TGF-β1 and TNF-α strongly stimulated secretion of ET-1 by the cells, whereas PDGF displayed the opposite effect. After application of CTGF or IFN-γ, statistically significant but nevertheless small increases of ET-1 levels in culture supernatants were observed.

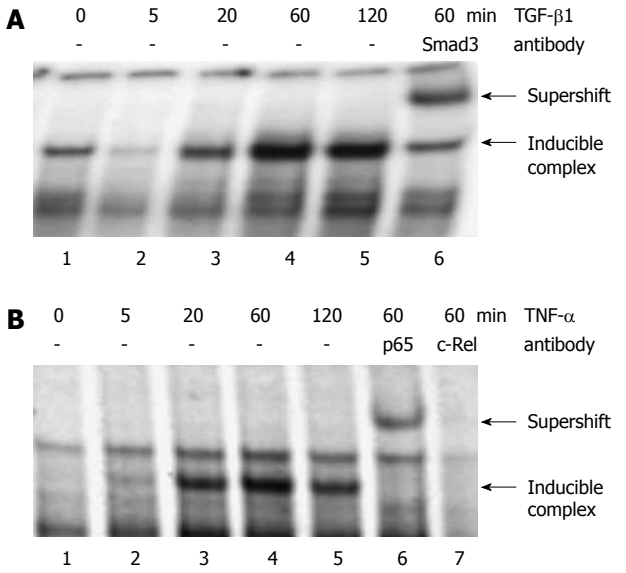

Figure 6 Effects of TGF-β1 and TNF-α on protein binding to the ET-1 promoter. Serum-starved PSC growing in 6-well plates were incubated with (A) TGF-β1 (5 μg/L) and (B) TNF-α (10 μg/L) for the indicated periods of time. Nuclear extracts were subjected to EMSA analysis using [^{32}P]-labelled oligonucleotides derived from the ET-1 promoter as described in the "Materials and Methods" section. Supershift analysis was performed by incubating binding reactions with the indicated protein-specific antibodies. Inducible complexes and supershifted bands (lane 6 in A and B) are pointed out by arrows. Addition of c-Rel antibody (B, lane 7) weakened protein binding to the oligonucleotide, suggesting the presence of the c-Rel protein in the complex (competitive binding of antibody and oligonucleotide to c-Rel). Results in (A) and (B) are representative of three independent experiments.

To study the molecular mechanisms underlying the effects of TGF-β1 and TNF-α, EMSA experiments using oligonucleotides derived from the rat ET-1 promoter were performed. TGF-β1 induced the binding of a transcription factor complex containing Smad3 to a DNA sequence that corresponds to the TGF-β response element of the human ET-1 gene[17] (Figure 6A). TNF-α stimulation of PSC was associated with enhanced protein binding to a putative NF-κB site in the ET-1 promoter. Antibodies to the NF-κB subunits p65RelA and c-Rel shifted the protein/DNA complex or weakened DNA/protein interaction, suggesting the presence of both proteins (Figure 6B).

DISCUSSION

PSC are the principal effector cells in pancreatic fibrosis[2,21]. Progressive replacement of pancreatic parenchyma by connective tissue promotes the development of exocrine and endocrine organ failure in the course of chronic pancreatitis. Furthermore, paracrine interactions between stroma cells and pancreatic cancer cells have been implicated in accelerated tumour growth and resistance to chemotherapeutics[22-24]. PSC are therefore considered attractive targets for the adjuvant treatment of pancreatic cancer based on inhibition of fibrogenesis. One important prerequisite for such an antifibrotic therapy is to understand the molecular processes underlying PSC activation in pancreatitis and cancer.

We and others have recently shown that ET-1 acts on PSCs in an autocrine fashion[11-13]. In agreement with a previous study[13], we have now found that application of ET-1 to PSC cultures promotes migration. Furthermore, our novel data indicate that ET-1 directly fosters exhibition of an activated, myofibroblastic phenotype, since α-SMA expression in PSC was enhanced. As previously suggested by Masamune *et al*[13], exogenous ET-1 did not stimulate cell proliferation. Nevertheless, the dual-specific ET receptor antagonist bosentan significantly inhibits PSC growth[11], suggesting a contribution of endogenous ET-1 to the induction of mitogenesis. Together, these findings clearly point to a profibrogenic role of the ET-1/ET receptor axis in PSC. Additional support for this conclusion comes from our studies at the molecular level: ET-1 induced an activation of the MAP kinases ERK1/2, p38 and the transcription factor AP-1. All these signalling molecules have previously been identified as key components of the intracellular network triggering PSC activation[14,15,25]. Furthermore, ET-1 significantly stimulated expression of two cytokines that have previously been suggested as autocrine enhancers of PSC activation, IL-1β and IL-6[18,19]. Since both cytokines are well-established pro-inflammatory mediators, our data also implicate, for the first time, ET-1 in the enhancement of local inflammatory reactions in the pancreas.

In the present study, experiments aimed at elucidating regulation of ET-1 gene expression in PSC revealed that TGF-β1 and TNF-α strongly enhanced the release of ET-1 by culture-activated PSC. Based on our molecular studies, Smad transcription factors and NF-κB are likely to be involved in the induction of ET-1 expression by TGF-β1 and TNF-α, respectively. Although both cytokines have previously been shown to stimulate ET-1 synthesis in other types of cells[17,26], this finding is interesting since it suggests the existence of a regulatory network in which TGF-β1 and TNF-α exert their biological effects on stellate cells in part through the ET-1/ET receptor axis. Given that ET-1 induces expression of IL-1β/IL-6, and TGF-β1 is secreted by PSC[27], a vicious cycle may develop which leads to enhanced inflammation and progressive fibrosis.

Unexpectedly, IFN-γ displayed a small stimulatory effect on ET-1 release by PSC, although it inhibited expression of the ET-1 gene in a cell line of immortalized PSC[28]. Here and in the case of PDGF, which diminished ET-1 synthesis, the underlying molecular mechanisms warrant further investigation.

As indicated by the results of our previous study[11], interruption of autocrine and paracrine loops at the level of ET-1 action represents a suitable approach to target activated PSC. However, the antifibrotic efficiency of the ET receptor antagonist bosentan in our rat model of severe chronic pancreatitis[29] was quite limited. Based on our previous and novel data, we therefore suggest that in future preclinical studies ET-1 receptor antagonists should be combined with drugs interfering with a different critical step of fibrogenesis and inflammation, such as transduction of profibrogenic signals in PSC.

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COMMENTS

Background

Fibrosis is a key feature of chronic pancreatitis and pancreatic cancer. The extensive deposition of extracellular matrix (ECM) proteins fosters the development of an exocrine and endocrine organ insufficiency, and accelerates progression of the tumour. Pancreatic stellate cells (PSC) are the principal effector cells in pancreatic fibrosis. They are activated by profibrogenic stimuli, which include, for example, cytokines and ethanol metabolites. So far, there are no specific therapies available to interfere with dysregulated fibrogenesis in the diseased pancreas.

Research frontiers

The molecular mechanisms underlying induction and maintenance of PSC activation are incompletely understood. This deficit hampers to some degree the development of therapeutic approaches aimed at the inhibition of pancreatic fibrosis in pancreatitis and cancer.

Innovations and breakthroughs

The results of this study provide molecular insights into endothelin-1 (ET-1) action in PSC. The data indicate that ET-1 stimulates exhibition of an activated stellate cell phenotype. The intracellular signalling molecules found to be activated by ET-1, ERK1/2, p38 and AP-1, have previously been linked to the process of stellate cell activation. With IL-1β and IL-6, two proinflammatory cytokines were identified as target genes of ET-1, suggesting for the first time a direct link between inflammation and fibrosis at the level of ET-1 action. In addition, studies on the regulation of ET-1 expression revealed that transforming growth factor-β1 and tumour necrosis factor-α strongly stimulate ET-1 secretion by PSC. Together, these data suggest that ET-1 is part of a network of proinflammatory and profibrogenic mediators that fosters interactions between inflammatory cells and PSC, ultimately enhancing inflammation and fibrosis.

Applications

The results encourage further studies aimed at the inhibition of fibrogenesis by targeting the ET-1/ET receptor axis. The authors suggest that combinations of ET receptor antagonists with drugs interfering with independent steps of stellate cell activation should be tested in animal models of pancreatic fibrosis.

Terminology

Pancreatic fibrosis refers to the process of progressive replacement of pancreatic tissue by connective tissue. PSC are fibroblast-like cells that produce most of the ECM in the diseased organ. ET-1 is a polypeptide originally identified as an endothelial cell-derived hormone with vasoconstrictive activity. Additional cellular origins and functions of ET-1 have been described.

Peer review

The authors revealed alteration of several factors involved in inflammation and fibrogenesis focusing on ET-1 action in isolated PSC. The study is well designed and provided some interesting results of scientific value, implying future therapeutic strategy using ET-1 receptor antagonists against chronic pancreatitis.

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ORIGINAL ARTICLES

Measuring Ca^{2+} influxes of TRPC1-dependent Ca^{2+} channels in HL-7702 cells with Non-invasive Micro-test Technique

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Abstract

AIM: To explore the possibility of using the Non-invasive Micro-test Technique (NMT) to investigate the role of Transient Receptor Potential Canonical 1 (TRPC1) in regulating Ca^{2+} influxes in HL-7702 cells, a normal human liver cell line.

METHODS: Net Ca^{2+} fluxes were measured with NMT, a technology that can obtain dynamic information of specific/selective ionic/molecular activities on material surfaces, non-invasively. The expression levels of TRPC1 were increased by liposomal transfection, whose effectiveness was evaluated by Western-blotting and single cell reverse transcription-polymerase chain reaction.

RESULTS: Ca^{2+} influxes could be elicited by adding 1 mmol/L $CaCl_2$ to the test solution of HL-7702 cells. They were enhanced by addition of 20 μmol/L noradrenalin and inhibited by 100 μmol/L $LaCl_3$ (a non-selective Ca^{2+} channel blocker); 5 μmol/L nifedipine did not induce any change. Overexpression of TRPC1 caused increased Ca^{2+} influx. Five micromoles per liter nifedipine did not inhibit this elevation, whereas 100 μmol/L $LaCl_3$ did.

CONCLUSION: In HL-7702 cells, there is a type of TRPC1-dependent Ca^{2+} channel, which could be detected *via* NMT and inhibited by La^{3+}.

Key words: Non-invasive Micro-test Technique; Ca^{2+} channels; Transient Receptor Potential Canonical 1; Gene expression; HL-7702 cells

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INTRODUCTION

Changes in the concentration of Ca^{2+} in the cytoplasmic space play a central role in intracellular signaling pathways in liver cells, including glucose, fatty acid, amino acid and xenobiotic metabolism, bile acid secretion, protein synthesis and secretion, the movement of lysosomes and other vesicles, the cell cycle and cell proliferation, and apoptosis and necrosis[1-4]. In earlier reports, it has been shown that ligand-gated, store-operated, receptor-activated, and stretch-activated Ca^{2+}-permeable channels are expressed in hepatocytes and in liver cell lines. No voltage-operated Ca^{2+} channels (VOCCs) have been detected[5-8]. There is increasing evidence that members of the canonical subgroup of Transient Receptor Potential (TRP) proteins constitute tetramers of both receptor-activated and store-operated Ca^{2+} channels (SOCs)[9-11], and Transient Receptor Potential Canonical 1 (TRPC1) is considered as one of the most likely candidates in forming Ca^{2+} channels in mammalian cells[12-15].

The Non-invasive Micro-test Technique (NMT) was developed in the late 20th century, and is a new

technology for obtaining dynamic information on specific ionic/molecular activities on material surfaces, non-invasively. This technique incorporates different temporal and spatial resolution domains from other traditional methods, and its 3-dimensional measurement capability enables us to observe the physiological characteristics of biological phenomena that would be difficult or even impossible with other techniques[16]. To date, Ca^{2+}, H^+, K^+, Cl^-, NO^-, Mg^{2+}, Cd^{2+}, Al^{3+}, and O_2 have be detected as sensors for ionic/molecular species.

In the present study, we used NMT to study the Ca^{2+} influxes elicited by extracellular elevations of Ca^{2+} concentration, and the inhibitory effects of several Ca^{2+} channel blockers, to investigate the role of TRPC1 in regulating Ca^{2+} fluxes in HL-7702 cells.

MATERIALS AND METHODS

Materials

Nifedipine, noradrenalin, protease inhibitor Cocktail, Fast Red TR, Naphthol AS-MX phosphate, and Calcium Ionophore I [Cocktail A were bought from the Sigma-Aldrich Company (Catalog Number: 21048)]. Lipofectamine 2000 was purchased from Invitrogen. The TRPC1 polyclonal antibody was acquired from the Abnova Company. Peroxidase-conjugated secondary antibody was obtained from the Beijing Zhongshan Golden Bridge Co.. All the other reagents were of reagent grade.

Cell culture, plasmid construction and transfection

The human liver cell line, HL-7702, bought from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, was maintained in RPMI-1640 containing 10% FBS, 1% Penicillin, and Streptomycin. Plasmid pBS-T-TRPC1 was constructed and verified as previously described[17]. HL-7702 cells were grown to 75%-80% confluence in 35 mm dishes in advance and transfection was carried out with 1 μg/mL of the recombinant plasmid and Lipofectamine 2000 according to the manufacturer's protocol.

Measurements of extracellular Ca^{2+} influxes

Measurements of net influxes of Ca^{2+} were performed using NMT (BIO-001A, Younger USA Sci. & Tech. Co., Amherst, MA, USA; Applicable Electronics Inc., Forestdale, MA, USA; and ScienceWares Inc., East Falmouth, MA, USA). The electrode was controlled to move with an excursion of 10 μm at a programmable frequency in the range of 0.3-0.5 Hz; this minimized mixing of the bathing saline.

To construct the microelectrodes, borosilicate micropipettes (2-4 μm aperture, XYPG120-2, Xuyue (Beijing) Science and Technology Co., Ltd., Beijing, China) were silanized with tributylchlorosilane and the tips filled with Calcium Ionophore I - Cocktail A. An Ag/AgCl wire electrode holder (XYEH01-1) was inserted in the back of the electrode to make electrical contact with the electrolyte solution. Only electrodes with Nernstian slopes > 25 mV were used. Ca^{2+} fluxes were calculated by Fick' law of diffusion: $J_0 = -[D \times (dC/dX)]$ where J_0 represents the net Ca^{2+} flux (in μmol/cm per second), D is the self-diffusion coefficient for Ca^{2+} (in cm^2/s), dC is the difference value of Ca^{2+} concentrations between the two positions, and dX is the 10 μm excursion over which the electrode moved in our experiments. Data and image acquisition, preliminary processing, control of the three-dimensional electrode positioner, and stepper-motor-controlled fine focus of the microscope stage were performed with ASET software.

Single cell reverse transcription-polymerase chain reaction (RT-PCR)

Single cell RT-PCR was performed to determine whether the cells measured by NMT were successfully transfected, using a previously described method, with some modifications[18]. Directly after the Ca^{2+} influx assay, the contents of tested cell were aspirated into a microelectrode. The tip of the electrode was then broken in a PCR tube and stored at -80℃ until use. Reverse transcription was carried out using a kit from TIANGEN (Beijing, China) according to the manufacturer's instructions. The first PCR was performed using specific primers (Forward: GCAATGATACCTTCCATTCGTTC; Reverse: CGATGCACTAGGCAGCAGATC) and the following conditions: pre-denaturation at 94℃ for 3 min; followed by 30 cycles of denaturation at 94℃ for 30 s and annealing at 60℃ for 30 s, then synthesis at 72℃ for 60 s; the last step was extension at 72℃ for 5 min. After the first PCR, 1 μL of the reaction products was used as the template for a secondary PCR with the same conditions as above and 25 cycles. The predicted size of the TRPC1 amplicons were 455 bp and reaction products were confirmed and analyzed by agarose gel electrophoresis.

Western blotting

Total proteins were obtained from cultured cells by using lysis buffer (35 mmol/L Tris at pH 7.4, 0.4 mmol/L EDTA, 10 mmol/L $MgCl_2$, 0.1% protease inhibitor Cocktail). For western blotting analysis, 20 μg proteins were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto to polyvinylidene difluoride membranes for 2 h at 20 V. The membranes were then blocked for 2 h with blocking solution (5% bovine serum albumin) and probed with anti-TRPC1 antibodies. The primary antibodies were incubated for 1 h at room temperature and, after washing, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h. Finally, the proteins on the membranes were dyed by staining solution containing Fast Red TR and Naphthol AS-MX phosphate. Immunoblots were then scanned to obtain images.

Statistical analysis

Data were expressed as mean ± SD of n cells from at least six cell culture dishes. The statistical significance of diversities between means was determined using the DUNNET t-test. A value of $P < 0.05$ was considered significant.

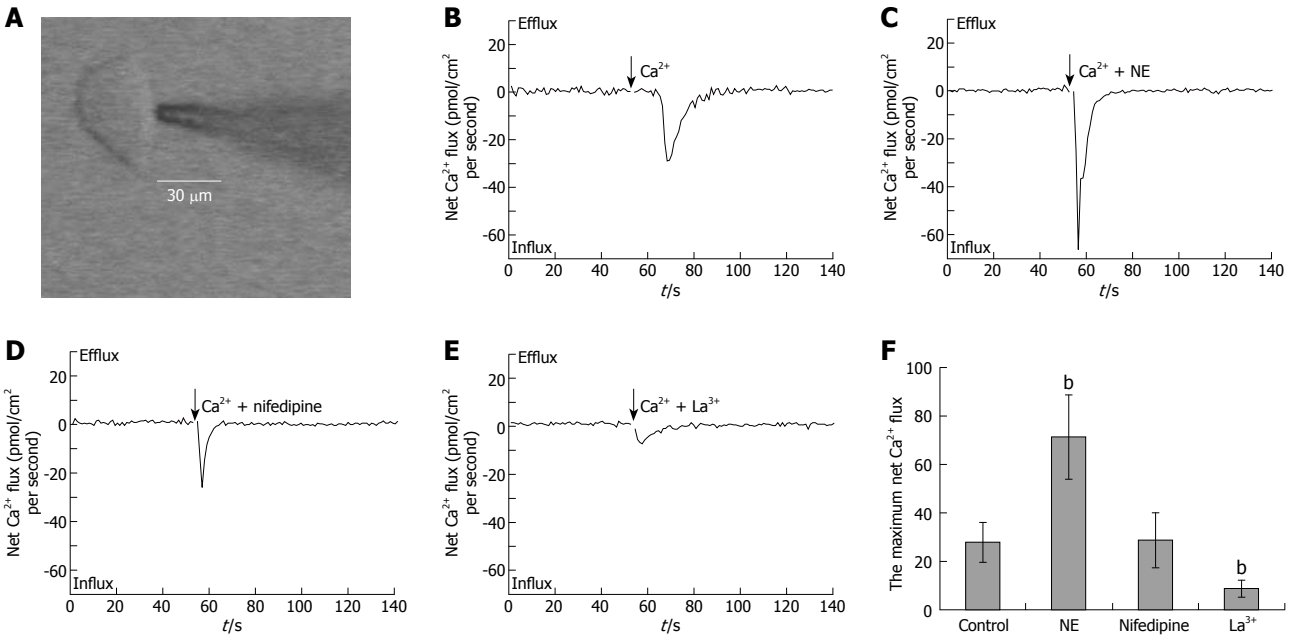

Figure 1 The Ca^{2+} fluxes of HL-7702 cells. A: A screen-printed picture of a cell measured by NMT; B: Net Ca^{2+} fluxes of a HL-7702 cell. The maximum values were 28.2 ± 8.2 pmol/cm^2 per second (n = 6); C: Net Ca^{2+} fluxes of a noradrenalin-treated HL-7702 cell. The maximum values were 71.7 ± 17.5 pmol/cm^2 per second (n = 6); D: Net Ca^{2+} fluxes of a nifedipine-treated HL-7702 cell. The maximum values were 29.0 ± 11.3 pmol/cm^2 per second (n = 6); E: Net Ca^{2+} fluxes of a La^{3+} -treated HL-7702 cell. The maximum values were 9.0 ± 3.5 pmol/cm^2 per second (n = 6); F: Bar graph of the maximum net Ca^{2+} influxes in four groups. $^bP < 0.01$, control group *vs* noradrenalin-treated or La^{3+}-treated group.

RESULTS

The Ca^{2+} influxes of HL-7702 cells were measured by NMT

Before the experiment, 35 mm dishes with pre-dispersed normal HL-7702 cells were perfused with test solution containing (in mmol/L): 2.3 $NaHCO_3$, 27 Na_2SO_4, 9.7 KCl, 61.1 $MgCl_2$, and 366.7 NaCl. The Ca^{2+} influxes were then measured by NMT. The background noise was recorded for three min before 1 mmol/L $CaCl_2$ was added to elicit an inward Ca^{2+} current. As shown in Figure 1A, the Ca^{2+} selective microelectrode moved between two positions close to the tested cells constantly to acquire experimental data. Net Ca^{2+} fluxes are depicted in Figure 1B, a giant wave trough emerged shortly after $CaCl_2$ was added.

Effects of noradrenalin, nifedipine and La^{3+} on Ca^{2+} influxes of HL-7702 cells

To further identify the property of the Ca^{2+} influxes of HL-7702 cells, three drugs were selected as tools in the following experiments. After background noise was recorded for three min, a Ca^{2+} channel agonist, noradrenalin (20 μmol/L), or two types of inhibitors, nifedipine (5 μmol/L) or $LaCl_3$ (100 μmol/L) were added into the test solution together with 1 mmol/L $CaCl_2$. The effects of the three drugs on Ca^{2+} influxes are shown in Figure 1C-E. Net Ca^{2+} fluxes were significantly influenced by noradrenalin and La^{3+}; however, nifedipine did not induce any change. A bar graph of the maximum net Ca^{2+} fluxes in the four groups (three drug-treated groups and control group) is depicted in Figure 1F; these experiments were repeated six times (n = 6).

Effects of TRPC1-transfection on Ca^{2+} influxes of HL-7702 cells

When TRPC1-transfected HL-7702 cells were measured by NMT, the maximum net Ca^{2+} influxes increased to 48.9 ± 6.4 pmol/cm^2 per second (n = 6) and a deeper wave trough was observed (Figure 2A), The bar graph shown in Figure 2B shows that the statistical difference between the TRPC1-transfected group and the control group was significant ($P < 0.01$). Single cell RT-PCR and western blotting were performed after NMT experiments. The results of agarose gel electrophoresis and SDS-PAGE are shown in Figure 2C and D, respectively. They demonstrated that TRPC1-expressions in tested cells were elevated after transfection.

Effects of nifedipine and La^{3+} on Ca^{2+} influxes of TRPC1-transfected HL-7702 cells

TRPC1-transfection induced increases in Ca^{2+} influxes. To investigate the effects of nifedipine and La^{3+} on these increased Ca^{2+} influxes, the two drugs were added into the test solution together with 1 mmol/L $CaCl_2$. As shown in Figure 3A-C, the maximum net Ca^{2+} fluxes maintained an average of 44.0 ± 5.7 pmol/cm^2 per second (n = 6) when 5 μmol/L nifedipine was applied, whereas 100 μmol/L $LaCl_3$ made reduced the maximum value to 7.6 ± 1.9 pmol/cm^2 per second (n = 6). A significant statistical difference only existed between the TRPC1 + La^{3+}-treated group and the TRPC1-transfected group ($P < 0.01$). Single cell RT-PCR and western blotting were performed after NMT experiments to confirm the increased TRPC1 expression (data not shown).

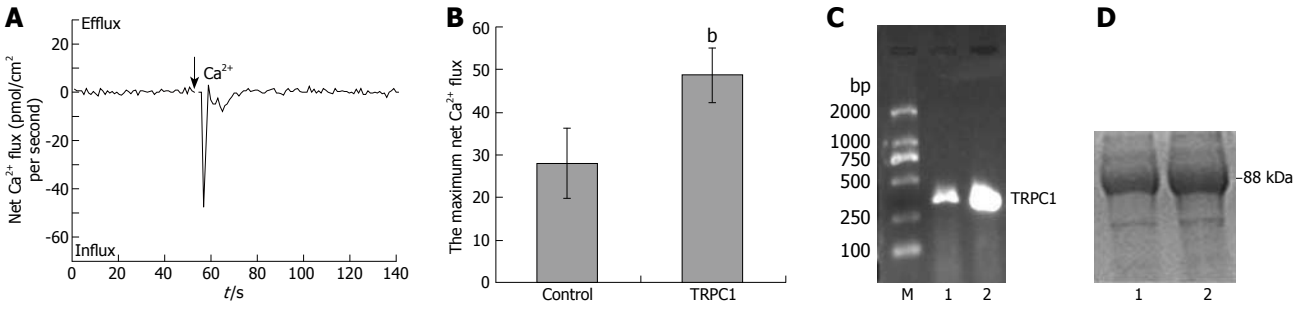

Figure 2 TRPC1-transfection influences Ca^{2+} influxes of HL-7702 cells. A: Net Ca^{2+} fluxes of a TRPC1-transfected cell. The maximum values were 48.9 ± 6.4 pmol/cm^2 per second (n = 6); B: Bar graph of the maximum net Ca^{2+} fluxes in the control group and the TRPC1-transfected group. The statistical difference between the two groups was significant ($^bP < 0.01$); C: Single cell RT-PCR products from cultured HL-7702 cells and TRPC1-transfected cells using primers for human TRPC1 (455 bp); lane M: DNA marker, lane 1: TRPC1 amplified from HL-7702 cells, lane 2: TRPC1 amplified from transfected cells; D: TRPC1 protein was detected in cultured HL-7702 cells and TRPC1-transfected cells using western-blotting analysis; lane 1: TRPC1 protein extracted from HL-7702 cells, lane 2: TRPC1 protein from transfected cells. The molecular mass of TRPC1 is 88 kDa.

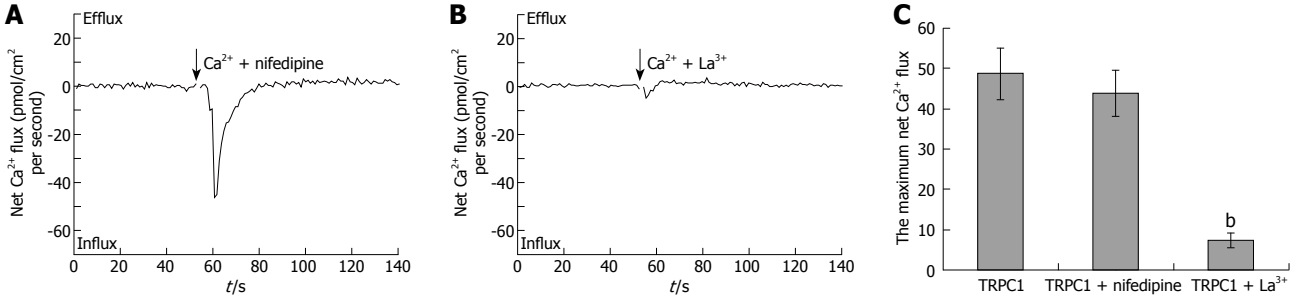

Figure 3 Nifedipine and La^{3+} influence Ca^{2+} influxes of TRPC1-transfected HL-7702 cells. A: Net Ca^{2+} fluxes of a TRPC1 + nifedipine-treated cell. The maximum values were 44.0 ± 5.7 pmol/cm^2 per second (n = 6); B: Net Ca^{2+} fluxes of a TRPC1 + La^{3+}-treated cell. The maximum values were 7.6 ± 1.9 pmol/cm^2 per second (n = 6); C: Bar graph of the maximum net Ca^{2+} fluxes in the TRPC1-transfected group, TRPC1 + nifedipine-treated group, and TRPC1 + La^{3+}-treated group. A significant statistical difference only existed between the TRPC1-transfected group and the TRPC1 + La^{3+}-treated group ($^bP < 0.01$).

DISCUSSION

Initially, we detected Ca^{2+} influxes in HL-7702 cells using NMT when 1 mmol/L $CaCl_2$ was added to the test solution and the extracellular Ca^{2+} concentration markedly changed. Ca^{2+} influx could be influenced by two drugs, noradrenalin, and $LaCl_3$; however nifedipine did not induce any change.

As a known neurotransmitter, noradrenalin can act through α_1-adrenoceptors to activate phospholipase C. This generates inositol 1,4,5-trisphosphate (IP_3) within the cell, which in turn mediates the rise of Ca^{2+} concentration by release from intracellular stores[19,20] and opening of receptor operated Ca^{2+} entry at the plasma membrane[21-23]. Thus, the increase in Ca^{2+} influx induced by noradrenalin together with 1 mmol/L external $CaCl_2$ could be explained if receptor operated Ca^{2+} channels played a central role in these experiments. On the other hand, Ca^{2+} influxes were prominently inhibited by La^{3+}. Analogs often effect their action through competitive inhibition with their common receptors or channels. La^{3+} has a similar size of ionic radius to that of Ca^{2+}, which enables La^{3+} to compete with Ca^{2+}, which makes La^{3+} a non-selective Ca^{2+} channel blocker. In addition, a study showed that La^{3+} could inhibit both influx and efflux of Ca^{2+} in lacrimal cells[24], which was consistent with the present study. Most importantly, nifedipine, as an antagonist of L-type Ca^{2+} channels, did not induce any change in Ca^{2+} influxes, which would indicate that no VOCCs existed or that transmembrane Ca^{2+} influxes elicited by external Ca^{2+} did not pass through VOCCs in HL-7702 cells.

TRPC1 is one of seven members of the TRPC subfamily of non-selective cation channels and is expressed in a wide variety of cell types and tissues[25-27]. It is likely that TRPC1 plays a significant part in intracellular Ca^{2+} homeostasis. In salivary gland cells, the current through the endogenous SOCs was the same as the membrane current which was activated by the depletion of intracellular Ca^{2+} stores in cells in which TRPC1 was ectopically expressed and the endogenous SOCs were decreased by transfection with antisense TRPC1[28]. In kidney epithelial cells, a new receptor-operated channel formed by heteromeric assembly of TRPP2 and TRPC1 subunits was discovered[29]. In T cells, intracellular Ca^{2+} elevation induced by Δ9-tetrahydrocannabinol was attributable entirely to extracellular Ca^{2+} influxes, which were not dependent on store depletion, but mediated through TRPC1 channels[30]. The results of the second part of our study do not conflict with these reports. In HL-7702 cells, overexpression of TRPC1 causes an increase of Ca^{2+} influxes induced by adding external Ca^{2+}, and the non-selective Ca^{2+} channel blocker, La^{3+}, can attenuate this elevation. In summary, there is a TRPC1-dependent Ca^{2+}-type channel(s), either receptor-activated or store-operated present in HL-7702 cells, which can be inhibited by La^{3+}.

Taken together, NMT is a powerful tool for ion

channel research, which has been effectively applied in various systems[31-33]. We used NMT to explore the properties of Ca^{2+} channels, and found that there was a TRPC1-dependent Ca^{2+}-type channel(s), which could be detected *via* NMT and inhibited by La^{3+}, in HL-7702 cells.

COMMENTS

Background

Ca^{2+} plays an important role in intracellular signaling pathways and Transient Receptor Potential Canonical 1 (TRPC1) is considered as one of the most likely candidates in forming Ca^{2+} channels in mammalian cells. As a technology to obtain dynamic information of specific ionic/molecular activities on material surfaces non-invasively, Non-invasive Micro-test Technique (NMT) is being increasingly applied to study characters of ion channels.

Research frontiers

TRPC1 has been verified as a molecular candidate or a regulator of Ca^{2+} channels in several mammalian cells. However, the role of TRPC1 in normal human liver cells has not been elucidated. In this study, the authors demonstrate that a TRPC1-dependent Ca^{2+}-type channel(s) exists in HL-7702 cells, using NMT, a non-invasive technique.

Innovations and breakthroughs

This is the first study to investigate the role of TRPC1 in regulating Ca^{2+} channels in normal human liver cells. Furthermore, measuring Ca^{2+} influxes was performed non-invasively, which cannot be accomplished with other traditional techniques.

Applications

Cytoplasmic Ca^{2+} overloading might cause damage to liver cells. By understanding the role of TRPC1 in mediating extracellular Ca^{2+} influxes, this study might represent a future strategy for preventing or treating diseases induced by dysfunctions of Ca^{2+} channels in the clinic, such as hepatic ischemia-reperfusion injury.

Terminology

The canonical transient receptor potential (TRPC) channel subfamily consists of seven mammalian cation channels and is expressed in almost every tissue, including the liver. The TRPC1 channel is permeable to Ca^{2+} and is the most likely candidate for receptor-operated Ca^{2+} channels. In addition, TRPC1 also plays a dominant role in mediating store-operated Ca^{2+} channels in many types of cells.

Peer review

The authors recorded Ca^{2+} influxes elicited by adding external Ca^{2+} into a test solution in several different conditions. It revealed that there is a TRPC1-dependent Ca^{2+}-type channel(s), which can be detected *via* NMT, in normal human liver cells. This is an interesting study.

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BRIEF ARTICLES

Effect of early preoperative 5-fluorouracil on the integrity of colonic anastomoses in rats

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Abstract

AIM: To determine the effect of chemotherapy on wound healing by giving early preoperative 5-fluorouracil (5-FU) to rats with colonic anastomoses.

METHODS: Sixty Albino-Wistar male rats (median weight, 235 g) were used in this study. The rats were fed with standard laboratory food and given tap water *ad libitum*. The animals were divided into three groups: Group 1: Control group (chemotherapy was not administered), Group 2: Intraperitoneally (IP) administered 5-FU group (chemotherapy was administered IP to animals at a dose of 20 mg/kg daily during the 5 d preceeding surgery), Group 3: Intravenously (IV) administered 5-FU group. Chemotherapy was administered *via* the penil vein, using the same dosing scheme and duration as the second group. After a 3-d rest to minimize the side effects of chemotherapy, both groups underwent surgery. One centimeter of colon was resected 2 cm proximally from the peritoneal reflection, then sutured intermittently and subsequently end-to-end anastomosed. In each group, half the animals were given anaesthesia on the 3rd postoperative (PO) day and the other half on the 7th PO day, for *in vivo* analytic procedures. The abdominal incisions in the rats were dissected, all the new and old anastomotic segments were clearly seen and bursting pressures of each anastomotic segment, tissue hydroxyproline levels and DNA content were determined to assess the histologic tissue repair process.

RESULTS: When the IV group was compared with the IP group, bursting pressures of the anastomotic segments on the 3rd and 7th PO days, were found to be significantly decreased, hydroxyproline levels at the anastomotic segment on the 7th PO day were significantly decreased ($P < 0.01$).

CONCLUSION: In this study, we conclude that early preoperative 5-FU, administered IV, negatively affects wound healing. However, IP administered 5-FU does not negatively affect wound healing.

Key words: 5-fluorouracil; Neoadjuvant therapy; Rats; Wound healing

Peer reviewer: Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

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INTRODUCTION

Colorectal cancer is a common malignancy in most developed countries worldwide[1]. Metastasis frequently occurs before clinical detection of the primary tumour. Despite the advances in surgical techniques, this characteristic of the malignancy prevents a significant

improvement in cure rates for colorectal cancers[2]. While cancer therapy was limited to surgery in the past, nowadays therapy includes the combination of surgical radiotherapy, chemotherapy, hormonal and biological therapies[3,4]. 5-fluorouracil (5-FU) treatment has been accepted as standard chemotherapy for colorectal cancers for a long time. Recently, high recurrence rates, the presence of distant metastasis, the possibility of complete resection, and the removal of circulating tumour cells after curative resection of colorectal cancers constitute a new therapeutic approach known as neo-adjuvant chemotherapy[5-7]. While the integrity of anastomoses after colorectal cancer resection is an important parameter on mortality and morbidity, the effects of preoperative chemotherapy on wound healing and anastomoses are also important and have not been clearly outlined.

This study investigates the effects of early preoperative administration of 5-FU, given intravenously (IV) and intraperitoneally (IP), on wound healing in colon anastomoses.

MATERIALS AND METHODS

Sixty male Wistar-Albino rats weighing between 225 and 315 g were used in this study. All rats were clinically healthy and were fed with standard laboratory food and water. The animals were numbered at the beginning of the study and weighed every day during the study. There were three groups: Group Ⅰ, a control group (n = 20); group Ⅱ which received 5-FU IP (n = 20); group Ⅲ which received 5-FU IV (n = 20). The study was approved by the local Ethics Committee of Haydarpaşa Numune Hospital.

Drug administration

When pilot studies and related literature were taken into account, a dose of 20 mg/kg 5-FU was calculated to be the maximum non-lethal dose[8]. Chemotherapy was not administered to the control group. The second group was administered 5-FU IP at a dose of 20 mg/kg in saline at a concentration of 5 mg/mL each day prior to surgery. The third group was given 5-FU *via* the penile vein at a dose of 20 mg/kg in saline at a concentration of 2 mg/mL each day for 5 d before surgery. Both groups underwent surgery on the 3rd d after chemotherapy to reduce the adverse effects of chemotherapy.

Operative procedure

The same operative procedure was performed in all groups by the same surgeon. 10 mg/kg of ketamine was given subcutaneously to rats under ether anaesthesia. After shaving the frontal abdominal wall, this area was cleaned with povidone iodine and covered with sterile cloths. The abdomen was entered through a 3 cm mid-line incision, 1 cm of colon 2 cm proximal of the peritoneal reflection was resected, and a side-to-side anastomosis was made using ten intermittent sutures with 6/0 polypropylene (Ethicon). Muscles of the front abdominal wall and skin were closed by continuous suture with 3/0 silk. Half the animals in each group were anaesthetized again either on day 3 or 7 after surgery for *in vivo* analytic procedures. The animals were then killed by haemorrhage for *in vitro* analytic procedures.

Analytic procedures

After making an abdominal incision, macroscopic evaluations of the anastomotic segment were performed. Adhesions surrounding the anastomoses were not cut, and bursting pressure was measured for every anastomotic segment during the internal passage of 200 mL/h saline. For this purpose, a 10/0 silicon catheter was passed *via* the anus to 2 cm distal of the anastomosis and the colon was ligated with silk suture above the catheter. The colon was cut 3 cm proximal to the anastomotic segment. The catheter, which had its end fixed to a standard sphygmomanometer (Petaş, Turkey), was moved 1 cm to the anastomotic segment from the cut end of the colon and the colon was ligated with silk suture around the catheter. The perfusator (Becton Dickinson, France) was maintained at a speed of 200 mL/h and saline was given continuously through the catheter situated in the anus. Increased pressure on the sphygmomanometer was observed. Pressure values of the first leakage from the anastomotic segments, when increased pressure on the sphygmomanometer stopped and the time of falling pressure were recorded as bursting pressures. These bursting pressures were recorded in mmHg for each animal.

After measuring the anastomotic pressures and just before the animals were sacrificed, 5 mL blood samples were taken from the inferior vena cava to determine white blood cell, haemoglobin and platelet counts.

Anastomotic segments were isolated from the surrounding tissues. One cm of colonic segment, including the anastomotic area was resected and was longitudinally separated into two parts. One of the segments was frozen at -45℃ for hydroxyproline measurements (hydroxyproline was used as a marker of collagen content) which were calculated as nanograms per gram of tissue. Anastomotic hydroxyproline content was measured by spectrophotometric determination using the method described by Bergman *et al*[9]. The other segment was embedded in paraffin for DNA content measurement and histological assay. Tissue DNA content was determined by flow cytometry using Mod-Fit Ver 5.01 software[10].

Tissue sections from routinely embedded paraffin blocks were stained with hematoxylin-eosin and examined by light microscopy. Histological examination was evaluated using the criteria determined by de Roy van Zuidewijn *et al*[11]. Slides were evaluated twice by the same observer in a blind fashion. Granulation tissue was evaluated as; 1-low; 2-medium; 3-high (intense) and histological parameter scores related to muscular tissue were evaluated as; 1-negative; 2-medium; 3-complete. According to a seven-point scale, mucosal re-epithelization scores were as follows; 1-negative, 2-little-one line cubic, 3-lot-one line cubic, 4-nearly one line cubic, 5-finished-one line cubic, 6-one line glandular, 7-normal glandular mucosa.

Table 1 Leukocyte, platelet and haemoglobin values of blood samples in each group (mean ± SE)

Groups	Leukocytes (10^9/L)		Platelets (10^9/L)		Haemoglobin (g/dL)	
	3rd d	7th d	3rd d	7th d	3rd d	7th d
Control	9.0 ± 3.04	7.6 ± 3.4	1117 ± 169	1270 ± 229	11 ± 1.7	12.6 ± 1.2
IP	9.1 ± 3.3	9.7 ± 2.4	808 ± 203	1228 ± 194	10.5 ± 1.7	11.9 ± 0.45
IV	7.4 ± 4.7	15.9 ± 5.2	736 ± 243	1542 ± 289	9.6 ± 1.13	11.16 ± 1.9

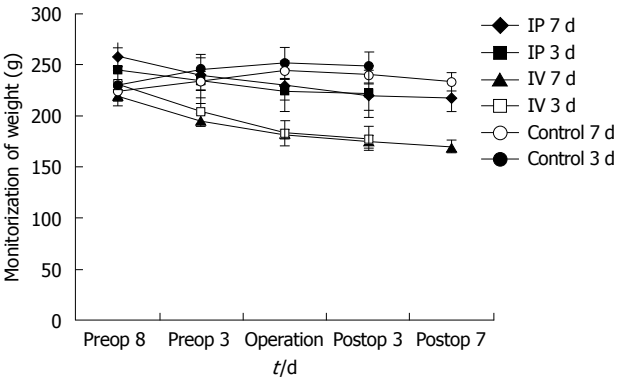

Figure 1 Weights of rats during experimental procedure. Preop: Pre-operation; Postop: Post-operation.

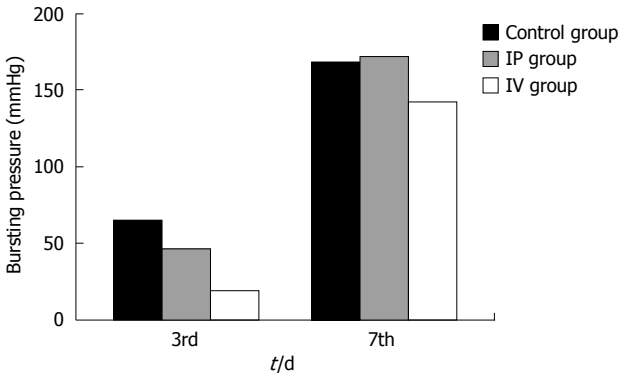

Figure 2 Bursting pressure values of experimental groups on postoperative 3rd and 7th d.

Statistical analysis

All data are presented as means ± SE. Non-parametric Kruskall Wallis variant analysis was used for multiple group comparisons of statistical analysis and subgroup comparisons were performed with Dunn's test. Pair group analysis was evaluated using Mann-Whitney *U* test.

RESULTS

During the experimental procedure, two animals died on the 3rd and 7th postoperative (PO) days in the IV group. The cause of death was leakage from the anastomotic site. No mortality was observed in the control and IP groups. Dead rats were replaced with new animals.

Weight measurements

Rats were weighed at the beginning of the study, during the chemotherapy period and in the postoperative period. Weight loss was observed in all animals in the chemotherapy groups (IP and IV) compared with baseline values, whereas control animals gained weight during the preoperative period. Animals in the control group also lost weight during the PO period. Weight loss in the rats is shown in Figure 1. On the 3rd PO day, the IV 5-FU group weighed significantly less than the control group ($P < 0.01$) and the IP group ($P < 0.01$). Weight loss in the IV 5-FU group on the 7th PO day was found to be significantly lower than the control group ($P < 0.01$) and the IP group ($P < 0.001$).

Hematological effects

Haemoglobin, white blood cell and platelet counts in the blood samples taken on the 3rd and 7th PO days after chemotherapy are shown in Table 1. There was no statistically significant difference between the groups for haemoglobin counts on the 3rd and 7th PO days. On the 3rd PO day in the IV 5-FU group, the platelet count was found to be significantly lower than the control group ($P < 0.05$).

Bursting pressure

Bursting pressures were measured at anastomotic segments on the 3rd and 7th PO days (Figure 2). Bursting pressure values were significantly lower than the control ($P < 0.01$) and IP group on ($P < 0.05$) the 3rd PO day in the IV 5-FU group. On the 7th PO day, bursting pressure values in the IV and IP groups were not significantly different from the control group. However, bursting pressure values in the IV 5-FU group were significantly lower when compared with the IP group ($P < 0.01$).

Tissue hydroxyproline

Anastomotic hydroxyproline values were statistically significantly higher in the IP group compared to the control and IV group on the 3rd PO day ($P < 0.01$). There were no statistically significant differences between the IV and control groups on PO day 3, regarding hydroxyproline levels. Hydroxyproline levels in the IP group were the highest and anastomotic hydroxyproline levels in the IV group were significantly lower than the IP group on PO day 7 ($P < 0.01$) (Figure 3).

DNA analysis

DNA contents in the anastomotic segments were evaluated using a flow cytometer technique in this study[10]. When the anastomotic segments were examined, a decrease in cell proliferation was noted on the 3rd PO day in the IV group, when compared to the control group, and on the 7th PO day between the control and the IP group. However, this decrease was not statistically significant.

Table 2 Histological scores of anastomotic colon healing in all groups on the 3rd and 7th postoperative days (mean ± SE)

Groups	3rd d			7th d		
	Control	IP	IV	Control	IP	IV
Granulation	1.66 ± 0.70	2.14 ± 0.89	1.42 ± 0.53	2.66 ± 0.50	2.71 ± 0.48	2.57 ± 0.53
Re-epithelization	1.55 ± 1.66	2.71 ± 2.21	1.00 ± 0.00	4.77 ± 1.20	5.28 ± 0.95	2.42 ± 2.14
Muscular tissue	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.22 ± 0.44	1.42 ± 0.44	1.14 ± 0.37

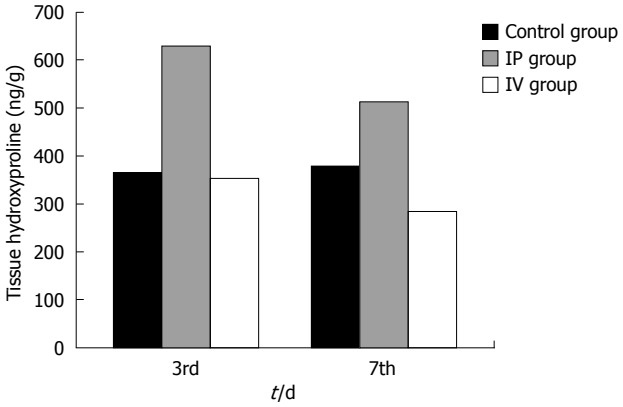

Figure 3 Hydroxyproline levels in anastomotic segment.

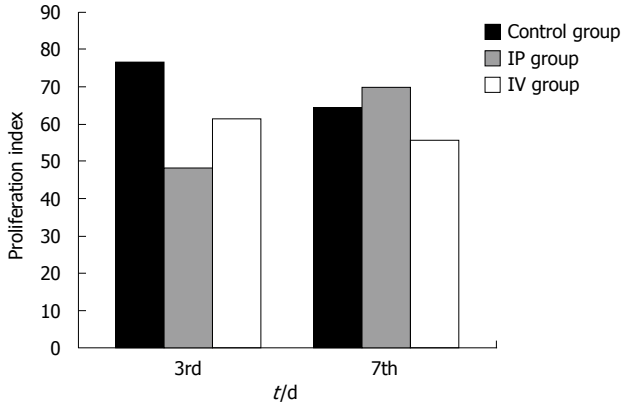

Figure 4 Proliferative cell rates of anastomotic segments.

The tissue proliferation index of anastomotic segments is shown in Figure 4.

Histological analysis

Scores for the histological parameters in the groups are given in Table 2. The best granulation tissue was observed on the 3rd and 7th d in the IP 5-FU group. On the 3rd d of re-epithelization, there was no statistically significant difference between the control and both 5-FU administered groups. However, on the 7th d there was a statistically significant difference between the control and the IV 5-FU group ($P < 0.05$), and between the IP 5-FU and IV 5-FU groups ($P < 0.05$). On multiple comparisons between the groups, the statistically significant difference was maintained ($P < 0.05$). Muscle tissue formation was not complete on the 3rd and 7th d and related values were not statistically different between the groups.

DISCUSSION

Colorectal cancer is the second most frequent type of cancer in industrialized countries. Despite improvements in surgical techniques, almost half of patients with colorectal cancer will eventually die of recurrent disease.

When colon cancer recurs after surgical resection, local disease is found in 25%-40% of patients, peritoneal implants in 12%-28% of patients, and liver metastases in 40% of patients. Hepatic and peritoneal metastases have been reported to be the most frequent failure patterns in resected colorectal cancer patients[12,13].

Solomon *et al*[14] reported on the incidence of colorectal cancer cells on peritoneal surfaces. Overall, 15% of patients had positive cytology for cancer cells in the peritoneal or bowel surface. Stage Ⅱ and Ⅲ disease is treated with wide surgical resection in combination with adjuvant or neo-adjuvant chemoradiation. The combined modality of chemotherapy and surgery increases overall survival and the disease-free period[15-18]. 5-FU was first reported to be effective for colorectal cancer in the 1950s. For more than 10 years, 5-FU was the only adjuvant drug given as a single agent. Since its introduction, 5-FU has remained the cornerstone of adjuvant treatment for colorectal cancer. A number of clinical and experimental data are available on the effects of 5-FU, either alone or in combination with other chemotherapeutic agents, on wound healing[19-22].

In the adjuvant therapy of colorectal cancers, 5-FU was used alone in prolonged systemic regimens or in combination with other agents[22-25]. Fundamentals of preoperative chemotherapy were based on the results of Cole *et al*[5]. According to these results, numerous malignant cells pass into the peripheral circulation during surgical manipulations for localized carcinomas. Preoperatively administered cytotoxic agents may allow tumour resectability and decrease the incidence of distant or local metastases[26].

5-FU can be injected IV and hepatic concentrations can also be achieved intraportally and IP[22,27,28]. In addition, intravenous administration may not allow sufficient penetration in the abdominal cavity. This could be the reason why chemotherapeutic agents may not be effective enough to eliminate micrometastases, especially at the resection site and peritoneal surfaces, which are high risk sites for local recurrence. When the drug is administered *via* the intraperitoneal route, high local and hepatic concentrations can be achieved[28]. Local peritoneal recurrence and haematological toxicity were lower when 5-FU was administered IP. A prospective trial showed that 5-FU administered IP reduced peritoneal failure significantly more than intravenous 5-FU[29]. There

is an increased interest in the use of intraperitoneal chemotherapy with 5-FU to treat advanced colon cancer. Randomised clinical trials have reported a reduction in local recurrence rate with either intraperitoneal 5-FU alone or combined with intravenous 5-FU[30].

Toxic side effects are the major dose-limiting factors in chemotherapy. In patients with advanced primary colon cancer, a significantly higher dose of 5-FU can be given by the intraperitoneal route than by the intravenous route[29]. We used the intraperitoneal approach to deliver high concentrations of 5-FU into the peritoneal cavity without increasing the risk of systemic toxicity. In addition, previous clinical and experimental studies have shown that immediate postoperative 5-FU given IP has no adverse effect on outcome[8].

In 1994, Kelsen *et al*[31] reported a phase I trial of postoperative intraperitoneal floxuridine and leucovorin plus systemic 5-FU and levamisole after resection of high-risk colon cancer. Intraperitoneal therapy appeared to be well tolerated, with no substantial increase in postoperative morbidity and no operative mortality.

In 1998, Scheithauer *et al*[30] accepted 241 patients with resected stage Ⅲ or high-risk stage Ⅱ colon cancer into a trial, comparing intravenous 5-FU and levamisole given for a period of 6 mo with a treatment program consisting of leucovorin plus 5-FU given IV and IP. In patients with stage Ⅲ disease, a significant improvement in disease-free survival and overall survival rates was observed using the systemic plus intraperitoneal treatment, with an estimated 43% reduction in mortality rate. Intraperitoneal and systemic chemotherapy markedly reduced local regional recurrences[30].

It is stated that the highest non-lethal dose of 5-FU is 20 mg/kg for rats and this was the dose administered in our previous trial[11]. The 500 mg/m^2 human dose is equal to the animal dose used in this study. A 3 d interval between the last day of chemotherapy and the surgical procedure was approved to reduce the strong side effects of chemotherapy[6,22].

Weight reductions were detected both during 5-FU injections and after surgery. The weight of the rats was recorded during the 5-FU injection period and days after the operation. All rats lost weight compared with baseline values during 5-FU administration and after the operation, which was statistically significant in the IV group. These results were concordant with reports from the literature[6,8,32]. Weight loss after 5-FU is related to anastomotic healing.

The burst pressure values of the anastomotic segments were measured *in vivo* on the 3rd and 7th PO days in rats receiving and not receiving 5-FU in this study, similar to that in the study by Kuzu *et al*[6]. Anastomotic burst pressures have been measured *in vitro* in most previous similar reports[8,22]. We think that *in-vivo* measurements reflect the clinical status much better. The bursting sites we found in this study are in accordance with those in other studies which reported that the anastomosis is the most common bursting point[22,33]. On the 3rd PO day, bursting pressure values for the IV 5-FU group were found to be significantly lower than the control and IP groups. On the 7th PO day, bursting pressure values in the IV 5-FU group were significantly lower than the IP group. Similar results have been found in previously reported studies[22,32,33].

Graf *et al*[32] reported that early postoperative 5-FU administration had a negative impact on the bursting strength of colonic anastomosis.

5-FU administered preoperatively may also have a negative influence on the ability of fibroblasts to proliferate and synthesize collagen. Reduced collagen synthesis can lead to anastomotic dehiscence[22].

Collagens which guarantee tissue continuity in the tissue repair period contain high proportions of glycine, proline and hydroxyproline. Tissue hydroxyproline levels are important parameters in the tissue repair process[34,35]. Some studies have found that 5-FU decreases the hydroxyproline content of wounds[19,21]. There were significant differences in hydroxyproline levels at the anastomotic segments on both the 3rd and 7th PO days in the IV group compared to the IP group. The decrease in collagen content was correspondant with the decrease in bursting pressures.

It is already known that the chemotherapeutic agent 5-FU inhibits DNA synthesis and cell proliferation, by affecting the cell cycle[36]. Based on this mechanism of action, the effect of chemotherapy on cell proliferation at the anastomotic segment was evaluated by DNA analysis. An obvious decrease in cell proliferation was found at the anastomotic segment of the IV chemotherapy group, compared with the IP and control groups on the 7th PO day. This decrease was not statistically significant. The effects of 5-FU on cancer kinetics determined by DNA analysis, was reported to stress the importance of neo-adjuvant chemotherapy. It was thought that available parameters could be used to determine the anti-tumour effects of 5-FU[37]. The DNA proliferation index can be used as a parameter to determine the effect of chemotherapy on tissue repair; however, further clinical and experimental studies are required.

We looked at histological aspects of the colon during a 7-d period. Although there were no statistically significant differences between the granulation tissues on the 3rd and 7th PO days in our study, the decrease in re-epithelization, as a sign of a mucosal improvement, was statistically significant in the IV group compared with the control and IP groups. According to the results of de Roy van Zuidewijn *et al*[11], completion of colonic muscular tissue repair takes about 21 d. Consistent with our study, muscular repairs were not complete in any of the groups at the 3rd and 7th d of the early tissue repair period, and no difference was found between the control and the experimental groups. Our histological findings suggested that IP 5-FU administration may be preferred over IV administration which was in accordance with our previous results.

All anastomoses were examined macroscopically during the second laparotomy, before the bursting pressure

measurements. Intra-abdominal adhesions were not classified but the integrity of anastomoses, the existence of perianastomotic abscess or peritonitis and the formation of adhesions were less frequent in the IP group, when compared to the control and IV groups.

5-FU derivatives inhibit DNA synthesis by inhibiting thymidylate synthetase, and reduce the biological activity of RNA in both human cells and growing bacteria[38,39].

When applied locally, 5-FU seems to behave like an antibiotic. This confirms certain studies which implied an increasing bactericidal effect when antimicrobial drugs and antineoplastic drugs were administered together. Nyhlén *et al*[40] reported that against two of the three tested strains of *Staphylococcus epidermidis*. the combination of ciprofloxacin and 5-FU resulted in a synergistic prolongation of the postantibiotic effect (PAE) in comparison with the PAE induced by the drugs alone. However, these results need to be confirmed clinically.

In conclusion, our results demonstrate that, early preoperative IV administration of 5-FU has a negative effect on anastomotic healing of the colon. However, the IP route of 5-FU administration has no adverse effect on the healing process of colon anastomoses and burst pressure. This study was performed on healthy animals and may not reflect the exact situation in the case of malignant tumors.

COMMENTS

Background

For cancers of the large bowel, multi-institutional trials have demonstrated a significant reduction in mortality with adjuvant chemotherapy compared with surgery alone. 5-fluorouracil (5-FU) was therefore used as a neoadjuvant therapy in the present study where it had no adverse effect on anastomotic healing and burst pressure.

Research frontiers

5-FU is the mainstay of systemic treatment for colorectal cancer. Intravenous administration may not allow sufficient penetration in the abdominal cavity. This may be the reason why chemotherapeutic agents are not effective enough to kill all micrometastases, especially at the resection site and peritoneal surfaces. Because of these theoretical advantages, neoadjuvant intraperitoneal chemotherapy may be a new treatment option for colorectal cancer. This study investigated the effect of preoperative 5-FU on the healing of colorectal anastomoses in the rat.

Innovations and breakthroughs

5-FU can be injected not only intravenously, but also intraportally and intraperitoneally (IP). When administered by the intraperitoneal route, high local and hepatic concentrations can be achieved. Whether 5-FU compromises wound healing is still controversial. Previous clinical and experimental studies have shown that immediate postoperative 5-FU given IP has no adverse effect on outcome. Therefore, 5-FU was used as neoadjuvant therapy in the present study.

Applications

This study may represent a future strategy for neoadjuvant chemotherapy in colonic cancer.

Terminology

5-FU is the most widely used chemotherapeutic agent in the adjuvant treatment of colon cancer. 5-FU acts during synthesis by interfering with normal pathways. 5-FU might also have a negative influence on the ability of fibroblasts to proliferate and synthesize collagen. Reduced collagen synthesis could lead to anastomotic dehiscence.

Peer review

It is an interesting topic for the readers of *WJG*. The authors described pre- and postoperative variables after two methods of 5-FU administration.

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Fentanyl inhibits glucose-stimulated insulin release from β-cells in rat pancreatic islets

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Abstract

AIM: To explore the effects of fentanyl on insulin release from freshly isolated rat pancreatic islets in static culture.

METHODS: Islets were isolated from the pancreas of mature Sprague Dawley rats by common bile duct intraductal collagenase V digestion and were purified by discontinuous Ficoll density gradient centrifugation. The islets were divided into four groups according to the fentanyl concentration: control group (0 ng/mL), group Ⅰ (0.3 ng/mL), group Ⅱ (3.0 ng/mL), and group Ⅲ (30 ng/mL). In each group, the islets were co-cultured for 48 h with drugs under static conditions with fentanyl alone, fentanyl + 0.1 μg/mL naloxone or fentanyl + 1.0 μg/mL naloxone. Cell viability was assessed by the MTT assay. Insulin release in response to low and high concentrations (2.8 mmol/L and 16.7 mmol/L, respectively) of glucose was investigated and electron microscopy morphological assessment was performed.

RESULTS: Low- and high-glucose-stimulated insulin release in the control group was significantly higher than in groups Ⅱ and Ⅲ (62.33 ± 9.67 μIU *vs* 47.75 ± 8.47 μIU, 39.67 ± 6.18 μIU and 125.5 ± 22.04 μIU *vs* 96.17 ± 14.17 μIU, 75.17 ± 13.57 μIU, respectively, $P < 0.01$) and was lowest in group Ⅲ ($P < 0.01$). After adding 1 μg/mL naloxone, insulin release in groups Ⅱ and Ⅲ was not different from the control group. Electron microscopy studies showed that the islets were damaged by 30 ng/mL fentanyl.

CONCLUSION: Fentanyl inhibited glucose-stimulated insulin release from rat islets, which could be prevented by naloxone. Higher concentrations of fentanyl significantly damaged β-cells of rat islets.

Key words: Fentanyl; Inhibition; Insulin release; Islets

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Qian TL, Wang XH, Liu S, Ma L, Lu Y. Fentanyl inhibits glucose-stimulated insulin release from β-cells in rat pancreatic islets. *World J Gastroenterol* 2009; 15(33): 4163-4169 Available from: URL: http://www.wjgnet.com/1007-9327/15/4163.asp DOI: http://dx.doi.org/10.3748/wjg.15.4163

INTRODUCTION

In recent years, anesthetists and surgeons have had to manage an increasing number of diabetic patients. A concern for the anesthetist is how to control blood glucose and protect islet function in the clinical setting. Because the risk of surgery and anesthesia is higher for these patients, it is particularly important to maintain whole-body glucose homeostasis during the perioperative period. It is well known that many drugs used during surgery for anesthesia or pain relief have effects on pancreatic islets, for example, one study has demonstrated that β-endorphin, an endogenous opioid peptide, can inhibit insulin secretion[1]. Therefore, when administering drugs to diabetic patients, the surgeons and anesthetists must consider the protection of islet function in these patients. Fentanyl was first synthesized 60 years ago and is currently the most popular opioid used in the preoperative period because of its safety and efficacy. In addition,

fentanyl transdermal patches have been developed to manage chronic pain associated with diabetic neuropathy. These patches have been designed to provide continuous, rate-controlled systemic delivery of the fentanyl base for up to 72 h[2,3].

The minimum effective plasma concentration of fentanyl is 0.63 ng/mL after intravenous administration and the therapeutic plasma concentration is 1-2 ng/mL. However, the plasma concentration often exceeds 3 ng/mL because many practitioners prefer to administer high doses of fentanyl[4,5]. In most cancer cases secondary to disease progression, the initial median transdermal fentanyl dose is generally 60-70 μg/h (release rate), which increases to about 170 μg/h over time[6]. Furthermore, a small proportion of patients require doses of between 400 and 1000 μg/h in the latter stages of therapy and the mean treatment period is often longer than 50 d. These large doses are associated with high plasma concentrations (up to 14.5 ng/mL) which are linearly related to the dose[7,8]. Furthermore, fentanyl has been commonly used for patients undergoing cardiac operations because high doses of fentanyl can stabilize the cardiovascular circulatory system during operations. After administration of 50-100 μg/kg fentanyl, the plasma concentration of fentanyl in these patients is commonly above 20-30 ng/mL[9]. However, high plasma concentrations of fentanyl are associated with clinical side effects such as nausea and vomiting, constipation, skin itching and respiratory depression. So far, the potential effects of fentanyl on pancreatic islet β-cells remain unknown.

Because some opioid receptor agonists affect insulin release[10,11], it would be expected that high-doses of fentanyl would have an effect on islet insulin secretion. Studies have suggested that some opiates inhibit insulin secretion[12]. In mouse pancreatic islets incubated under static conditions, glucose-stimulated insulin release is inhibited by β-endorphin and endomorphin-1, endogenous opioid receptor agonists. This inhibition could be prevented by naloxone, an opioid receptor antagonist[13]. Therefore, we hypothesized that fentanyl would affect insulin release. Therefore, we investigated the effect of fentanyl on rat islets in a static culture model and believe the results have important implications on the use of fentanyl in clinical situations, particularly in people with diabetes.

MATERIALS AND METHODS

Animals

Male SD rats weighing 250-300 g were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences and housed under constant conditions of temperature (20-22℃) and artificial lighting (12-h light-dark cycle) before taking part in the study. The study was carried out in accordance with the Guidelines for Animal Experimentation, Tongji University of Shanghai, China, and all the tests were approved by the Animal Experimentation Committee of Tongji University of Shanghai.

Islet preparation

After male adult rats were anesthetized with 50-75 mg/kg sodium pentobarbital, the abdominal wall was cut open and 10 mL of Hank's buffered saline solution (HBSS) containing collagenase V 1.0-1.2 mg/mL (Sigma Chemical Co., St Louis, MO, USA) was injected into the common bile duct of the rat. The pancreas, which was swollen with the digestion solution, was quickly excised and immersed into a plastic culture bottle containing HBSS and incubated with shaking for 13-15 min at 37℃. The digested suspension was obtained by filtering the suspension through a 0.5-mm metal mesh and washed with HBSS containing 2% bovine serum albumin (BSA). A total of 300-400 islets were obtained from each rat by discontinuous Ficoll density gradient centrifugation (density: 1.100, 1.077) (Ficoll, Sigma Chemical Co., St Louis, MO, USA). After being washed with HBSS containing 2% BSA, the islets were cultured for 24 h with 5% CO_2 and collected for further tests[14]. The islets were identified by dithizone (Sigma, USA) staining. Cells stained red under light microscopy were considered to be islets[15]. The combination dyes acridine orange (AO) and propidium iodide (PI) (AO: 0.67 μmol/L; PI: 75 μmol/L) were applied to differentiate between viable and non-viable whole islets; the dyes stained living cells green and dead cells red using minimal background fluorescence[16].

Groups

The islets were divided into four groups according to the fentanyl concentration used: control group (0 ng/mL), group Ⅰ (0.3 ng/mL), group Ⅱ (3.0 ng/mL), and group Ⅲ (30 ng/mL). In each group, the islets were co-cultured for 48 h with drugs as follows: fentanyl alone, fentanyl + 0.1 μg/mL naloxone and fentanyl + 1.0 μg/mL naloxone. There were 12 wells in each group with 30 IEQ (islet equivalent, the diameter of an islet of 150-200 μm equates to 1.7 IEQ) islets/well. An additional two groups were co-cultured with naloxone alone at concentrations of 0.1 or 1.0 μg/mL.

Islet viability

The effect of fentanyl on cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, USA) assay to display a direct correlation with cell metabolism. After co-culturing with fentanyl for 48 h, 100 μL MTT solution was added to each well to a final concentration of 0.5 mg/mL per well and the plates were cultured for 4 h at 37℃. The supernatant was then removed by the addition of DMSO to each well to dissolve the deposition, and the optical density (OD) disparity was read at 490 nm using a spectrophotometer microplate reader (Labsystems, Finland). The inhibition of islet cells caused by the different concentrations of fentanyl was calculated using the formula: inhibition rate (%) = $(1 - OD_{\text{drug exposure}}/OD_{\text{control}}) \times 100$[17,18].

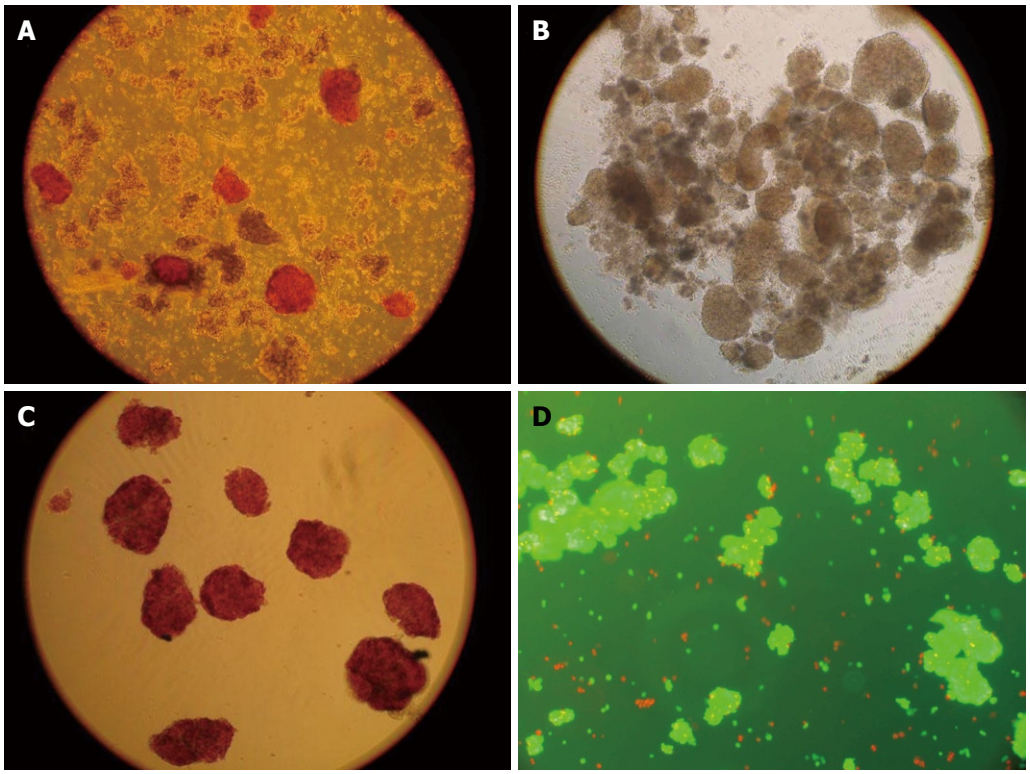

Figure 1 The procedure of islets preparation. A: The islets were stained red by DTZ in digested pancreatic tissue; B: Pure islets isolated from digested pancreatic tissue; C: Pure islets stained by DTZ; D: The dye AO-PI stained living cells green and dead cells red in minimal background fluorescence (× 100).

Glucose-stimulated insulin release assay in static culture

After incubating with the drugs for 48 h, the islets were washed with serum-free and glucose-free medium twice. The insulin release stimulation test was performed by first incubating the islets in low (2.8 mmol/L) and then high (16.7 mmol/L) concentrations of glucose in static culture medium for 1 h each. Supernatant from each well was collected and the insulin level was determined using a rat insulin radioimmunoassay kit (Linco Research, Inc, St Charles, MO, USA). The stimulation index (SI) was calculated by dividing the value of high glucose-stimulated secretion by the value of low glucose-stimulated secretion[19,20].

Electron microscopy studies

After co-culturing with fentanyl for 48 h, the islets in the control group and those in group Ⅲ were cut into thin sections and mounted on slides. Samples were stained with 2% uranyl acetate and lead citrate. The sections were viewed and photographed on a JEOL JEM-1230 transmission electron microscope (Jeol Ltd., Japan) at 80 kV.

Statistical analysis

Statistical and graphic analyses were performed with SPSS 13.0 software. Differences between groups were evaluated with one-way ANOVA or Kruskal-Wallis H tests, as appropriate, and the differences between two groups were analyzed with the LSD test or Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

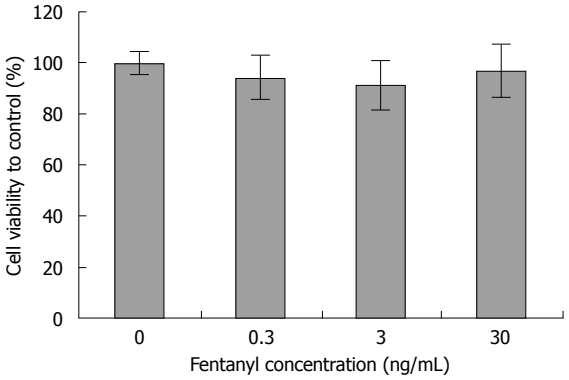

Figure 2 The cell viability after co-culture with fentanyl. The viability measured by MTT was 100% in the control group. The viability of islets exposed to 0.3, 3 and 30 ng/mL fentanyl was 94.3%, 91.3% and 96.9%, respectively. There was no difference between groups. The data represent means ± SE.

RESULTS

Islet viability assessment

In this study, the rat islets were isolated from the rat pancreas with good quality. The viability was about 90% and the purification rate was about 95% (Figure 1). Fentanyl had no effect on cell viability (Figure 2).

Insulin release test

Fentanyl significantly inhibited the low and high concentration glucose-stimulated insulin release in a concentration-dependent manner ($P < 0.01$), and the insulin release was lowest at the concentration of 30 ng/mL ($P < 0.01$). After adding 0.1 μg/mL naloxone, fentanyl still significantly inhibited glucose-stimulated insulin release ($P < 0.01$). However, after adding 1 μg/mL naloxone,

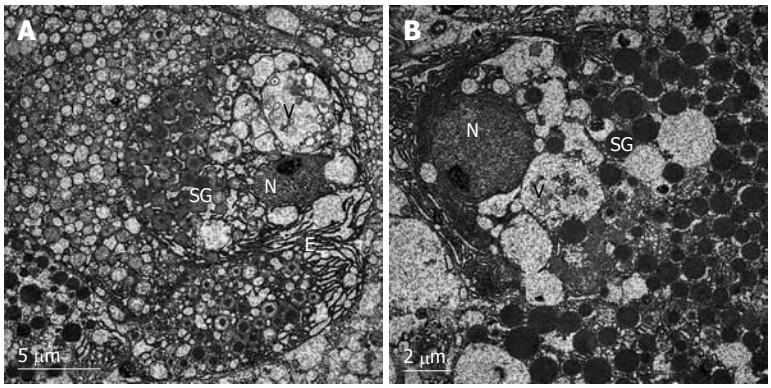

Figure 3 Electron micrographs of β-cells in rat pancreatic islets of control group. N: Nucleus; SG: Secretory granule; V: Vesicle; E: Endoplasmic reticulum. The typical β-cells had an abundance of cytoplasmic granules and endoplasmic reticulum. The electron density of the granules increased when the granules were maturating in the vesicles. The mature secretory granules displayed a highly electron-dense core surrounded by a wide electron-lucent halo. The granules had a space between the core and the membrane. The vesicles showed a normal round outline (A, × 6000). There were many nascent granules in the β-cells; subsequent maturation involved further condensation of the matrix constituents and a reduction in granule diameter (B, × 8000).

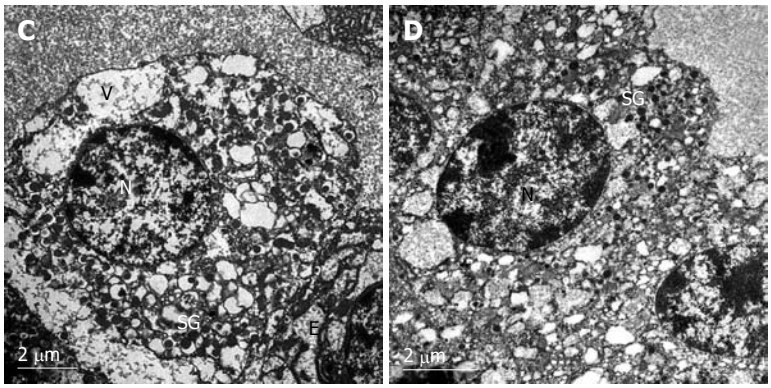

Figure 4 Electron micrographs of rat pancreatic islet β-cells after exposure to 30 ng/mL fentanyl for 48 h. N: Nucleus; SG: Secretory granules; V: Vesicle; E: Endoplasmic reticulum. Chromatin margination and severe cytoplasmic vacuolization and degeneration were observed in the islets. Large and small vacuoles are present in the cytoplasm. The secretory granules and endoplasmic reticulum were much smaller than in the control group (A, × 10 000). In addition, endoplasmic reticulum and normal vesicles were almost never observed (B, × 12 000).

Table 1 The glucose-stimulated insulin release (μIU) in response to fentanyl with different concentrations of naloxone (mean ± SD, n = 12)

	Fentanyl alone		Fentanyl + 0.1 μg/mL naloxone		Fentanyl + 1 μg/mL naloxone	
	Low glucose	High glucose	Low glucose	High glucose	Low glucose	High glucose
Control (0 ng/mL)	62.33 ± 9.67	125.5 ± 22.04	62.33 ± 9.67	125.5 ± 22.04	62.33 ± 9.67	125.5 ± 22.04
Ⅰ (0.3 ng/mL)	54.75 ± 5.93[a]	118.17 ± 16.81	54.33 ± 8.99	110.4 ± 15.69	61.5 ± 8.13	126.75 ± 16.48
Ⅱ (3 ng/mL)	47.75 ± 8.47[b]	96.17 ± 14.17[b]	45.92 ± 7.63[b]	88.25 ± 11.22[d]	59.75 ± 8.42	118.33 ± 21.09
Ⅲ (30 ng/mL)	39.67 ± 6.18[b]	75.17 ± 13.57[b]	36.33 ± 5.79[b]	68.67 ± 11.99[d]	61.08 ± 8.07	126.0 ± 15.54
Naloxone			61.67 ± 9.16	120.3 ± 18.04	60.67 ± 9.15	123.0 ± 20.89

[a]P < 0.05, [b]P < 0.01 *vs* control group (Mann-Whitney U test); [d]P < 0.01 *vs* control group (LSD test). Low- and high-glucose-stimulated insulin release in the 3 and 30 ng/mL fentanyl groups was significantly lower than in the control group (P < 0.01), and was lowest with 30 ng/mL fentanyl (P < 0.01). After adding 0.1 μg/mL naloxone, insulin secretion in the 3 and 30 ng/mL fentanyl groups remained significantly lower than the control group (P < 0.01) and was lowest with 30 ng/mL fentanyl (P < 0.01).

the insulin release showed no difference from that of controls. Naloxone had no effect on insulin release (Table 1). There was no difference in SI between any of the groups (Table 2).

Evaluation of electron microscopy studies

Electron micrography of β-cells in rat islets from the control group showed no pathological changes (Figure 3). After exposure to 30 ng/mL fentanyl for 48 h, the β-cells were in a poor morphological condition, and exhibited chromatin margination and severe cytoplasmic vacuolization and degeneration (Figure 4).

Table 2 Stimulation index at different concentrations of fentanyl and naloxone (mean ± SD, n = 12)

	Fentanyl	Fentanyl + 0.1 μg/mL naloxone	Fentanyl + 1 μg/mL naloxone
Control (0 ng/mL)	2.01 ± 0.21	2.02 ± 0.21	2.02 ± 0.21
Ⅰ (0.3 ng/mL)	2.17 ± 0.36	2.07 ± 0.35	2.08 ± 0.26
Ⅱ (3 ng/mL)	2.04 ± 0.26	1.95 ± 0.23	1.99 ± 0.34
Ⅲ (30 ng/mL)	1.89 ± 0.16	1.89 ± 0.19	2.08 ± 0.27
Naloxone		1.97 ± 0.29	2.04 ± 0.28

DISCUSSION

Many studies have focused on islet isolation, application and function in the diabetes field. Many factors affect the isolation of rat pancreatic islets; therefore, the yields and function of the isolated islets vary considerably, despite the introduction of novel or improved methods. Large variations in yields and function have been found, even if the same sources of collagenase are used[21]. In our study, the islets were isolated from the rat pancreas with good quality (viability rate was about 90% and purification rate was about 95%). Generally, we obtained 300-400 islets from each rat. This indicates that our method is efficient and provides high yields of islets.

The high yields and favorable function of the isolated islets were attributed to the pancreatic tissue being infused and fully digested. Low yields of islets typically result from insufficient digestion; therefore, we suggest that the digestion time and collagenase concentration should be optimized precisely for islet isolation.

Over the last two decades, despite the development of more potent, safer, faster onset, and shorter- and longer-lasting opioids, fentanyl has remained the mainstay of anesthesiologists and Certified Registered Nurse Anesthetists in the perioperative period, and for physicians involved in pain management. Because diabetic patients undergo higher risk procedures during the period of operation and anesthesia, anesthetists and surgeons should protect islet function and try to minimize any harmful medical effects on islets during the procedure. Although some studies have suggested that some opiates inhibit insulin secretion, it was unknown whether fentanyl has a similar effect[12].

Before our current study, a number of studies have demonstrated direct effects of endogenous and selective opioid receptor agonists on insulin release[10,11]. Some opioid receptor agonists, such as methadone, were found to improve multiple-low-dose streptozotocin-induced type 1 diabetes in mice, which suggests that the opioid receptor agonists improve insulin release *in vivo*[22]. However, other studies have suggested that some opiates inhibit insulin secretion[12]. β-endorphin seems to inhibit glucose-induced insulin secretion but, conversely, an excitatory effect has been reported in many studies[11,23]. These observations have yielded conflicting results with marked variations between species. Therefore, it could not be hypothesized whether fentanyl inhibits insulin release[13].

Our results clearly demonstrate that fentanyl inhibited glucose-stimulated insulin release from islets *in vitro*. Electron microscopy studies showed that the cells in the control group exhibited good viability but, after the cells were exposed to 30 ng/mL fentanyl for 48 h, chromatin margination and severe cytoplasmic vacuolization were observed, indicating that the cells were in a poor condition. Thus, our test suggests that the islets were injured by exposure to fentanyl at the concentrations tested.

We know that glucose uptake by tissues is as likely to play a decisive role as does the release of insulin. In the presence of insulin, opioid receptors in the pancreas have been reported to regulate plasma glucose and the activation of mu-opioid receptors, which could increase the utilization of glucose in peripheral tissue to lower the plasma glucose[24,25]. *In vivo*, a potent opiate was shown to lower glucose levels by enhancing peripheral glucose utilization without improving insulin release[26]. Also, serum glucose levels are responsible for the altered potency of mu-opioid agonists only during the early stages of diabetes[27]. Thus, the plasma glucose-lowering response induced by mu-opioid receptor activation *in vivo* is not attributed to improved insulin release.

Endogenous opioid receptors are expressed in endocrine pancreas and studies have indicated that endogenous opioid peptides and selective opiate receptor agonists suppress insulin release[28]. Thus, the effect of fentanyl on insulin release might be related to opiate receptor activation. Activation of the opioid receptor initiates a cascade of events that result in an array of biological effects, which inhibit insulin release[29,30]. It seems that fentanyl-induced mu-opioid receptor activation leads to the inhibition of glucose-stimulated insulin release. This inhibitory effect could be reversed by high doses of naloxone, an opioid receptor antagonist. Therefore, the activation of opioid receptors induced by fentanyl appears to be a route by which insulin release is inhibited.

There are two processes that take place during insulin secretion: hormone biosynthesis and release. Many drugs affect insulin secretion, although the exact mechanism varies. Many drugs have different effects on either of the two processes. For example, long-chain fatty acids powerfully increase insulin release and inhibit glucose-stimulated pro-insulin biosynthesis[31,32]. In the present study, it is unknown which of these processes are affected by fentanyl. Because fentanyl may be involved in both insulin release and biosynthesis[33,34], the role of opioid receptor activation on insulin secretion requires further investigation.

There are certain limitations in our experiment. The pharmacology of the mu receptor signaling pathway involves G-protein coupling which, when activated, closes Ca^{2+} channels and opens K^+ channels, resulting in a decline in intracellular Ca^{2+} levels and hyperpolarization; all of which are short-term effects. The magnitude of the insulin response to glucose is related not only to the absolute level of glucose but also to the rate of change in the glucose level. There are two phases of insulin release. An acute increase in glucose level elicits a rapid and transient secretion of insulin, called the first or acute-phase response, which subsides within 10 min. The second phase response begins when glucose levels increase slowly and progressively for up to 4 h[35]. As in many other studies, we measured the insulin release over 60 min in static culture after incubating in low (2.8 mmol/L) and then high (16.7 mmol/L) glucose concentrations[19,20,36]. Therefore, the effect of fentanyl on glucose-stimulated insulin release in rat islets in dynamic culture also needs to be investigated. In addition, perfusion of islets with glucose provides a dynamic profile of the characteristics of glucose-stimulated insulin release, which could be used to fully determine the effect of fentanyl on the inhibition of insulin release, and the ability of the cells to down-regulate insulin secretion after a fentanyl challenge are a focus of our future studies.

In conclusion, fentanyl inhibited glucose-stimulated insulin release from islets, and the inhibition could be reversed by naloxone. Because islet function varies between animal species, translation of our results to the

clinical setting will necessitate further studies. However, our results indicate the potential for fentanyl to inhibit insulin release; thus, it is necessary to test the plasma insulin level and the islet function regularly in patients treated with fentanyl, particularly patients treated with fentanyl for a long term and/or with high doses of fentanyl. Further studies are needed to explore the exact mechanism by which fentanyl affects islet insulin release.

COMMENTS

Background

Fentanyl citrate is a potent synthetic narcotic analgesic extensively used for anesthesia and analgesia in the operating room and intensive care unit. In recent years, anesthetists and surgeons have had to manage an increasing number of diabetic patients. However, the effects of fentanyl on the glucose-stimulated insulin secretion capacity of rat β-cells *in vitro* has remained unclear. Although some studies have suggested that some opiates inhibit insulin secretion, it could not be concluded whether fentanyl has a similar effect, because the earlier studies yielded conflicting results, with marked species variation. Therefore, this study investigated the effects of fentanyl on rat islets in static culture.

Research frontiers

This study is a novel field in diabetes investigation that has been largely overlooked. The research team used freshly isolated islets to study the effects of the opiate fentanyl on insulin release, which is an important subject for anesthetists and surgeons. In this study, the results demonstrated that fentanyl inhibited glucose-stimulated insulin release in rat islets and higher concentrations of fentanyl significantly damaged rat islets.

Innovations and breakthroughs

The study of effects of anesthetic and analgesic drugs on pancreatic islets is a novel field in diabetes management. This is the first study to report that fentanyl inhibits the release of insulin from rat islets. These findings would be helpful for clinicians who administer fentanyl.

Applications

The results may stimulate further investigation of diabetes management in the anesthesia field. These findings suggest that it is important to regularly test the plasma insulin level and islet function in patients treated with fentanyl for the long term and/or at high doses, and could help to develop novel approaches to help people with diabetes to maintain whole-body glucose homeostasis during the perioperative period.

Peer review

The author has used rat islets as a model to study the effect of the mu-opioid agonist fentanyl on glucose-stimulated insulin release. Generally, this is a topic that has been overlooked in the past, thus this study is important and unique. Also, the islet preparation and EM technology were good.

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BRIEF ARTICLES

Indicators of prognosis after liver transplantation in Chinese hepatocellular carcinoma patients

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Abstract

AIM: To identify prognostic factors of patients with hepatocellular carcinoma (HCC), who were treated by orthotopic liver transplantation (OLT).

METHODS: From January 2000 to October 2006, 165 patients with HCC underwent OLT. Various clinicopathological risk factors for actuarial and recurrence-free survival were identified using the Kaplan-Meier method with the log-rank test. The Cox proportional hazards model was used to identify independently predictive factors for actuarial and recurrence-free survival, which were used to propose new selection criteria. We compared the outcome of the subgroup patients meeting different criteria. Survival analysis was performed using the Kaplan-Meier method with the log-rank test.

RESULTS: The median follow-up was 13.0 mo (2.8-69.5 mo). Overall, 1-, 2-, 3- and 5-year actuarial survival was 73.3%, 45.6%, 35.4% and 32.1%, respectively. One-, 2-, 3- and 5-year overall recurrence-free survival was 67.0%, 44.3%, 34.5% and 34.5%, respectively. In univariate analysis, number of tumors, total tumor size, lobar distribution, differentiation, macrovascular invasion, microvascular invasion, capsulation of the tumor, and lymph node metastasis were found to be associated significantly with actuarial and tumor-free survival. By means of using the multivariate Cox proportional hazards model, total tumor size and macrovascular invasion were found to be independent predictors of actuarial and tumor-free survival. When the selection criteria were expanded into the proposed criteria, there was no significant difference in 1-, 2-, 3- and 5-year actuarial and tumor-free survival of the 49 patients who met the proposed criteria (97.6%, 82.8%, 82.8% and 82.8%, and 90.7%, 82.8%, 68.8% and 68.8%, respectively) compared with that of patients who met the Milan or University of California, San Francisco (UCSF) criteria.

CONCLUSION: Macrovascular invasion and total tumor diameter are the strongest prognostic factors. The proposed criteria do not adversely affect the outcome of liver transplantation for HCC, compared with the Milan or UCSF criteria.

Key words: Hepatocellular carcinoma; Prognosis; Liver transplantation; Outcome assessment; Survival

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INTRODUCTION

In the 1980s, outcomes for orthotopic liver transplantation (OLT) for hepatocellular carcinoma (HCC) were discouraging. There were high recurrence rates and a low patient survival of 30% at 3 years[1]. After the Milan criteria (single tumor up to 5 cm or up to three tumors up to 3 cm) were introduced by Mazzaferro *et al*[2] in the

early 1990s, recurrence rate fell to 8%, and tumor-free patient survival at 4 years was 83%. With worldwide adoption of these criteria, 5-year survival rates rose to 60%-80%[3-5].

Recently, there have been numerous proposals for expanding the Milan criteria. The University of California, San Francisco (UCSF) proposed an expansion of transplantation criteria[6]. The expanded criteria includes having a single tumor < 6.5 cm in diameter, or having no more than three tumors, the largest of which is < 4.5 cm in diameter, and with a total tumor diameter of < 8 cm. Using these criteria, 1- and 5-year recurrence-free survival rates were 90% and 75.2%, respectively. Marsh *et al*[7] have studied 407 cases who underwent OLT at the University of Pittsburgh between 1981 and 2002, and found that patients who exceeded the Milan criteria had a 49.7% recurrence-free survival rate at 5 years. Todo *et al*[8] have reported that patients with unresectable HCC who underwent living donor liver transplantation under the expanded indication criteria had 3-year survival and disease-free survival rates of 60.4% and 52.6%, respectively. In Kyoto[9], the only OLT exclusion criteria are for patients with extrahepatic metastasis or macroscopic vascular invasion, and there are no restrictions based upon on the number or size of tumors. There was no difference in the 4-year survival rate in the Kyoto study between patients with HCC who fit (66%) or do not fit (60%) the Milan criteria. Therefore, it has been suggested that there are differences in OLT criteria between the East and West, which may be correlated with race or combinations of underlying hepatic diseases.

Worldwide, 55% of HCC cases and deaths occur in China[10]. Over 90% of HCC patients in China are also infected with hepatitis B virus (HBV). These rates are different from those in Europe, America, and even Japan. To explore these issues, we evaluated the OLT outcomes for HCC patients at the Liver Transplantation Center of the West China Hospital, Chengdu, China. We aimed to analyze the effect of HCC prognostic factors on actuarial and recurrence-free survival after OLT, and to reevaluate the prediction criteria that are the basis for OLT selection.

MATERIALS AND METHODS

Patients

Between January 2000 and October 2006, a total of 424 OLTs were performed at the Liver Transplantation Center of the West China Hospital. Among these, 165 (38.9%) patients were diagnosed with HCC, and 148 underwent follow-up. Follow-up excluded 17 patients who died of complications during the 3-mo period after the operation. Of the 148 HCC patients, 146 were diagnosed with HCC before liver transplantation. In two patients, HCC was found incidentally by pathological examination of the explanted liver after transplantation. Preoperative clinical data of the 148 HCC patients are shown in Table 1. Liver transplantation was considered for patients with HCC if the tumor was determined to be unresectable because of its location, or because of concomitant liver disease and HCC without extrahepatic spread.

Table 1 Clinical data for 148 patients

	n (%)
Sex	
Male	133 (89.9)
Female	15 (10.1)
Age (yr)	Range 17-68 (median 45)
Etiology	
Hepatitis B	137 (92.6)
Hepatitis B, C	2 (1.4)
Alcoholic	1 (0.7)
Idiopathic	8 (5.4)
Pre-transplantation AFP (ng/mL)	
≤ 400	60 (40.5)
> 400	88 (59.5)
Child-Pugh Class	
A	76 (51.4)
B	57 (38.5)
C	15 (10.1)
Meld score	
< 14	107 (72.3)
≥ 14	41 (27.2)
Pre-transplantation therapy	
Positive	47 (31.7)
Negative	101 (68.2)
Tumor stage	pTNM
Ⅰ	27 (18.2)
Ⅱ	40 (27.0)
Ⅲa	66 (44.6)
Ⅲb	10 (6.8)
Ⅲc	5 (3.4)

Diagnosis

Preoperative diagnosis of HCC was made using artery high-flow perfusion and imaging examinations, which showed intrahepatic tumors. Examinations included at least two of the following three methods: ultrasound, contrast-enhanced computed tomography (CT) and magnetic resonance imaging (MRI). Macroscopic vascular invasion was observed using Doppler ultrasonography and contrast-enhanced CT before operation, and was validated through pathological examination of the impaired liver after operation. Diagnosis of HCC was confirmed by fine-needle aspiration cytology or biopsy of all known tumors. Pretransplant studies in patients with HCC included abdominal, thoracic, and head CT scans and bone scintigraphy to rule out extrahepatic tumor spread.

Follow-up

The patients were followed for tumor recurrence with CT scans and α-fetoprotein level (AFP) every 6 mo for 5 years. Annually thereafter, or as the clinical situation dictated, suspicious lesions in the liver or lung were biopsied. Bone lesions were not biopsied routinely but were observed for bone pain and progression of growth. A rising AFP level alone was not taken to be confirmatory

of tumor recurrence, but the date of recurrence was taken as the time that the AFP level began to rise once tumor recurrence had been confirmed.

Immunosuppression and antiviral protocols

Immunosuppression consisted of cyclosporine or tacrolimus and corticosteroids, with or without azathioprine and mycophenolate. In cases in which acute rejection was suspected, a liver biopsy was performed, and steroid pulse therapy was conducted after the rejection diagnosis was made. The steroids were withdrawn 3-6 mo after surgery to minimize the risk of tumor recurrence[8,11]. Lamivudine was administrated to hepatitis-B-surface-antigen-positive patients before and after surgery, and hepatitis B immune globulin was administered to HBV-DNA-positive patients before, during and after surgery.

Statistical analysis

Cumulative survival time was calculated from the date of transplantation to the date of death, from the date of transplantation to the date of final follow-up, or from the date of transplantation to the date of loss to follow-up. The latter two conditions were tabulated from censored data. Tumor-free survival (TFS) time was calculated from the date of transplantation to the date when tumor recurrence and metastases were discovered, from the date of transplantation to the date of final follow-up, or from the date of transplantation to the date of loss to follow-up. The latter two conditions were also tabulated from censored data. Statistical analysis software (SPSS 13.0) was used for data processing and analysis. The Kaplan-Meier method was used to calculate the cumulative survival rate (CSR), TFS rate, and to present the corresponding survival curves in graphical form. The log-rank test was used to compare the differences between groups. The univariate Cox proportional hazard regression model was used to analyze each factor that might have influenced liver transplantation prognosis in HCC patients, and to identify factors with statistical significance. The multivariate Cox proportional hazard regression model was used to analyze and confirm the independent prognostic factors for OLT in HCC patients. Statistical significance was defined as $P < 0.05$.

RESULTS

Pathological characteristics of explanted liver

The pathological data on HCC tumors and potential indicators are shown in Table 2. In general, OLT was offered when liver function was impaired, or when the HCC become unresectable. HCC was diagnosed before transplantation in 148 patients, and in two, we identified an incidental tumor during pathological examination of the explanted liver.

Survival

The median follow-up time for the 148 patients was 13.0 mo (2.8-69.5 mo). The follow-up time of 126 of these patients was ≥ 6 mo. Eighty-one patients survived, with a median follow-up of 15.5 mo (2.8-69.5 mo). Sixty-two patients died, with a median follow-up of 11.0 mo (3.0-38.0 mo). Five patients were lost to follow-up, with a median follow-up of 16.0 mo (14.0-23.0 mo).

Table 2 Pathological data from 148 HCC patients

Characteristics	*n* (%)
Tumor	
≤ 3	101 (68.2)
> 3	47 (31.8)
Total tumor size (cm)	
≤ 5	24 (16.2)
5–9	30 (20.9)
> 9	94 (63.5)
Lobar distribution	
Unilobar	80 (54.1)
Bilobar	68 (45.9)
Differentiation	
Well	35 (23.6)
Moderate	100 (67.6)
Poor	13 (8.8)
Vascular invasion	
None	47 (31.8)
Micro	49 (33.1)
Macro	52 (35.1)
Lymph node status	
Negative	143 (96.6)
Positive	5 (3.4)
Cirrhosis	
Negative	13 (8.8)
Positive	135 (91.2)

The mean total cumulative survival time of the entire group was 33.6 mo (95% CI: 27.5-39.6 mo). The CSR was 73.3% at 12 mo, 45.6% at 24 mo, 35.4% at 36 mo, and 32.1% at 60 mo. The average TFS time of the entire group was 32.8 mo (95% CI: 26.4-39.1 mo). The TFS rate was 67.0% at 12 mo, 44.3% at 24 mo, 34.5% at 36 mo, and 34.5% at 60 mo.

Predicators of actuarial survival

Based on the results of the univariate Cox regression model analysis, the following 10 variables significantly affected CSR: (1) age; (2) largest tumor size; (3) total tumor size; (4) tumor number; (5) bilobar disease; (6) macrovascular invasion; (7) microvascular invasion; (8) lymph nodes positive; (9) differentiation; and (10) capsule invasion. The above 10 variables were analyzed using the multivariate Cox proportional hazard regression model and the stepwise regression method, to identify the independent factors that influenced total survival rate. The results showed that total tumor size and macrovascular invasion were the two risk factors that affected total survival rate.

Predictors of recurrence-free survival

According to the results of the univariate Cox regression model analysis (Table 3), the following eight variables significantly affected tumor-free survival rate: (1) total tumor size; (2) tumor number; (3) bilobar tumor; (4) tumor differentiation; (5) macrovascular invasion; (6) microvascular invasion; (7) tumor capsular invasion;

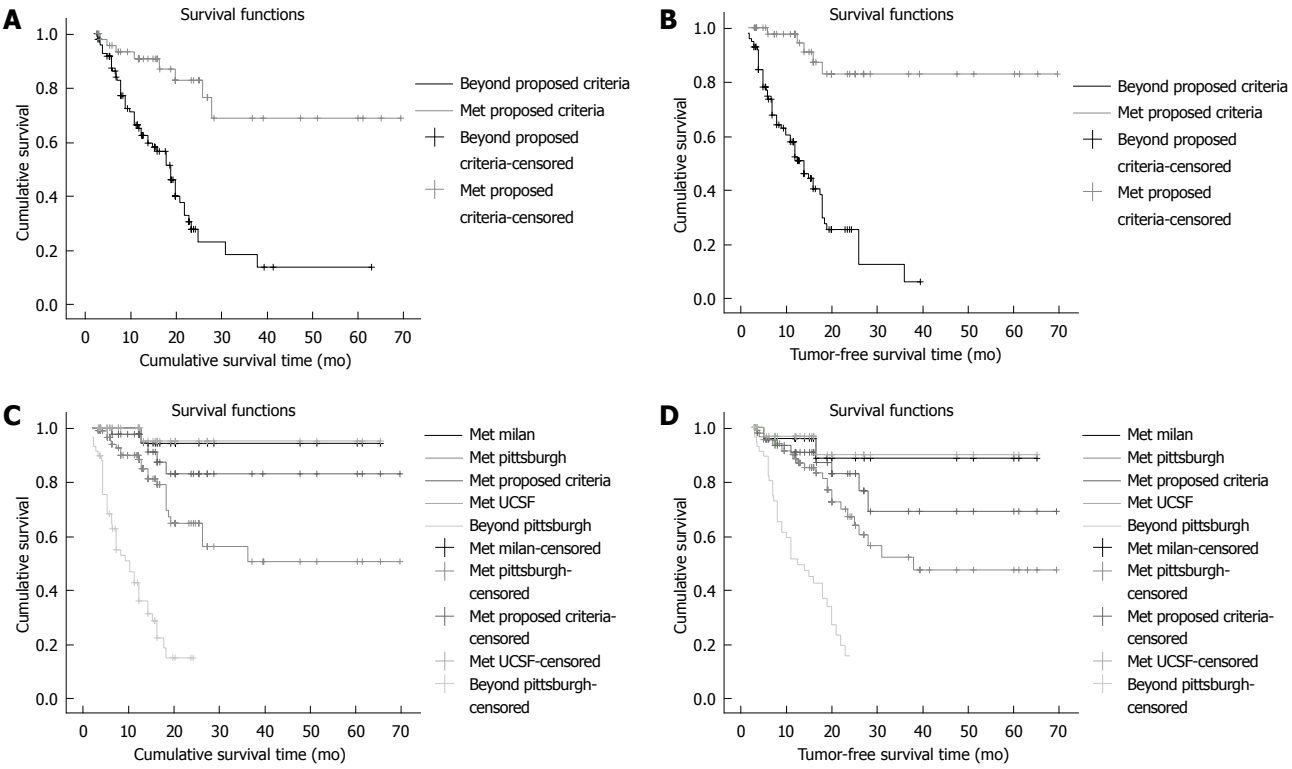

Figure 1 Proposed prognostic criteria. A: CSR for proposed criteria. Log-Rank test: $P < 0.001$; B: TFS rate for proposed criteria. Log-Rank test: $P < 0.001$; C: Comparison of CSR for different criteria; D: Comparison of TFS rate for different criteria.

Table 3 Univariate analysis of all variables that significantly affected tumor-free survival

Predictors	Relative risk	95% CI	*P*
Total tumor size (≤ 9 cm *vs* > 9 cm)	2.291	1.654-3.173	0.000
Tumor number (≤ 4 *vs* > 4)	1.127	1.052-1.207	0.003
Bilobar disease	2.206	1.327-3.666	0.002
Macrovascular invasion	2.951	2.160-4.032	0.000
Microvascular invasion	4.479	2.126-9.436	0.000
Positive lymph nodes	12.472	4.457-34.898	0.000
Differentiation	1.641	1.014-2.654	0.044
Capsule invasion	1.462	1.092-1.957	0.011

and (8) lymph nodes positive. The above eight variables were analyzed using the multivariate Cox proportional hazard regression model and the stepwise regression method, to identify independent factors that influenced the TFS rate. The results showed that total tumor size, macrovascular invasion and lymph node status were the independent predictors of TFS.

Proposed criteria

Macrovascular invasion and total tumor size were prognostic factors that independently influenced CSR and TFS. Our proposed criteria for OLT selection included patients with total tumor size ≤ 9 cm and who were without macrovascular invasion or extrahepatic metastases, regardless of the number of tumor lesions.

For the cases which conformed to the proposed criteria ($n = 49$), the total survival rate at 1, 2, 3 and 5 years was 97.6%, 82.8%, 82.8% and 82.8%, respectively (Figure 1A). The average survival time was 53.7 mo (43.8-63.6 mo). Among these 49 patients, 18 (16.3%) died by the end of follow-up, and seven (9.5%) died of recurrence. There were 41 cases of TFS (83.7%). The average TFS rate at 1, 2, 3 and 5 years was 90.7%, 82.8%, 68.8% and 68.8%, respectively (Figure 1B). The average TFS time was 60.0 mo (52.3-67.7 mo).

Comparisons of the CSR and the TFS at 1, 2, 3 and 5 years for different patient subgroups are shown in Figure 1C and D. CSR and TFS were similar in patients who met the proposed criteria and Milan criteria ($P = 0.321$, $P = 0.331$), and UCSF criteria ($P = 0.229$, $P = 0.257$). However, the patient number was increased more in the proposed criteria ($n = 49$), than the Milan ($n = 24$) or UCSF ($n = 33$) criteria. What's more CSR was similar in patients who met the proposed criteria and the Pittsburgh criteria ($P = 0.158$). Although the patient number was greater in the Pittsburgh ($n = 90$) than the proposed criteria,the TFS for the Pittsburgh criteria was lower than that for the proposed criteria ($P = 0.027$).

DISCUSSION

Numerous studies have shown that tumor size is an important prognostic factor for liver transplantation in patients with HCC[8,12,13]. Bismuth *et al*[12] have found that HCC patients with two or fewer tumors, each with a diameter ≤ 3 cm, had an 83% 3-year CSR and TFS following liver transplantation and hepatectomy. Roayaie *et al*[14] have reported that HCC patients with tumor diameters > 7 cm (12 cases) and 5-7 cm (32 cases) had 5-year tumor-free rates of 34% and 55%, respectively,

following transplantation. Tumor diameter > 7 cm and the presence of vascular invasion were correlated with HCC recurrence. In this study, total tumor size was shown to influence prognosis in the univariate Cox regression model analysis, and was shown to be an independent prognostic factor in the multivariate analysis. Compared to largest tumor size, tumor number can better predict the OLT prognosis in HCC patients. Univariate Cox regression analysis showed that the 2-year CSR and TFS rates were 73.5% and 75.6%, respectively. These rates were significantly different compared to those in cases where total tumor size was > 9 cm. Tumor number and size are believed to have a combined effect on HCC recurrence probability, and this effect is taken into consideration during selection of HCC OLT recipients in many institutions[2,6]. The total tumor size in the present study also reflects a combined effect of tumor number and size.

Many other studies have shown that macrovascular invasion is a primary factor that influences prognosis after OLT in HCC patients[12,13,15-18]. Shetty *et al*[18] have concluded that macrovascular invasion and AFP levels were prognostic factors that influenced CSR and TFS. Moreover, microvascular invasion had no significant influence on prognosis. Bismuth *et al*[12] have reported that 10 of 60 (16.7%) HCC patients who underwent OLT had tumor thrombus in the main trunk of the portal vein, and had a 3-year survival rate of only 20%. Thus, they proposed that tumor thrombus in the main trunk of the portal vein was a major risk factor for OLT. Other studies have proposed that when HCC is accompanied by vascular invasion, tumor cells are more likely to be present in the circulation, rather than being restricted to the liver[19,20]. While circulating tumor cells may not develop into distant metastases, immunosuppressive treatment after OLT may increase the possibility of tumor recurrence. Currently, most transplantation centers advocate exclusion of HCC patients with tumor thrombus in the main trunk or right and left branches of the portal vein, for these reasons. In the present study, multivariate Cox regression analysis showed that macrovascular invasion was an independent risk factor that affected CSR and TFS. In the 52 cases with macrovascular invasion, the recurrence rate was as high as 71.2% during follow-up. The 1-year CSR was 53.5%, and TFS was 14.4%. The 2-year CSR and TFS were 34.2% and 12.9%, respectively. Therefore, OLT patients with macrovascular invasion had poor long-term prognosis.

Univariate analysis identified hepatic lobar distribution, differentiation, and capsule invasion as prognostic factors that influenced CSR and TFS in the present study. However, multivariate analysis showed that only total tumor size and macrovascular invasion were independent prognostic factors that influenced CSR and TFS. Therefore, hepatic lobar distribution, differentiation, and capsule invasion may have a relationship with total tumor size or macrovascular invasion. We believe that these three indicators may reflect malignant tumor invasion to a certain degree, since HCC patients who have bilobar distribution, low differentiation or capsular (or non-capsular) tumor invasion have a relatively high incidence of tumor recurrence and metastasis.

Our data showed that age was related significantly to CSR by univariate Cox regression analysis, with a relative risk of 0.963 (95% CI: 0.936-0.991). The younger the onset of HCC, the greater the possibility of tumor recurrence was after liver transplantation. An early age of HCC onset may lead to greater malignancy and faster disease development. An understanding of this mechanism requires further exploration.

Positive lymph nodes and distant metastasis are considered to be absolute contraindications for liver transplantation in HCC patients[21]. Marsh *et al*[22] have found that the average TFS time for 231 HCC liver transplantation cases with negative local lymph nodes was 140.6 ± 6.8 mo. The TFS time was significantly less, 5.3 ± 1.0 mo, for six cases with positive local lymph nodes. In the present study, five HCC cases (3.4%) had positive lymph nodes in the porta hepatis. Four of these patients died, and all five had tumor recurrence. The average survival time of these five patients was only 7.0 mo, and the average TFS time was 4.2 mo, in accordance with previous studies. Detection of lymph nodes can be observed in HCC patients with hepatitis, who have enlarged, inflammatory lymph nodes. Therefore, careful observations should be conducted during surgery, and all enlarged lymph nodes should be sent to the pathology department for intraoperative frozen-section examination. Liver transplantation should not be carried out in patients with confirmed positive lymph nodes.

China has the greatest incidence of liver cancer worldwide[10]. Each year, over half of liver cancer detections and deaths occur in China. Liver cancer (mainly HCC) has been the primary indication for liver transplantation. It is worth noting that the hepatic disease background and epidemiology of HCC is unique to China and is considerably different from that in Europe and America. Therefore, prognostic factors for liver transplantation in Chinese HCC patients are likely different from those in other countries. It is therefore necessary to explore the novel criteria for HCC liver transplantation in China.

Here, we studied independent prognostic factors, and developed proposed criteria for liver transplantation in HCC patients. The proposed criteria are as follows: total tumor size ⩽ 9 cm, and there should be no macrovascular invasion, positive lymph nodes or extrahepatic metastases, regardless of tumor number and distribution. According to the proposed criteria, we screened HCC patients with liver transplantation. The postoperative 2- and 5-year CSR was 82.8% and 68.8%, respectively, using our proposed criteria. There was no significant difference between the proposed and the Milan criteria (both 88.5%) (P = 0.321). Using the proposed criteria, the 2- and 5-year TFS rates were both 82.8%.

Compared to the Milan criteria (both rates 94.1%),

there was no significant difference ($P = 0.331$). We compared the HCC cases that fitted the proposed criteria (49 cases) and those that exceeded the criteria (99 cases) (Figure 1A and B). The 2- and 5-year CSR of the cases that fitted the proposed criteria were 82.8% and 68.8%, respectively, while the CSR for cases exceeding the criteria were 27.9% and 14.0%, respectively ($P < 0.001$). The 1- and 2-year TFS of the cases that fitted the proposed criteria were 97.6% and 82.8%, respectively, while the TFS for cases exceeding the criteria were 52.5% and 25.7%, respectively ($P < 0.001$). Use of our proposed criteria included 16.9% more cases than the Milan criteria and 10.8% more cases than the UCSF criteria. In this study, we also analyzed cases that fitted the proposed criteria, but exceeded the Milan criteria (25 cases). Our results showed that the 1-, 2- and 5-year CSRs were 85.2%, 77.4% and 48.4%, respectively, and the corresponding TFS rates were 95.0%, 70.4% and 70.4%, respectively. It is agreed generally in the field of transplantation that HCC patients whose 5-year, post-transplantation, survival rate is $\geqslant 50\%$ qualify as transplant candidates[23,24]. The present study showed that the 5-year CSR and TFS of the HCC patients were 68.8% and 82.8%, respectively. The 5-year CSR and TFS of the HCC patients that fitted our proposed criteria but exceeded the Milan criteria (25 cases) were 48.4% and 70.4%, respectively. The CSR and TFS rate curves of these patients were not significantly different compared to those of patients who fitted the Milan criteria ($P = 0.105$ and $P = 0.115$, respectively).

In summary, we suggest that the proposed criteria function to predict the prognosis of liver transplantation in patients with HCC. The proposed criteria allow us to increase the range of indicators for HCC patients in need of liver transplantation, and decrease the exclusion rate, and they allow more patients to receive therapeutic liver transplantation in China.

COMMENTS

Background

In the 1980s, the outcome of orthotopic liver transplantation (OLT) for hepatocellular carcinoma (HCC) was discouraging. After the Milan criteria (single tumor up to 5 cm, or up to three tumors up to 3 cm) were introduced by Mazzaferro *et al* in the early 1990s, recurrence rate fell to 8%, and tumor-free patient survival at 4 years was 83%. The Milan criteria have long been considered to be the classical criteria for selection of patients with HCC for liver transplantation. Recently, there have been numerous proposals for expanding the Milan criteria, such as the University of California, San Francisco (UCSF) criteria.

Research frontiers

The Milan criteria have long been considered to be the classical criteria for selection of patients with HCC for liver transplantation. China has the greatest incidence of liver cancer worldwide. Each year, over half of liver cancer detections and deaths occur in China. A substantial proportion of adult Living donor liver transplantation patients not fulfilling the Milan or UCSF criteria have been found to survive longer than expected after transplantation. Therefore, it seems reasonable to attempt further reduction of unnecessary dropouts arising from the strict application of narrow selection criteria.

Innovations and breakthroughs

In the present study, the authors identified various clinicopathological risk factors for actuarial and recurrence-free survival of 165 patients with HCC who underwent OLT using the Kaplan-Meier method with the log-rank test. They found that macrovascular invasion and total tumor diameter, as assessed on explanted liver, were the strongest prognostic factors.

Peer review

The authors reviewed their single-center experience with 148 OLTs for HCC and proposed a new prognostic score.

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BRIEF ARTICLES

Overexpression of polo-like kinase1 predicts a poor prognosis in hepatocellular carcinoma patients

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Abstract

AIM: To elucidate the role of overexpressed polo-like kinase1 (PLK1) in hepatocellular carcinoma (HCC).

METHODS: We prospectively collected clinicopathological, immunohistochemical and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) data from 135 HCC patients undergoing successful hepatectomy. The correlations between PLK1 mRNA expression and clinicopathologic variables were analyzed by Mann-Whitney *U* test. Prognostic factors were identified by univariate and multivariate analyses.

RESULTS: Immunohistochemical results showed overexpression of PLK1 was mainly found in tumor tissues compared with tumor-free tissue. A similar mRNA result was obtained by semi-quantitative RT-PCR. A total of 111 samples were positive for PLK1 mRNA expression. The positive expression was correlated with venous invasion, tumor nodules and Edmondson grade. Furthermore, 1, 3, 5-year survival rates in the positive expression group were significantly lower than the negative control group. Multivariate analysis showed that positive PLK1 expression was an independent risk factor for HCC.

CONCLUSION: PLK1 could be a potential biomarker for diagnosis and therapy for HCC.

Key words: Hepatocellular carcinoma; Immunohistochemistry; Reverse transcription-polymerase chain reaction; Survival analysis; Polo-like kinase 1

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the deadliest of all cancers, ranking third among all cancer-related mortalities[1]. Surgical resection plays a major role in the treatment of HCC. However, less than 30% of HCC patients are surgical candidates[2] due to limiting factors such as severe impairment of hepatic functional reserve, bilobar tumor distribution and extra-hepatic metastasis. No single-agent or combination chemotherapy regimen has been found to be particularly effective in HCC[3]. Locoregional treatment is not the first choice for HCC[4] and is reserved for non-surgical candidates.

The study of carcinogenesis mechanisms may provide new treatment regimens for cancer. Many key carcinogenetic pathways, such as increased angiogenesis, aberrant signal transduction and dysregulated cell cycle control, appear to be involved in tumor development[5]. As a cell cycle control kinase, polo-like kinase1 (PLK1) and its overexpression are highly associated with many human cancers, including bladder[6], breast[7-10], colorectal[11,12], endometrial[13,14], esophageal[15-17], gastric[18-21], glioma[22], hepatoblastoma[23], hepatocellular carcinoma[24], head and neck[25], leukemia and lymphoma[26,27], melanoma[28,29], non-small-cell lung[30], ovarian[31], papillary[32], pancreatic[33,34], prostate[35] and thyroid cancer[36]. However, up to now, as far as we know, there are few studies available on the overexpression of PLK in HCC. The objective of our study was to understand the relevance of PLK1 expression in HCC.

MATERIALS AND METHODS

Patients

There were 135 successful hepatectomy procedures for HCC performed from January 2003 to September 2008 in our department. None of these patients were given preoperative transarterial chemoembolization as a neoadjuvant treatment, but they all regularly received postoperative chemoembolization by hepatic artery infusion. All specimens from 135 cases were collected, immediately frozen in liquid nitrogen and subsequently stored at -70℃ for reverse transcription-polymerase chain reaction (RT-PCR). Five cases of liver regenerating nodule samples were obtained from patients with liver cirrhosis as controls. The clinicopathologic variables were recorded in detail including gender, age, liver cirrhosis, hepatitis B surface antigen, α-fetoprotein (AFP), venous invasion, Edmondson stage, tumor size (cm) and number of tumor nodules. Two expert pathologists who were blinded to the other results of the study, scored the HCC samples. The study protocol was approved by the Ethics Committee of Central South University, and written informed consent was provided by all participants prior to initiation of the study.

Follow-up

All patients were involved in our follow-up system, and were reviewed at 1-2 mo intervals. Routine post-operative medical examinations were carried out every 2 mo, including liver function, serum AFP level, B-ultrasound and CT. Follow-up ranged from 3-62 mo and ended on December 31, 2008. The median follow-up time was 18 mo.

Semi-quantitative RT-PCR

Total RNA was extracted from tumor and tumor-free tissues using the TRIzol reagent (Gibco BRL) according to the manufacturer's instructions (Gibco BRL). cDNA synthesis performed using the reverse transcription system. The primers were as follows: PLK1, 5'-GATTCC ACGGCTTTTTCGAG-3', 5'-CCCACACAGGGTCTTC TTCC-3' (product size, 296 bp); β-actin: 5'-CGCGAGAA GATGACCCAGAT-3', 5'-GCACTGTGTTGGCGTAC AGG-3' (product size, 550 bp). The following PCR cycling parameters were employed: at 95℃ for 5 min, followed by 35 cycles at 95℃ for 45 s, 56℃ for 1 min, at 72℃ for 1 min and then 72℃ for 7 min. The PCR products were resolved on a 1.5% agarose gel. All experiments were carried out in triplicate.

Immunohistochemical tissue slides

Immunohistochemical reaction against PLK1 (BD Transduction, a monoclonal mouse antibody) was performed in 5 μm paraffin sections. Negative controls were processed without primary antibody. For antigen retrieval, deparaffinized slides were placed in 0.01 mol/L sodium citrate buffer, pH 6.0 and boiled for 5 min in a pressure cooker. Then, slides were allowed to cool down for an additional 5 min in the same buffer. After several rinses in TBS and pre-treatment with blocking reagent (Dako, Glostrup, Denmark) for 5 min, slides were incubated with primary antibody diluted 1:500 (PLK1) and 1:50 (Ki-67) in antibody diluent solution (Zymed, San Francisco, CA, USA) for 20 min at room temperature and then at 4℃ overnight. After slides were washed in TBS,. The slides were visualized using Aquatex (Merck, Gernsheim, Germany).

Statistical analysis

Quantitative values were presented as mean ± SD or median (range). Independent Student's *t*-test was used to compare PLK1 mRNA expression in HCC and non-cancer samples. The Mann-Whitney *U* test was used for correlations between PLK1 mRNA expression and clinicopathologic variables. The Kaplan-Meier method was employed to calculate survival and the log-rank test to compare survival among two patient groups. Cox regression was adopted for multivariate analysis of prognostic predictors. The statistical software package SPSS16.0 (SPSS Inc, Chicago, IL) was applied for all analyses. A statistically significant *P* value was defined as < 0.05.

RESULTS

Immunohistochemical analysis of PLK1 protein expression

The pattern of PLK1 protein expression was examined by means of immunohistochemical analysis. Overexpression was detected in tumor tissues, especially in cytoplasm, compared with the tumor-free tissue. A more varied morphology of the cells was reflected by high expression in the cytoplasm. (Figure 1A and B). Tumor tissues had sharp margins under the microscope (Figure 1B and D). No or sporadic expression was observed in adjacent normal tissue or regenerating nodules (Figure 1C and D).

Expression of PLK1 mRNA in HCC

To compare the expression levels of PLK1 mRNA between neoplasm, adjacent normal tissue in the experimental group and regenerating nodules in the control group, total RNA was extracted for RT-PCR. The results indicate that HCC tissues expressed a significantly higher level of PLK1 mRNA than adjacent normal tissue and regenerating nodule (0.53 ± 0.05 *vs* 0.23 ± 0.04 and 0.20 ± 0.02, respectively, $P < 0.01$). When regenerating nodules were compared with adjacent normal tissue, the mRNA levels of regenerating nodules were slightly elevated, but the difference was not statistically significant (0.23 ± 0.04 *vs* 0.20 ± 0.02, $P > 0.05$) (Figure 2).

There were 111 samples (111/135, 82.22%) in which the OD values of PLK1 mRNA in HCC tissues were higher than those of adjacent normal tissue ($P < 0.05$). These were named the PLK1 positive group. The remaining 24 samples (24/135, 17.78%) were named the PLK1 negative group. There were statistically significant differences in PLK1 mRNA levels between the positive and negative groups ($P = 0.003$).

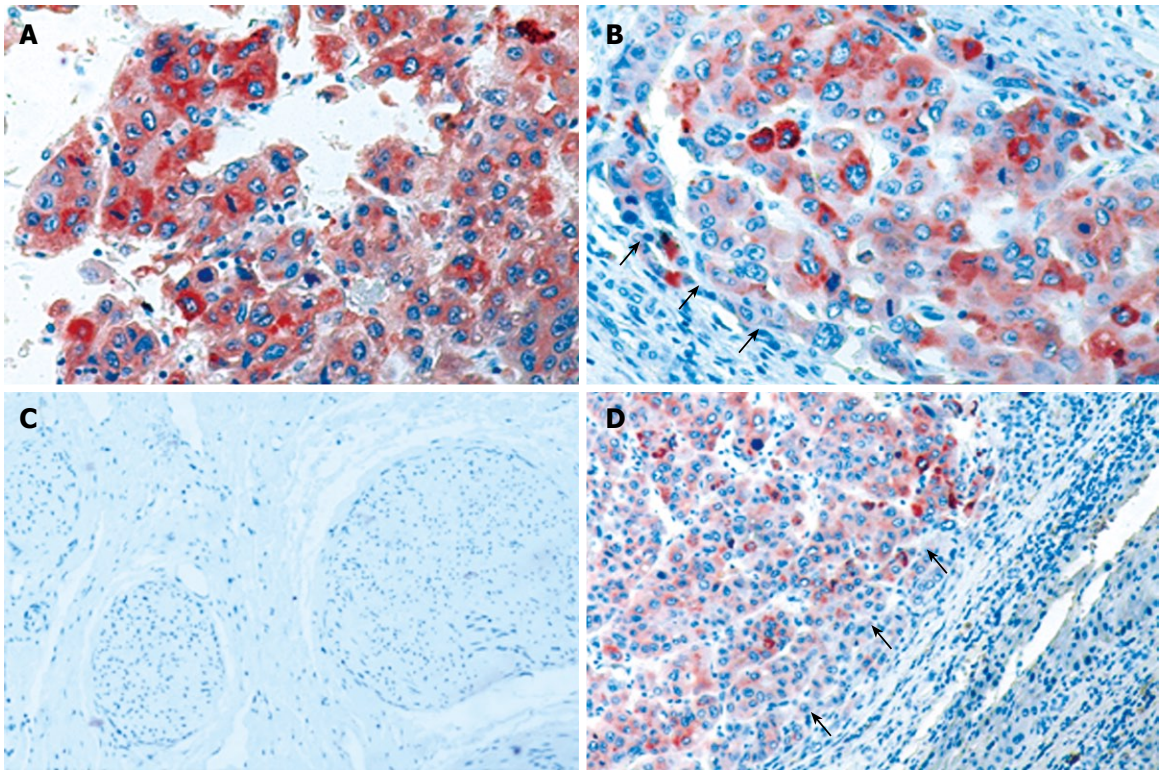

Figure 1 Expression of PLK1 in HCC tissue specimens. A: Expression of PLK1 in HCC tissue, especially in cytoplasm; B, D: Tumor tissue margins seen under the microscope (black arrows); C: Liver regenerating nodule sample; D: Adjacent normal tissue examined for PLK1 expression. Original magnifications, A and B × 200; C and D × 100.

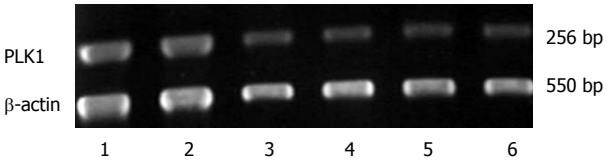

Figure 2 Expression of PLK mRNA in HCC, adjacent normal and regenerating nodule tissues. Lanes 1 and 2, HCC tissues; Lanes 3 and 4, adjacent normal tissue; Lanes 5 and 6, regenerating nodule tissues. RT-PCR for β-actin was used to monitor the quality of the RNA sample. RT-PCR was performed in triplicate.

Correlations between PLK1 positive expression group and clinicopathologic variables

The relationship between PLK1 positive expression groups and clinicopathologic data was analyzed by statistical software (Table 1). By the Mann-Whitney *U* test, the positive expression group with multiple tumor nodules had values significantly higher than that with solitary tumor nodules ($P = 0.002$). The high Edmondson-Steiner grade (Ⅲ, Ⅳ) in the positive expression group was also significantly stronger than those of low grade tumors (Ⅰ, Ⅱ) ($P = 0.022$). Furthermore, venous invasion was significantly correlated with the positive expression group ($P = 0.042$). However, this study also showed that positive expression had no significant relationship with gender, age, liver cirrhosis, HBsAg, AFP or tumor size ($P > 0.05$) (Table 1).

Survival analysis of prognostic factors

As mentioned above, 135 cases of HCCs were divided into positive and negative expression groups. The Kaplan-Meier method was employed to analyze the correlation of PLK1 expression level and the prognosis of HCC patients. Our results indicated that the positive expression group correlated with a shorter survival time than the negative group. The median survival time was 22.53 mo *vs* 60.88 mo. In addition, the 1, 3 and 5 year survival rates for the patients with positive and negative expression in HCC were 91.3%, 67.1% and 50.3% and 67.8%, 42.3% and 20.9%, respectively. The overall survival rates in the two groups was significantly different ($P = 0.003$, log-rank test) (Figure 3). At the same time, all the clinicopathologic variables were also analyzed with the Kaplan-Meier method. The results showed that a high Edmondson grade, venous invasion and multiple tumor nodules were correlated with a poor prognosis in HCC while the other clinicopathologic variables did not provide any independent prognostic information (Table 2).

By multivariable Cox regression analysis, PLK1 mRNA positive expression (RR, 3.507; 95% CI: 1.386-8.874, $P = 0.008$), high Edmondson grade (RR, 1.929; 95% CI; 1.069-3.482, $P = 0.029$), multiple tumor nodules (RR, 2.377; 95% CI: 1.384-4.082, $P = 0.002$) and venous invasion (RR, 4.848; 95% CI: 2.649-8.871, $P < 0.001$) were found to be independent prognostic factors for survival (Table 3).

DISCUSSION

Polo-like kinase 1 (PLK1) belongs to a family of

Table 1 Correlations between PLK1 mRNA expression and clinicopathologic variables by Mann-Whitney *U* test

Factors	*n*	PLK1 mRNA		*P*
		Positive	Negative	
Gender				
Male	114	93	21	0.650
Female	21	18	3	
Age (yr)				
≤ 40	65	53	12	0.842
> 40	70	58	12	
Liver cirrhosis				
Yes	109	93	16	0.055
No	26	18	8	
HBsAg				
Positive	96	80	16	0.598
Negative	39	31	8	
AFP (ng/mL)				
≤ 400	51	45	6	0.156
> 400	84	66	18	
Venous invasion				
+ (cases)	53	48	5	0.042
- (cases)	82	63	19	
Edmondson stage				
Ⅰ - Ⅱ	56	41	15	0.022
Ⅲ-Ⅳ	79	70	9	
Tumor size (cm)				
≤ 5. 0	36	28	8	0.417
> 5. 0	99	83	16	
Tumor nodule				
Single tumor nodule	77	70	7	0.002
Multiple tumor nodule	58	41	17	

HBsAg: Hepatitis B surface antigen; AFP: α-fetoprotein.

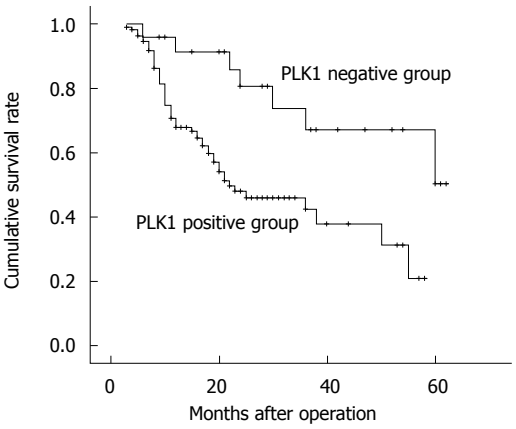

Figure 3 Log-rank test shows that HCC patients in the positive PLK1 mRNA expression group had a lower survival than those in the negative group.

conserved serine/threonine kinases involved in multiple mitotic processes[37], including functional maturation of centrosomes, establishment of the bipolar spindle[38], chromosome segregation[39] and response to DNA damage[40]. PLK1 is essential in the G_2/M-phase transition. The enzyme is able to activate CDC25c which in turn activates the CDC2/cyclin B1 complex leading to the import of cyclin B1 into the nucleus[41], and it is also able to phosphorylate cyclin B1 directly[42]. Moreover, PLK1 has been implicated in the regulation of anaphase-promoting complex/cyclosome[43]. Interestingly, overexpression of PLK1 has been associated with tumor development and can serve as a prognostic marker for some cancers[12,20,31]. However, few previous reports have examined the possible role of PLK1 in HCC.

Table 2 Correlation of several clinicopathological factors and of PLK1 mRNA expression group with patient survival (log-rank test)

Factors	*P*-value
Gender	0.155
Age	0.125
Liver cirrhosis	0.746
HBsAg	0.766
AFP	0.416
Venous invasion	< 0.001
Edmondson stage	0.001
Tumor size (cm)	0.183
Tumor nodule	0.018
PLK1 expression	0.003

Table 3 Multivariate survival analysis by Cox's proportional-hazard model for PLK1

	Relative risk	95.0% CI	*P*-value
Venous invasion	4.848	2.649-8.871	< 0.001
High Edmondson stage	1.929	1.069-3.482	0.029
Multiple tumor nodule	2.377	1.384-4.082	0.002
PLK1 positive expression	3.507	1.386-8.874	0.008

In our study, overexpression of PLK1 by immunohistochemical analysis was detected in tumor tissues,while no or sporadic expression were observed in adjacent normal tissue or regenerating nodules. Further investigation showed that the degree of PLK1 mRNA expression was also higher in HCC tissues than in adjacent normal tissues and regenerating nodules; elevated mRNA levels of PLK1 were detected in 82.22% of tumor samples. These data indicate that overexpression of PLK1 was a frequent event in hepatocellular carcinoma.

In investigating the association between PLK1 expression and clinicopathological data, PLK1 positive expression was correlated with multiple tumor nodules, high Edmondson-Steiner grade (Ⅲ, Ⅳ) and venous invasion; 1, 3 and 5 year survival rates of the positive expression group were 67.8%, 42.3% and 20.9%, respectively, lower than the negative group ($P < 0.01$); multivariate analysis showed that PLK1 was an independent prognostic factor. Thus, overexpression of PLK1 predicts a poor prognosis in HCC patients.

PLK1 is well known to be involved in cell proliferation. Was the PLK1 overexpression the cause of tumor formation or the consequence of high mitotic index during tumor cell proliferation? Others studies have shown that the overexpression of PLK1 in NIH3T3 leads to tumor formation[44], and knockdown of PLK1 causes inhibition of growth and induction of apoptosis in human esophageal cancer cells[17]. Furthermore, PLK1 was inhibited by DNA damage in the G2 phase of mitosis. When the conserved threonine residue in the T-loop was changed to aspartic acid, expression of these mutants

was found to override the G2 arrest induced by DNA damage[45]. In our studies, the level of PLK1 mRNA expression in HCC was obviously higher than in regenerating nodules, which also indicted that PLK1 might be a "proto-oncogene" for HCC.

In summary, this is the first attempt to clarify the relation of PLK1 expression and HCC. Our results indicate that high PLK1 expression predicts a poor prognosis in HCC patients. The data further suggest that PLK1 may serve as a potential biomarker for HCC. However, this is only a preliminary step. Further studies are warranted to understand the overexpression mechanism of PLK1 and to develop effective targeted interventions as a therapy for HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the deadliest of all cancers with as yet incompletely elucidated causes. As a cell cycle control kinase, polo-like kinase1 (PLK1) and its overexpression are highly associated with many human cancers. However, few studies are available on the overexpression of PLK1 in HCC.

Research frontiers

PLK1 is involved in cell proliferation. Its overexpression is associated with many human cancers. It is unclear whether the overexpression was the cause of tumor formation or the consequence of a high mitotic index during tumor cell proliferation. The trigger mechanisms of PLK1 overexpression in tumor formation are also unknown. Effective targeted interventions against PLK1 overexpression may be possible strategies as a therapy for HCC. All these questions are hotspots in the research field related to the article.

Innovations and breakthroughs

As a preliminary study of the relation between overexpression of PLK1 and HCC, it was found that overexpression of PLK1 was mainly found in tumor tissues compared with tumor-free tissue by immunohistochemical tissue slides and semi-quantitative reverse transcription-polymerase chain reaction analysis. In all HCC patients involved in the follow-up system, the survival rates in the PLK1 positive expression group were significantly lower than the negative control group. Theses results indicate that PLK1 could be a potential biomarker for diagnosis and therapy for HCC.

Applications

Overexpression of PLK1 is correlated with tumors. However the mechanism remains unclear. Their studies find overexpression of PLK1 in HCC, analyze the relationship between the prognosis of HCC and the overexpression, and provide the theoretical basis for targeted intervention against PLK1 as a means of treatment of HCC.

Terminology

PLK1: PLK1 is an important regulator of cell cycle progression during M-phase, involved in the assembly and dynamics of the mitotic spindle apparatus and in the activation and inactivation of CDK/cyclin complexes.

Peer review

This is a good descriptive study in which authors correlated polo-like kinase 1 expression in human HCC with clinicopathological findings. The data are very informative and deserve publication in the journal. The results are interesting and suggest that PLK1 could be a potential biomarker for diagnosis and therapy for HCC.

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Expression and clinical significance of S100A2 and p63 in esophageal carcinoma

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Abstract

AIM: To investigate the expression and clinical significance of S100A2 mRNA and protein, p63 protein in esophageal squamous cell carcinoma (ESCC) and their roles in carcinogenesis and progression of esophageal carcinoma (EC).

METHODS: Immunohistochemical staining (S-P method) for S100A2 and p63 protein were performed in 40 samples of ESCC and 40 samples of normal esophageal mucosa. *In situ* hybridization (ISH) was used to detect the expression of S100A2 mRNA.

RESULTS: Expression of S100A2 mRNA in ESCC was positive in 77.5% of samples, which was lower than that in normal mucosa (100%) by ISH (P = 0.002). The expression level of S100A2 mRNA was closely related to differentiation and and node-metastasis (P = 0.012, P = 0.008). Expression of S100A2 protein was positive in 72.5% of ESCC samples and expression of p63 protein was positive in 37.5% of ESCC samples, and was lower than that in normal mucosa (100%) (P = 0.000). The expression of S100A2 protein was correlated with the differentiation and node-metastasis (P = 0.007, P = 0.001), but no relationship was observed between the expression of p63 protein and clinical pathological manifestations. S100A2 protein was positively correlated with the expression of S100A2 mRNA, and negatively associated with the expression of p63 protein (P = 0.000, P = 0.002).

CONCLUSION: S100A2 and p63 protein both play important roles in the carcinogenesis of ESCC. An investigation into the combined expression of S100A2 and p63 may be helpful in early diagnosis and in evaluating the prognosis of ESCC.

Key words: Immunohistochemistry; *In situ* hybridization; Esophageal carcinoma; p63; S100A2

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INTRODUCTION

Esophageal carcinoma (EC) is one of the most common malignant diseases in China and has a poor survival rate. The carcinogenesis and development of EC is a complex process referring to multiple factors, stages and genovariations, and to numerous changes in genes and proteins at the molecular level. However, the biological roles of these changes in esophageal carcinogenesis are still vague. Up to now, no specific tumor marker for EC has been identified, and there are no biomarkers for use in screening, early diagnosis and judgment of the biological behaviour of EC.

As a tumor suppressor gene, *S100A2* is located on human chromosome 1q21[1] and its encoding protein is a calcium-binding protein constituted by 97 amino acids[2]. As a member of the S100 family, S100A2 is individual compared with other members of this family. Firstly, S100A2 protein is predominantly located in the nucleus rather than the cytosol like other S100 proteins. Moreover, *S100A2* is down-regulated in several tumors and may play a role in inhibiting tumor initiation or in suppressing tumor cell growth, whereas other members of the S100 family are up-regulated in tumors and may play a promotional role in carcinogenesis. Therefore, *S100A2* is considered a candidate tumor-suppressor gene[3].

p63 gene, a new member of the *p53* gene family, was identified and named in 1998 by Yang *et al*[4]. *p63* gene is located on human chromosome 3q27-29 and expresses

at least six protein isoforms, which can be divided into two groups -TAp63 and ΔNp63[5]. Among them, the transcription level of ΔNp63α is the highest. TAp63 is able to activate the transcription of specific target genes and induce cell cycle arrest and apoptosis. Similar to p53, TAp63 has anti-oncogene activity. ΔNp63 is unable to activate transcription and inhibit transcription activation by both p53 and TAp63. ΔNp63 has anti-apoptosis and proto-oncogene activity[6,7].

Normally, *p63* is mainly expressed in the basal lamina of many epithelial tissues and plays an important role in initiating epithelial stratification during development and in maintaining the proliferative potential of basal keratinocytes in mature epidermis. Recently, *p63* gene has been studied in the fields of tumorigenesis, cell apoptosis and tissue growth.

At present, few studies have been performed on the expression and relationship of *S100A2* and *p63* in EC. In this study, *in situ* hybridization (ISH) used to identify *S100A2* mRNA and immunohistochemical staining (S-P method) used to identify S100A2 and p63 protein, were performed in 40 samples of esophageal squamous cell carcinoma (ESCC) and 40 samples of normal esophageal mucosa. The purpose of this study was to investigate the expression of S100A2 mRNA, S100A2 protein and p63 protein in EC, and their relationship with clinical pathological features, and to explore their roles in the carcinogenesis and prognosis of EC.

MATERIALS AND METHODS

Patients and samples

Forty specimens of ESCC and matched adjacent normal mucosa were obtained from the Department of Pathology, First Affiliated Hospital, Anhui Medical University between 2005 and 2006. None of the patients had been treated with radiotherapy or chemotherapy prior to surgery. Samples were taken from tumor tissue without hemorrhage or putrescence, whereas the matched normal mucosa samples were taken from the surgical cutting edge, which was approximately 3-5 cm from the cancerous lesion. The clinical diagnosis in all 40 patients was confirmed by histological examination after surgery.

ISH and immunohistochemistry

ISH reagents were purchased from Boster Co. (Wuhan, China) and Zhong Shan Co. (Beijing, China). A digoxigenin-labeled oligonucleotide probe was used. The probe sequence of S100A2 is described as: 5'-TGATGTGCAG TTCTCTGGAGCAGGCGCTGGCTGTG-3'; 5'-ACTG TCATGTGCAATGACTTCTTCCAGGGCTGCCC-3'. ISH for S100A2 was performed as follows: The tissue sections were treated with 3% hydrogen peroxide and 10% pepsin (diluted with 3% citric acid), respectively, after deparaffinization and rehydration. The sections were prehybridized at 37℃ for 4 h with a prehybridization solution (Boster Co., China). Next, the sections were incubated in 100 μL hybridization solution/section containing 1 μL of denatured probe and 400 μL dilution of oligonucleotide probe (Boster Co., China) at 43℃ for 16-20 h. The slides were washed at 37℃ in 2 × SSC (5 min, three times), 0.5 × SSC (5 min, three times) and 0.2 × SSC (5 min, three times), respectively. During the color reaction procedure, the slides were incubated in sheep serum at 37℃ for 30 min and then incubated with mouse antidigoxigenin antibody at 37℃ for 60 min. After washing with PBS, the color was developed in DAB (3,3'-diaminobenzidine) (Zhongshan Co., China) for 15-30 min identified by occasional observation. Counterstaining of slides was then conducted with hematoxylin followed by a sealing procedure with neural gum.

S100A2 polyclonal antibody was purchased from Neomarker Co. (USA) and was used at a concentration of 1:100, and p63 monoclonal antibody was purchased from Maixin Co. (China). Immunohistochemistry for S100A2 and p63 were performed as follows: Deparaffinization and rehydration of sections (3 × 3 min with xylene; 3 × 2 min with 100% ethanol; 2 min with 95% ethanol, 2 min with 75% ethanol, and 2 × 1 min with distilled water), was performed using microwave repaired antigen. The samples were then washed 3 × 5 min with PBS. Endogenous peroxidases were blocked by soaking the slides in a solution of 3% H_2O_2 for 15 min at room temperature (RT). The samples were then washed 3 × 5 min with PBS, and 50 μL of nonimmune animal blood serum was added to each section for 15 min at RT. The samples were shaken and excess PBS was wiped off. Primary antibody (50 μL) was added to each section immediately, and incubated overnight at 4℃ in a humidified chamber. The slides were washed 3 × 10 min in PBS. Fifty microliters of biotin labeling secondary antibody was added to each section for 15 min at 37℃, then the slides were washed 3 × 5 min with PBS. (ABC was made according to the Vector protocol 30 min before use (mix 5 mL PBS with two drops of solution A and two drops of solution B). The samples were incubated for 15 min at RT, then washed 3 × 5 min in PBS. Freshly prepared DAB (100 μL) was added to the slides and the color change was observed approximately 5-20 min later. Coloration was stopped by flushing with water. The slides were then counterstained with hematoxylin followed by a sealing procedure with neural gum.

Normal epithelium showing strong S100A2 or a p63-positive section served as the positive control, and that without the antibody section served as the negative control.

ISH and immunohistochemistry evaluation

S100A2 mRNA and S100A2 protein were expressed in the nucleus or the nucleus and cytoplasm. The percentage of positive cells in each high power field was noted, and less than 10% of positive cells was considered negative and greater than 10% was considered positive. p63 protein was expressed in the nucleus. The grading of positive cells was as follows: no p63-positive cells was characterized as negative, less than 25% was weakly positive (+), 25%-75% was moderately positive (++), and greater than 75% was strongly positive (+++). The percentage of staining was estimated by two independent pathologists, respectively.

Table 1 Expression of S100A2 mRNA, S100A2 protein and p63 protein in ESCC and normal mucosa (n = 40) n (%)

	S100A2 mRNA		S100A2 protein	
	Negative	Positive	Negative	Positive
Normal mucosa	0	40 (100)	0	40 (100)
ESCC	9	31 (77.5)[a]	11	29 (72.5)[a]

[a]$P < 0.01$ *vs* normal mucosa. ESCC: Esophageal squamous cell carcinoma.

Table 2 Relationship between the expression of S100A2 mRNA and S100A2 protein, and clinical pathological features in ESCC n (%)

	n	S100A2 mRNA		S100A2 protein	
		Negative	Positive	Negative	Positive
Gender					
Male	31	7	24 (77.4)	8	23 (74.2)
Female	9	2	7 (77.8)	3	6 (66.7)
Age (yr)					
< 62	21	4	17 (81)	4	17 (81)
≥ 62	19	5	14 (73.7)	7	12 (63.2)
Clinical stage					
Ⅰ and Ⅱ	31	5	26 (83.9)	6	25 (80.7)
Ⅲ and Ⅳ	9	4	5 (55.6)	5	4 (44.4)
Tumor location					
Upper and middle segment	23	3	20 (87)	5	18 (78.3)
Inferior segment	17	6	11 (64.7)	6	11 (64.7)
Tumor size (cm)					
< 3.5	16	3	13 (81.3)	4	12 (75)
≥ 3.5	24	6	18 (75)	7	17 (70.8)
Depth of tumor invasion					
Not to serosa	20	4	16 (80)	4	16 (80)
To serosa	20	5	15 (75)	7	13 (75)
Degree of differentiation					
Well	15	1	14 (93.3)	2	13 (86.7)
Moderately	14	2	12 (85.7)	2	12 (85.7)
Poorly	11	6	5 (45.5)[a]	7	4 (36.4)[b]
Lymph node metastasis					
Negative	25	2	23 (92)	2	23 (92)
Positive	15	7	8 (53.3)[d]	9	6 (40)[d]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* the well and moderately differentiated group; [d]$P < 0.01$ *vs* the lymph node-negative group.

Statistical analysis

All data were analyzed with SPSS 13.0 software. Pearson's χ^2 test was used for data measurement. Using the Monte Carlo simulation method, the exact probability value was calculated for the data where the theoretical frequency was less than five. Non-parametric Spearman rank correlation analysis was used for correlation analysis of ranked data.

RESULTS

The expression of S100A2 mRNA and S100A2 protein in ESCC and normal mucosa

S100A2 mRNA and S100A2 protein were expressed in the nucleus or the nucleus and cytoplasm (Figure 1A and B, Figure 2A and B). All 40 samples of normal esophageal mucosa positively expressed S100A2 mRNA and S100A2 protein. In 40 ESCC samples, 31 positively expressed S100A2 mRNA, and 29 expressed S100A2 protein. The positive rates of S100A2 mRNA and S100A2 protein in ESCC (77.5% and 72.5%, respectively) were significantly lower than that in normal mucosa (100% and 100%, respectively) ($P < 0.01$) (Table 1).

Table 3 Relationship between S100A2 mRNA and S100A2 protein in ESCC

S100A2 protein	S100A2 mRNA		Total
	+	-	
+	27	2	29
-	4	7	11
Total	31	9	40

$r = 0.607$, $P = 0.000$.

The relationship between expression of S100A2 mRNA and S100A2 protein and clinical pathological features in ESCC

S100A2 mRNA was positive in 93.3% (14/15), 85.7% (12/14), and 45.5% (5/11) in the well differentiated, moderately differentiated, and poorly differentiated groups, respectively. The differences were significant among the three groups ($P < 0.05$). Moreover, the expression level of S100A2 mRNA was significantly higher in the well and moderately differentiated groups than that in the poorly differentiated group ($P < 0.05$). The expression of S100A2 was positively correlated with node metastasis ($P < 0.01$). S100A2 protein was positive in 86.7% (13/15), 85.7% (12/14), and 36.4% (4/11) in the well differentiated, moderately differentiated, and poorly differentiated groups, respectively. The expression level of S100A2 protein was related to differentiation and lymph node metastasis. The expression of S100A2 mRNA and S100A2 protein was not correlated with gender, age, clinical stage, tumor location, tumor size, and depth of tumor invasion ($P > 0.05$) (Table 2).

Relationship between S100A2 mRNA and S100A2 protein in ESCC

A positive correlation was found between the expression of S100A2 mRNA and S100A2 protein ($r = 0.607$, $P < 0.001$). Of 40 ESCC samples, 27 expressed S100A2 mRNA and S100A2 protein at the same time, and seven cases did not express these parameters at the same time. Four cases expressed S100A2 mRNA but not S100A2 protein. Two cases did not express S100A2 mRNA but expressed S100A2 protein (Table 3).

The expression of p63 protein in ESCC and normal mucosa

p63 protein was expressed in the nucleus (Figure 3A and B). In 40 samples of normal esophageal mucosa, 10 expressed p63 but none were strongly positive. In 40 ESCC samples, 21 expressed p63. Among these, 15 cases were strongly positive. p63 protein was positive in 52.5% and

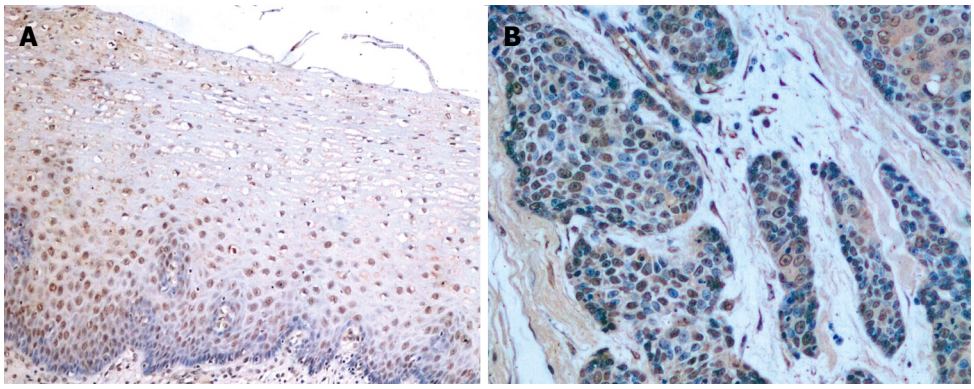

Figure 1 The expression of S100A2 mRNA. A: Normal mucosa (+) (ISH, × 100); B: ESCC (+) (ISH, × 200).

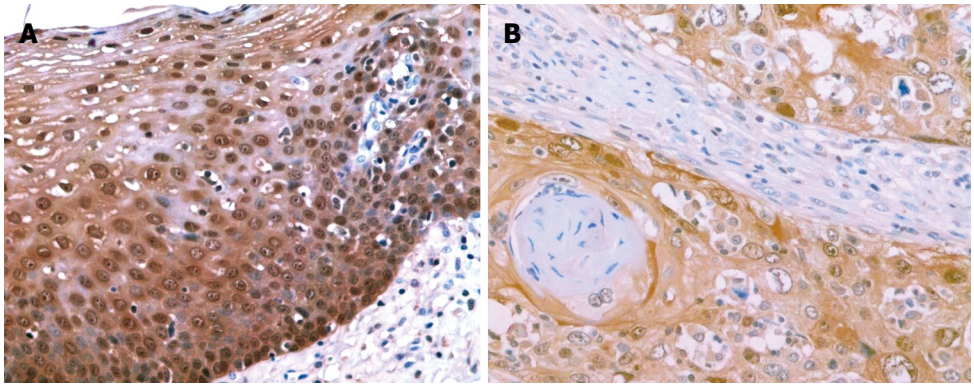

Figure 2 The expression of S100A2 protein. A: Normal mucosa (+) (S-P, × 200); B: ESCC (+) (S-P, × 200).

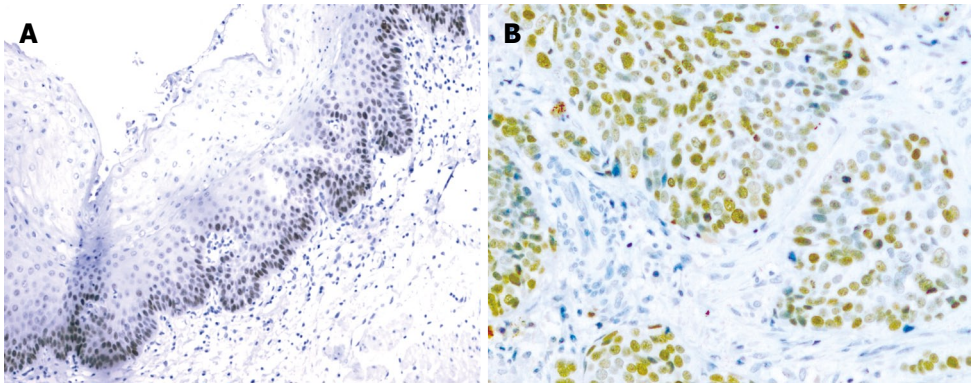

Figure 3 The expression of p63 protein. A: Normal mucosa (+) (S-P, × 100); B: ESCC (+++) (S-P, × 200).

Table 4 Expression of p63 protein in ESCC and normal mucosa

	n	p63 protein				Positive rate (%)	Strong positive rate (%)
		-	+	+ +	+ + +		
Normal mucosa	40	30	10	0	0	25.0	0.0
ESCC	40	19	2	4	15	52.5[a]	37.5[b]

[a]$P < 0.05$, [b]$P < 0.001$ *vs* normal mucosa.

strongly positive in 37.5% of ESCC samples, and was higher than that in normal mucosa (25.0%) ($P < 0.05$, $P < 0.001$) (Table 4).

Relationship of p63 protein expression with clinical pathological features of ESCC

The expression of p63 protein was not correlated with gender, age, clinical stage, tumor location, tumor size, depth of tumor invasion, degree of differentiation or lymph node metastasis ($P > 0.05$).

Relationship between S100A2 protein and p63 protein in ESCC

A negative correlation was found between the expression of S100A2 protein and p63 protein ($r = -0.474$, $P < 0.01$). Of 40 ESCC samples, 11 expressed S100A2 and p63 at the same time, and 1 did not express these parameters at the same time. 18 ESCC samples were S100A2-positive and p63-negative. 10 cases were S100A2-negative and p63-positive (Table 5).

DISCUSSION

Up to now, 21 genes encoding S100 calcium-binding proteins of the EF-hand type have been identified. Calcium ion (Ca^{2+}) plays an important role in the regulation of a number of cellular processes. The

Table 5 Relationship between S100A2 protein and p63 protein in ESCC

p63 protein	S100A2 protein		Total
	+	-	
+	11	10	21
-	18	1	19
Total	29	11	40

r = -0.474, P = 0.002.

second messenger role of Ca^{2+} is mediated, at least in part, by calcium-binding proteins which contain the EF-hand motif. Through modulating Ca^{2+} and interacting with the target proteins, S100 proteins have a multitude of biological functions *in vivo*. Many S100 genes were reported to be clustered in the epidermal differentiation complex in chromosome 1q21[8-12]. This region is involved in epidermal differentiation and proliferation and is also frequently rearranged in tumors[13]. Studies have shown that the S100 gene deregulated expression in human diseases, especially in tumors. Nowadays, S100 protein antibodies have widespread application for tumor diagnosis by immunohistochemistry.

As a member of the *S100* gene family, S100A2 is significantly downregulated in several malignant tumors, such as breast cancer[14], melanoma[15], prostatic carcinoma[16], and pulmonary carcinoma[17]. Moreover, S100A2 may be closely associated with the development and prognosis of tumors[18].

Using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), Ji *et al*[19] examined the differential expression of *S100A2* and another 15 *S100* genes in 62 cases of ESCC *vs* the corresponding normal esophageal mucosa. Their results showed that the S100A2 gene was significantly downregulated ($P < 0.05$) in ESCC *vs* normal esophageal mucosa. Moreover, the deregulation of *S100A2* gene was significantly correlated with the deregulation of *S100A8*, *S100A14* and *S100P*. Kyriazanos *et al*[20] examined the clinical significance of *S100A2* expression in 116 resected specimens of ESCC using immunohistochemistry. Their results showed that S100A2 was positive in 49 cases (42.2%) and its expression was significantly higher in large and well differentiated tumors. Lymph node-positive tumors had a lower expression of S100A2 protein in comparison to the corresponding lymph node-negative equivalents in each of the T stages, but the difference was statistically significant only for T1b tumors. S100A2 status became an independent predictor of patient survival in lymph node-negative cases. Node-negative ESCC patients without S100A2 expression may be a high-risk group with poor survival and will need further attention to design appropriate adjuvant therapy.

We used ISH for the first time to detect the expression of *S100A2* mRNA and used immunohistochemical staining for S100A2 protein in 40 ESCC samples and 40 samples of normal esophageal mucosa. Our results showed that *S100A2* mRNA and S100A2 protein although positive in ESCC were both lower than that in normal mucosa ($P < 0.01$). This indicated that S100A2 was downregulated in ESCC and that the *S100A2* gene is concerned with the carcinogenesis of EC. A positive correlation was found between the expression of *S100A2* mRNA and S100A2 protein ($P < 0.001$), which indicated that S100A2 protein expression is regulated mainly at the transcriptional level, and the decrease in S100A2 protein expression corresponded to the decrease in transcriptional activity. In addition, our findings on S100A2 mRNA are consistent with those of Ji *et al*[19]. We suggest that the positive rate in our study is higher than that in the study by Ji *et al*[19] due to the difference in case number (40 *vs* 62) and method (ISH *vs* RT-PCR).

Furthermore, we analyzed the relationship between S100A2 positive expression and the clinical pathological features of ESCC. We found that the expression level of S100A2 mRNA and S100A2 protein were both significantly higher in the well and moderately differentiated groups than in the poorly differentiated group ($P < 0.05$). These differences were also significant between the lymph node-positive group and the lymph node-negative group ($P < 0.01$), which is roughly consistent with the findings of Kyriazanos *et al*[20]. These results indicate that S100A2 plays an important role in tumor cell differentiation, and that S100A2 might be an important biomarker in the biological behaviour of EC.

A study on the course of EC, found that *p63* gene and its encoding protein played important roles in the early period of the physiological and pathological course of esophageal mucosa, compared with mutation of p53 which occurs in the last stage of cancerization lineage from metaplasia, atypical hyperplasia to adenocarcinoma. Glickman *et al*[21] detected p63 protein in EC by immunohistochemical staining. Their results showed that p63 protein was highly expressed in ESCC, but was not expressed in adenocarcinoma of the esophagus and colorectal cancer. This indicated that *p63* gene is upregulated in ESCC and is concerned with the development of ESCC.

We used immunohistochemical staining for p63 protein in 40 ESCC samples and 40 samples of normal esophageal mucosa. Our results showed that p63 protein in normal esophageal mucosa was positive in 25.0%, and the expression was weakly positive and localization in the basal cells or the bottom of the prickle cell layer of normal esophageal mucosa. This supports the view that p63 is mainly expressed in the corpus of many epithelial tissues, and plays an important role in the abstraction, differentiation and morphogenesis of many epithelial tissues[22]. Moreover, our results showed that p63 protein has a high expression rate in the tissue of EC, which indicates that p63 is closely related to the carcinogenesis of EC. In contrast, we found that the expression of p63 protein was not correlated with the clinical pathological features of ESCC.

Our study showed that the expression of S100A2 protein was reduced and the expression of p63 protein was increased, and a negative correlation was observed between them ($P < 0.01$). This indicated that S100A2 protein and p63 protein may both play important roles in the carcinogenesis of EC. An investigation into the combined expression of S100A2 and p63 may be use-

ful in early diagnosis and in evaluating the prognosis of ESCC.

COMMENTS

Background

Esophageal carcinoma (EC) is one of the most common malignant diseases in China and has a poor survival rate. Up to now, no specific tumor marker of EC has been identified. Recently, *S100A2* has been considered a candidate tumor-suppressor gene, and *p63* gene has been studied in the fields of tumorigenesis, cell apoptosis and tissue growth.

Research frontiers

S100A2 is down-regulated in several tumors and may play a role in inhibiting tumor initiation or in suppressing tumor cell growth. Therefore, S100A2 has been considered a candidate tumor-suppressor gene. *p63* gene, a new member of the *p53* gene family, is normally expressed in the basal lamina of many epithelial tissues and plays an important role in initiating epithelial stratification during development and in maintaining proliferative potential of basal keratinocytes in mature epidermis. Recently, *p63* gene has been studied in the fields of tumorigenesis, cell apoptosis and tissue growth.

Innovations and breakthroughs

At present, few studies have been carried out on the expression and the relationship of *S100A2* and *p63* in EC. In this study, *in situ* hybridization (ISH) to identify *S100A2* mRNA and immunohistochemical staining (S-P method) to identify S100A2 and p63 protein were performed in ESCC and normal esophageal mucosa samples.

Terminology

S100A2 gene is located on human chromosome 1q21 and its encoding protein is a calcium-binding protein constituted by 97 amino acids. S100A2 protein is predominantly located in the nucleus rather than the cytosol like other S100 proteins. S100A2 is down-regulated in several tumors. Therefore, S100A2 is considered a candidate tumor-suppressor gene.

Peer review

The study investigated S100A2 and p63 expression in EC tissue samples and paired normal mucosa samples. S100A2 expression was analysed both on the mRNA and protein level by ISH and IH, respectively, p63 expression was determined by IH. The authors described a decreased expression of S100A2 and an increased expression of p63 in tumor samples. Statistical analysis revealed an inverse correlation. This is a clear strength of this study.

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CASE REPORT

Dysplasia in perforated intestinal pneumatosis complicating a previous jejuno-ileal bypass: A cautionary note

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Abstract

We present the case of an elderly woman who developed a bowel perforation related to pneumatosis intestinalis, 33 years after a jejuno-ileal bypass for severe obesity. Final histological examination revealed the presence of dysplasia in the resected specimen. On the basis of our case and a review of the literature, we discuss the etiopathogenesis, the clinical aspects and the treatment of this rare condition.

Key words: Acute abdomen; Pneumatosis intestinalis; Peritonitis; Obesity; Dysplasia

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INTRODUCTION

Pneumatosis intestinalis (PI) is an unusual intestinal pathology characterized by a collection of multiple gas-filled cysts within the wall of the gastrointestinal tract. According to this general definition, PI may be seen in some critical events, including necrotizing enterocolitis in the newborn and ischemic bowel disease in the adult, but also in innocuous conditions which are associated with a wide spectrum of diseases and situations which do not require surgery[1-7]. Benign causes are statistically more prevalent; PI may resolve spontaneously, although recurrent episodes are reported and the long term outcome of patients is often unclear because of the absence of an exhaustive imaging follow up[1]. The lack of reliable data regarding the outcome predictors for patients with PI complicates management decisions.

A pneumoperitoneum and a pneumoretroperitoneum can be present regardless of causes and are generally considered a complication of PI. Peritonitis may be consequent, but this is not the rule and a lack of perforation is reported in the presence of mild clinical symptoms[3]. These conditions are difficult to manage, taking into account that some clinical conditions, such as immunodepression or obesity (not infrequent in these patients) may mask the clinical relevance of pneumoperitoneum so delaying treatment. On the other hand, an automatic referral to surgery may result in an unnecessary laparotomy.

Herein we present a case of PI associated with jejuno-ileal bypass performed many years previously and complicated by a pneumoperitoneum in an obese patient. The histological data, showing a dysplasia of the mucosa, are subsequently discussed.

CASE REPORT

C.D., a 73-year old woman, having undergone an end-to-side jejuno-ileal bypass for severe obesity 33 years previously, was admitted to the Medical Division of our hospital complaining of abdominal pain, constipation and mild dyspnea lasting 2 d. Relevant history included chronic obstructive pulmonary disease without dyspnea at rest, postsurgical hypothyroidism and atrial fibrillation with necessity of continuous warfarin administration recorded; no significant signs of digestive impairment were present. Weight was 105 kg and BMI was 41. At admission, the patient was oliguric, hypotensive (85/55 mmHg) and hypoxemic (PCO_2 65 mmHg, SpO_2 90%). On physical examination the abdomen appeared distended, with

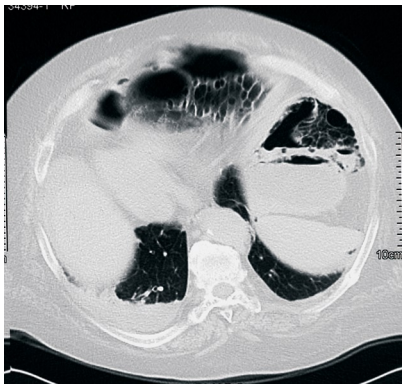

Figure 1 Unenhanced CT scan, showing the presence of multiple gas-filled parietal cysts and huge pneumoperitoneum.

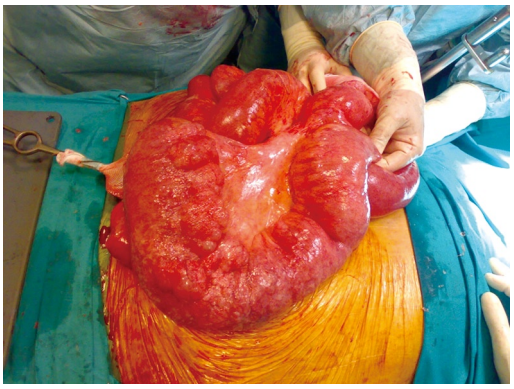

Figure 2 Intraoperative view of dilated and inflamed defunctionalized jejunal loop, with fibrous peritonitis. No clear evidence of enteric or biliary contamination was present.

diffuse pain and a mild rebound tenderness; no peristalsis at auscultation. Laboratory investigations revealed mild metabolic acidosis (pH 7.3, bicarbonate 15.4 mmol/L) with normal lactate value, leukocytosis (14 000/mL) and acute renal failure (creatinine 2.6 mg/dL); PCR was 459, albuminemia 2.7 g/dL. A marked reduction of prothrombin value (< 10%, INR > 8) was noted, requiring plasma transfusion. Thorax radiography was negative for pneumothorax or pneumomediastinum. Computed tomography (CT) without intravenous contrast medium showed some gas-filled cysts within the wall of the jejunum, ileum and mesentery, associated with massive free intraperitoneal air (Figure 1). A rapid worsening of the patient's general condition was observed, leading to urgent surgical intervention; the patient was classed as ASA four according to the American Society of Anesthesiology. At laparotomy, pneumatosis affected only the defunctionalized jejunal loop (Figure 2), which was abnormally dilated and distally anastomized end-to-side to the sigmoid loop. The first and the last segment of the bypassed bowel, including the ileocolic anastomosis, were relatively healthy. A transmural perforation was present in the antimesenteric border of the bowel along the tract with pneumatosis, in a context of fibrinous peritonitis, without evidence of free biliary or enteric fluid. A segmental 90 cm-long ileal resection with end-to-end anastomosis was performed, including the entire unhealthy bowel. The bacteriologic study on the peritoneal fluid showed the presence of *Difteroides*.

The macroscopic pathological analysis of the resected specimen showed a thickened bowel wall in the presence of multiple cysts without fluid, with a thin distended mucosa and absence of folded structure (Figure 3A and B). At the histological evaluation, the cysts were located in the submucosa and subserosa, with giant cells lining the cyst walls (Figure 3C and D). The mucosa was severely thinned, in some areas with a single cellular layer, and pseudo-atrophy of villi alternated with inflammation and ulceration. In some areas the epithelium was characterized by mild cytological atypia indicating low dysplasia (Figure 4A). Immunohistochemical staining with p53 (Thermo Fisher Scientific, Fremont, CA, USA, dilution 1:100) and p16 (Kit CINtec® Histology, MTM Laboratory, Germany) confirmed the diffuse positivity of epithelial cells in the focal mild dysplasia (Figure 4B and C). Marked flogistic reaction of the peritoneal serosa was reactive at the intestinal perforation.

The patient consequently became severely compromised during the whole surgical operation with prolonged hypotension and needed care in the intensive recovery room for 2 d. A progressive normalization of the septic signs and of renal function was then observed. Subsequently the patient's recovery was uneventful and she was discharged on post-operative day 14.

DISCUSSION

Pneumatosis intestinalis may occur in many different situations. The etiology actually favours iatrogenic conditions (surgery, endoscopy, enteric tube placement, positive pressure ventilation), immunodepression, collagen vascular disease and infectious agents[2,4,5,7,8]. The jejuno-ileal bypass has accounted for some cases of PI; this condition is generally considered benign with a favourable outcome, supporting the concept that "surgical indication is not indicated under the usual circumstances"[9]. The published experiences on this topic, generally in the form of single cases or small series of patients, refer to reports from the 1970s[9-15], when this type of bariatric surgery was adopted in many centres. PI occurred any time during the first postoperative phase (mean delay 5 mo, range 1-20 mo)[7] and patients recovered spontaneously in the majority of cases[9,11].

Although responsible for a small number of cases, the "model" of jejuno-ileal bypass is well within the supposed pathogenetic mechanisms of PI, because these patients really present several predisposing factors. The first is ascribed to surgery alone with the consequent modification in bowel pressure[16]. Another factor may be related to the marked obesity, as PI has been described in obese patients without a history of abdominal surgery[9]. The type of reconstruction of bowel continuity also seems to play a central role; increased colonic gas secondary to fermentation of undigested lactose may reflux into the excluded segment of the small bowel. This occurs in particular when an ileum-sigmoid anastomosis has been performed, so favoring, because of abnormal

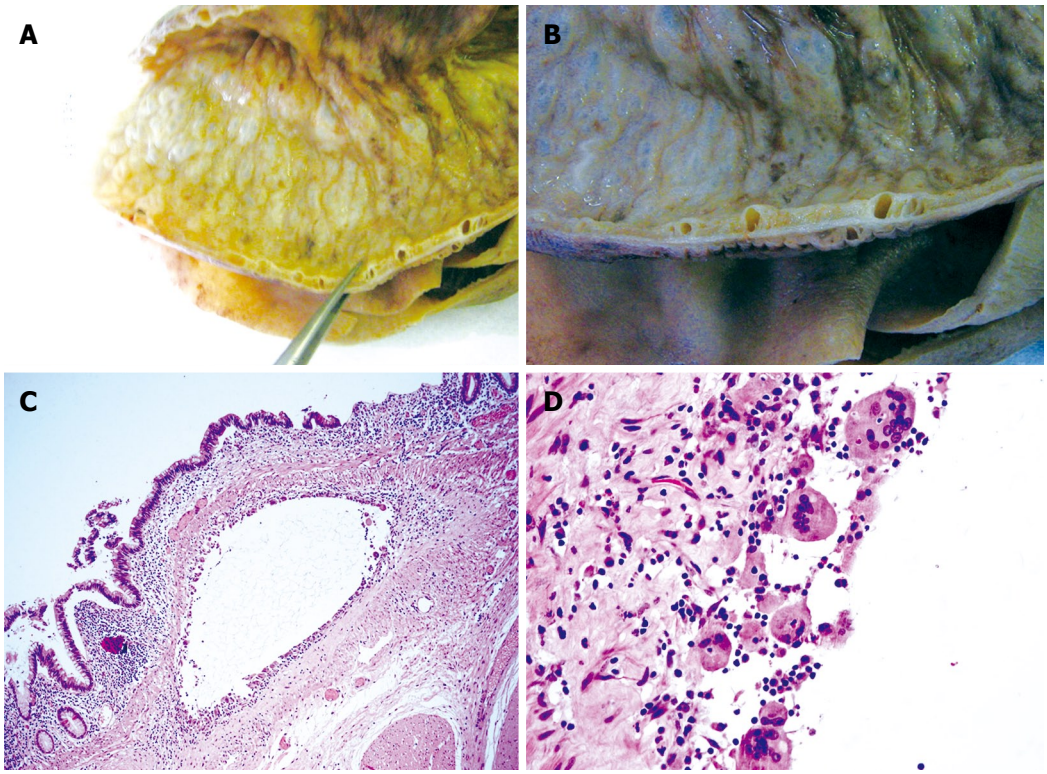

Figure 3 Pathological findings. A and B: Macroscopic appearance of surgical specimen with the typical cysts at different magnifications; C and D: Submucosal cysts (C, HE, × 20) lined by giant cells (D, HE, × 100).

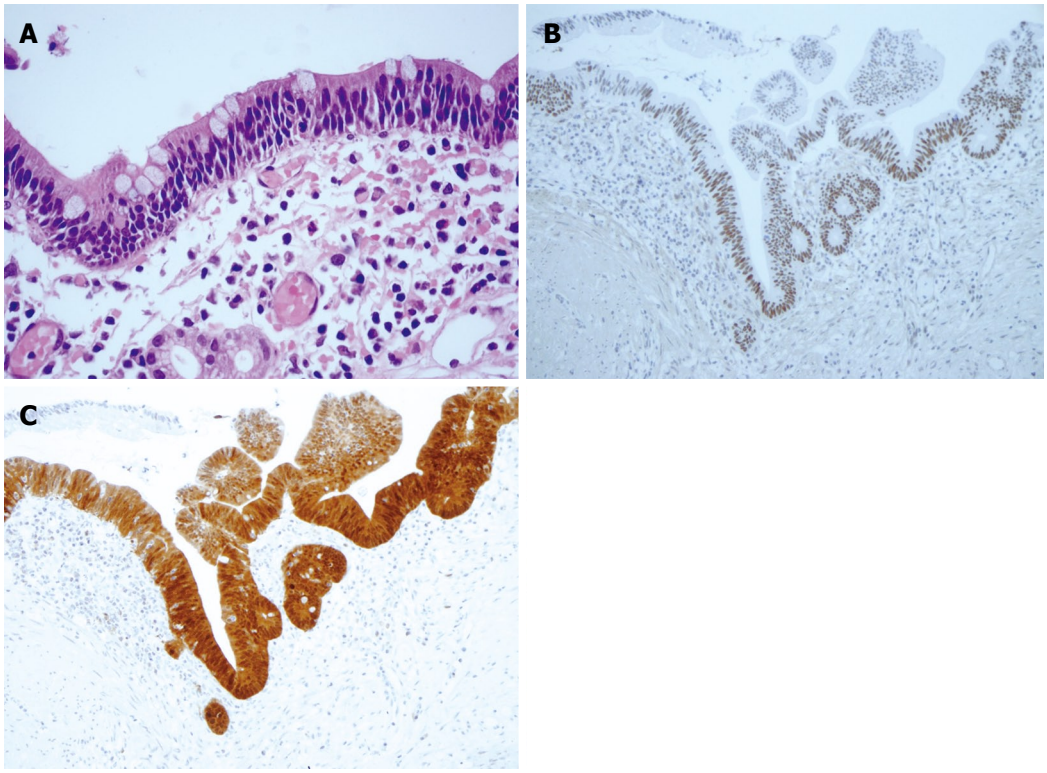

Figure 4 Histological appearance and immunohistochemical staining. Focal area of mild dysplasia (HE, × 60) (A), Positivity for P53 (B) and P16 (C).

pressure, the dissolution of gas into the bowel wall. This mechanism may be augmented by the concomitant bacteria overgrowth, most likely anaerobic, in the bypassed small bowel segment, so assigning the etiology of PI a mechanical - infectious process[12]. Our case tends to support this hypothesis. PI involved only the bypassed small bowel where the two mechanisms, high pressure and bacterial overgrowth, were more significant. An anastomosis had been built between the defunctionalized ileum and the sigmoid, the excluded small bowel was significantly dilated, with a clear discrepancy of size compared with that of the functioning bowel, and it was edematous. Histology showed a severe mucosal injury with marked inflammation and signs of a remodeling reactive process.

Pneumoperitoneum is a complication of PI, but not an automatic indication for surgery. An increasing list of conditions relating to pneumoperitoneum without

peritonitis are now recognized[17,18], PI being the most common. These cases have a benign progression without laparotomy[17-22]. They are the prevalent situations where no transmural perforation exists: as a consequence, no peritoneal contamination results from the rupture of the intramural sterile blebs. According to these considerations, the demonstration of PI in a patient with pneumoperitoneum may allow us to avoid the need for a laparotomy[19]. This assumption must be accepted with caution in our opinion. The presence of PI is not enough to consider pneumoperitoneum as a benign condition in every case, with a good spontaneous outcome. Surgical indication must rely above all on the clinical data; neither CT nor the amount of free air are reliable guides for therapy[20]. A negative abdominal examination could exclude signs of peritonitis, but clinical signs may be minimal or absent, for example in immunodepressed patients, or difficult to discover, as in obese patients. These two situations clearly define an increased risk of a delayed diagnosis of peritonitis, which can present with an ominous course in these frail patients. In our patient, peritonitis was not clear at the time of admission owing to the marked obesity of the patient, but the general signs of septic compromise (hypotension, oliguria, leukocytosis, high PCR value) suggested the clinical relevance of the radiological images. Laparotomy became urgent within a few hours. A complete perforation was discovered in the defunctionalized bowel without extravasation of enteric content, but the presence of gram-positive bacteria in the peritoneal fluid together with the histological demonstration of inflamed peritoneal serosa confirmed the contamination from the intestinal lumen. In the context of a fibrinous peritonitis a simple suture of the perforation might be advised, but this was not our choice because the bowel appeared very dilated and inflamed. The possibility of saving the proximal and distal segments, which were macroscopically normal, directed us towards performing the resection of the pathological bowel.

The histological examination of the resected bowel showed obvious pathological aspects of the PI with typical submucosal cysts surrounded by granulomatous reaction; the peculiarity of the case is the discovery of focal areas of mild dysplasia confirmed by the immunohistochemical evaluation with p53 and p16. These findings have never been reported before. Owing to the rare indication for surgery in these patients, an exhaustive histological evaluation of the pathologic bowel is rare but generally marked by an intact, pale and sometimes transparent mucosa[7]. These changes, together with the unusually long time between the intestinal bypass and the discovery of PI in our patient, support the hypothesis of a long standing remodeling process, promoting the concept of a preneoplastic condition. This possibility, together with the rare occurrence of malignant lymphomas developing in the excluded intestine[23], leads us to advise a long term follow up. When surgery is indicated for this complication, complete resection of the tract with pneumatosis, if technically possible in symptomatic patients, appears to us to be advisable.

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CASE REPORT

Neuroendocrine carcinomas arising in ulcerative colitis: Coincidences or possible correlations?

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Abstract

Patients with inflammatory bowel disease (IBD) are at increased risk of colorectal malignancies. Adenocarcinoma is the commonest type of colorectal neoplasm associated with ulcerative colitis (UC) and Crohn's disease, but other types of epithelial and non-epithelial tumors have also been described in inflamed bowel. With regards to non-epithelial malignancies, lymphomas and sarcomas represent the largest group of tumors reported in association with IBD, especially in immunosuppressed patients. Carcinoids and in particular neuroendocrine neoplasms other than carcinoids (NENs) are rare tumors and are infrequently described in the setting of IBD. Thus, this association requires further investigation. We report two cases of neoplasms arising in mild left-sided UC with immunohistochemical staining for neuroendocrine markers: a large cell and a small cell neuroendocrine carcinoma of the rectum. The two patients were different in age (35 years *vs* 77 years) and disease duration (11 years *vs* 27 years), and both had never received immunosuppressant drugs. Although the patients underwent regular endoscopic and histological follow-up, the two neoplasms were locally advanced at diagnosis. One of the two patients developed multiple liver metastases and died 15 mo after diagnosis. These findings confirm the aggressiveness and the poor prognosis of NENs compared to colorectal adenocarcinoma. While carcinoids seem to be coincidentally associated with IBD, NENs may also arise in this setting. In fact, long-standing inflammation could be directly responsible for the development of pancellular dysplasia involving epithelial, goblet, Paneth and neuroendocrine cells. It has yet to be established which IBD patients have a higher risk of developing NENs.

Key words: Colorectal cancer; Large cell carcinoma; Neuroendocrine carcinoma; Small cell carcinoma

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INTRODUCTION

It is well recognized that ulcerative colitis (UC) and Crohn's disease (CD) predispose to the development of colorectal adenocarcinoma (CRC). The duration and the anatomic extent of the disease have been shown to be independent risk factors for the development of CRC[1]. Thus, cancer is infrequently encountered when disease duration is less then 8-10 years, thereafter the risk rises at approximately 0.5%-1% per year[2]. Additional risk factors are the presence of primary sclerosing cholangitis and a family history of CRC. Younger age at diagnosis and the degree of endoscopic and histological activity may also play an important role[1]. A comprehensive meta-analysis of all published studies reporting CRC risk in UC up to 2001 showed that the risk for any patients with colitis was 2% at 10 years, 8% at 20 years and 18% after 30 years of disease[3].

Based on this evidence, guidelines for screening and surveillance of asymptomatic CRC in patients with inflammatory bowel disease (IBD) have been developed to detect either dysplasia or early cancer at a surgically curable stage, and to reduce CRC related mortality[1,2].

While adenocarcinoma is the most common colorectal epithelial malignancy associated with UC

and CD, other types of carcinomas such as squamous cell carcinoma, small cell carcinoma and "hepatoid" carcinoma have been described in inflamed bowel. With regards to non-epithelial malignancies, lymphomas and sarcomas represent the largest group of tumors reported in association with IBD, especially in immunosuppressed patients[4].

Carcinoid tumors and neuroendocrine neoplasms other than carcinoids (NENs) may also be associated with UC and CD of the colon based on the finding of increased numbers of neuroendocrine cells in inflamed mucosa, suggesting that long-standing inflammation is directly responsible for the development of this kind of neoplasia[4].

NENs of the colon and rectum are rare tumors, and are infrequently described in the setting of IBD. Here we report two cases of NENs arising in UC.

CASE REPORT

Case 1

A 35-year-old man with an 11-year history of mild distal colitis was admitted due to constipation, proctorrhagia, tenesmus and perianal pain. Twelve months before admission, a surveillance colonoscopy with biopsy showed endoscopic remission of the inflammatory disease. His treatment consisted of oral mesalamine 2.4 g/d and he had never received immunosuppressant drugs.

A second colonoscopy indicated a rectal substenosis caused by a large ulcerated mass originating about 2 cm above the anorectal junction, with a proximal extension of about 4 cm.

Histological examination showed a high grade large cell neuroendocrine carcinoma. Immunohistochemical staining was positive for chromogranin, synaptophysin, cytokeratin 7 and chorioallantoic membrane 5.2. Laboratory findings including chromogranin A and neuron-specific enolase were negative.

A staging computed tomography scan and somatostatin receptors imaging were negative for further spread of the tumor. The patient underwent a low anterior colorectal resection and distal rectal mucosectomy with colo-anal anastomosis and loop ileostomy. A definitive pathological investigation confirmed the previous histological findings, and the proliferative marker Mib1/Ki67 was positive in about 50% of the neoplastic cells. The TNM stage was $pT_3^{R1}pN_2^{17/30}M_0G3$. He was treated with adjuvant radiotherapy and chemotherapy with cisplatin and etoposide. One year after surgery, multiple liver metastases were observed. The patient died 15 mo after diagnosis.

Case 2

A 77-year-old man presented with bloody diarrhea, anorectal pain, mild anemia and a 3 kg weight loss. The patient had a 27-year history of left-sided colitis with mild activity. Ten months before admission, a surveillance colonoscopy with biopsy showed mild left-sided inflammation. He was receiving oral mesalamine 2.4 g/d.

Another colonoscopy was performed, which showed a large circular neoplasm of about 5 cm in length, 3 cm above the anorectal junction (Figure 1). Histological examination with immunohistochemical analysis excluded adenocarcinoma. A tumor with neuroendocrine differentiation and strong staining for synaptophysin was observed and a high grade small cell neuroendocrine carcinoma was diagnosed (Figures 2 and 3). The proliferative marker Mib1/Ki67 was positive in about 90% of the neoplastic cells. MR scanning and somatostatin receptors imaging were negative for further neoplastic localizations. The patient is scheduled to receive neo-adjuvant radiotherapy.

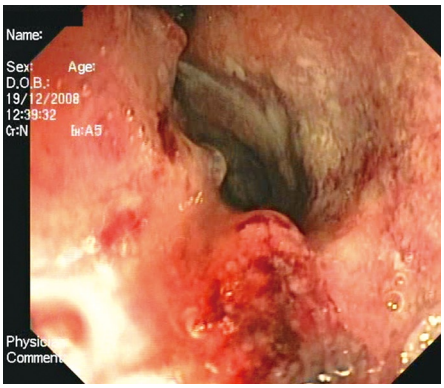

Figure 1 Small cell neuroendocrine carcinoma: endoscopic view.

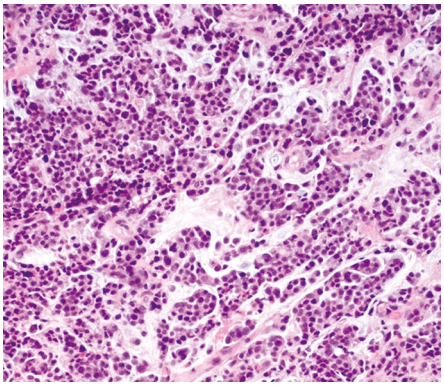

Figure 2 Cells with minimal cytoplasm, fusiform cell shape, finely granular chromatin, small, or absent, nucleoli (HE stain, original magnification × 20).

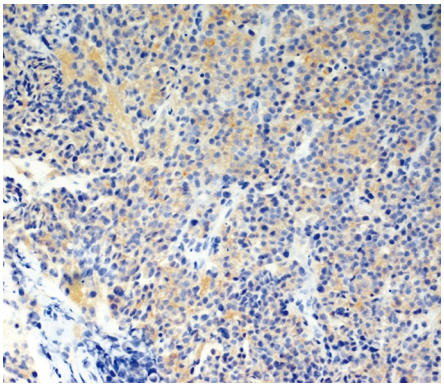

Figure 3 Immunohistochemical analysis reveals neuroendocrine differentiation with positive staining for synaptophysin (original magnification × 20).

DISCUSSION

The reported incidence of NENs is between 0.1% and 3.9% of all colorectal malignancies. Bernick *et al*[5], at New York Memorial Sloan-Kettering Cancer Center, reported an incidence of 0.6% in colon and rectal cancers.

NENs are subdivided on the basis of cytological-histological features and immunohistochemical findings into small cell neuroendocrine carcinoma (SCNC) and large cell neuroendocrine carcinoma (LCNC)[5]. The criteria for separating small cell from large cell variants are similar to those proposed for the classification of pulmonary neuroendocrine tumors[6]. SCNCs resemble their pulmonary homonyms, having minimal cytoplasm, fusiform cell shape, fine granular chromatin, small or absent nucleoli, and nuclear moulding. LCNCs are characterized by cells with more cytoplasm, a round or polygonal cell shape, prominent nucleoli, and a coarser chromatin pattern.

While the diagnosis of SCNC does not necessitate immunohistochemical documentation of neuroendocrine differentiation, the diagnosis of LCNC requires positive immunohistochemical staining for one of the three neuroendocrine markers (chromogranin, synaptophysin and neuron-specific enolase) in at least 10% of tumor cells[5].

Even though they are extremely rare, it is important to recognize NENs pathologically, since they have a particularly poor prognosis compared to colorectal adenocarcinoma. NENs shows a high rate of liver metastasis (50%) and the reported 1-year survival rate is about 40%[5]. Thus, patients could benefit from treatment with alternative cytotoxic chemotherapeutic agents[6].

There have been reports of neuroendocrine tumors associated with UC, but many of these tumors are carcinoids, often well-differentiated and clinical indolent, and are found incidentally at a different site from dysplastic areas after surgery for IBD[7]. Greenstein *et al*[7] believe that this association is coincidental, particularly since there are no significant demographic or clinicopathological differences between the carcinoids that occur in patients with and without IBD. Furthermore, the same authors point out that neuroendocrine cell hyperplasia, probably due to a trophic role of these cells in epithelial re-growth, is not itself an established association with carcinoids.

Sigel *et al*[8] reported the only series of NENs other than carcinoids arising in 14 IBD patients (eight CD; six UC). Similar to our findings, they observed that all the tumors arose in areas involved by IBD, and six of the 14 neoplasms affected the rectum. The NENs were well differentiated in 11 cases and poorly differentiated in three cases; two of these three patients died at 3 and 11 mo after tumor excision.

These authors suggested that neuroendocrine differentiation, different to carcinoids, might evolve from multipotential cells in dysplastic epithelium. This was based on their findings that dysplasia was found in adjacent mucosa in more than one-third of cases[8].

Our cases confirm the aggressive biological behavior and poor response to chemotherapy of NENs compared to colorectal adenocarcinoma. Both NENs in these patients arose in mild left-sided UC, however, the patients were different in age (35 years *vs* 77 years) and disease duration (11 years *vs* 27 years). This raises the question as to whether these carcinomas represent an incidental finding in the background of IBD or if there is a real, although rare, association between IBD and these tumors.

The hypothesis of a pancellular dysplasia involving epithelial, goblet, Paneth and neuroendocrine cells in chronic inflamed mucosa cannot be confirmed from the few published case reports[8-11]. In addition, the differences in terms of duration, extension, histological and endoscopic activity of the underlying IBD in our and other reported cases[8-11] do not allow the identification of a subgroup of patients at risk.

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CASE REPORT

Successful interferon desensitization in a patient with chronic hepatitis C infection

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Abstract

Treatment of hepatitis C, even when absolutely necessary, is almost impossible when interferon cannot be administered for any reason. We report a 65-year-old patient with chronic hepatitis C virus (HCV) infection and fibrosis, who was unable to receive interferon because of systemic hypersensitivity. The patient was desensitized successfully through gradual incremental exposure to interferon, and HCV infection was eradicated after a complete course of treatment, with no further allergic reactions. This case report that describes successful eradication of hepatitis C in a patient with advanced liver disease after desensitization to interferon revealed that desensitization may not necessarily damage the therapeutic efficacy of the drug.

Key words: Chronic hepatitis C; Desensitization; Drug hypersensitivity; Interferon a2b; Ribavirin

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection is one of the leading known causes of chronic liver diseases, including cirrhosis and hepatocellular carcinoma[1,2]. Risk factors associated with transmission of HCV include transfusion of infected blood products, injection drug use, employment in patient care or clinical laboratory work, exposure to an infected sex partner or household member, exposure to multiple sex partners, and low socioeconomic status[3].

HCV has a positive-sense, single-stranded RNA genome that has been classified into six different genotypes from 1 to 6[4,5]. The genotype determination is a relevant clinical practice, which not only helps predict the probability of sustained virological response (40%-45% for genotype 1 compared with 70%-80% for genotypes 2 and 3), but also is used routinely to determine duration of treatment (48 wk for genotypes 1 and 4 *vs* 24 wk for genotypes 2 and 3)[6,7]. Various genotypes have distinct geographical distributions around the world[8], and recent reports from Tehran and five cities from different locations in Iran, have shown that genotype 1a was predominant (47%), and 3a, 1b and 4 had a prevalence of 36%, 8% and 7%, respectively[9].

Interferon, in various forms or combinations, is the only proven effective treatment for hepatitis C. It has a fundamental, irreplaceable role in the treatment of patients with HCV infection. Treatment of hepatitis C, even when absolutely necessary, is almost impossible when interferon cannot be administered for any reason.

Here, we report a patient with chronic HCV infection and advanced fibrosis who was unable to receive interferon because of systemic hypersensitivity. The patient was desensitized successfully through gradual incremental exposure, and HCV infection was eradicated after a complete course of treatment, with no further allergic reactions.

CASE REPORT

The patient was a 65-year-old man who was referred to

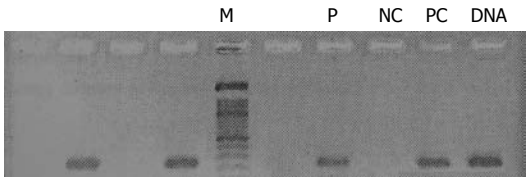

Figure 1 Reverse transcription polymerase chain reaction (RT-PCR) showing cDNA band of hepatitis C virus (HCV). P: Patient; PC: Positive control; NC: Negative control; M: Marker.

Table 1 Dosing protocol for the day 1

Dose number	Time from first dose (h)	Strength	Volume	Drug dosage (IU)
1	0	1/100000	0.1	3
2	1	1/100000	0.1	3
3	2	1/10000	0.1	30
4	3	1/10000	0.1	30
5	4	1/1000	0.1	300
6	5	1/1000	0.1	300
7	6	1/100	0.1	3000
8	7	1/100	0.1	3000
9	8	1/100	0.1	3000
10	9	1/100	0.1	3000
11	10	1/100	0.1	3000
12	11	1/100	0.1	3000
13	12	1/100	0.1	3000
14	13	1/100	0.1	3000
Cumulative dose				24666

our liver clinic with a positive anti-HCV antibody ELISA. The patient's main complaint was fatigue. Past medical history was positive for two coronary angioplasties for ischemic heart disease. No other medical diseases were present in his past history. General physical examination was normal except for mild hepatomegaly. Results of laboratory tests were within normal ranges except for a marginally low albumin level (3.4 g/dL) and mildly elevated aspartate aminotransferase (45-80 IU/mL) and alanine aminotransferase (46-78 IU/mL).

Reverse transcription polymerase chain reaction (RT-PCR) revealed a cDNA band that indicated the presence of HCV (Figure 1). Genotype-specific primers for HCV genotyping showed the virus to be of genotype 1b. Liver needle biopsy showed chronic hepatitis with moderate activity and advanced fibrosis (grade 9/18, stage 5/6).

The patient was selected for treatment with interferon plus ribavirin. The dosage of ribavirin was kept constant at 1200 mg/d throughout treatment. Since he was not able to afford the cost of pegylated interferon therapy, PDferon®, a brand of interferon α-2b produced in Iran (Pooyeshdarou Pharmaceutical Co., Tehran, Iran), was used.

After administration of the first dose of interferon α2b, the patient developed generalized maculopapular rash with severe itching and low-grade fever. As a result, treatment was stopped, with a diagnosis of hypersensitivity to interferon.

Since there was no other choice for treatment of hepatitis C, after careful discussion with the patient about possible benefits and side effects, a decision was made to proceed with a course of desensitization. The

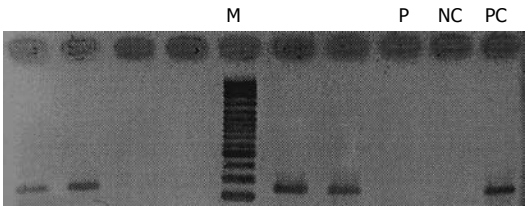

Figure 2 No evidence of cDNA band of HCV with RT-PCR after treatment with interferon plus ribavirin following desensitization. P: Patient; PC: Positive control; NC: Negative control; M: Marker.

Table 2 Dosing protocol for day 2

Dose number	Time from first dose (h)	Strength	Volume	Drug dosage (IU)
1	0	1/100	0.1	3000
2	1	1/100	0.1	3000
3	2	1/10	0.1	30000
4	3	1/10	0.1	30000
5	4	1/1	0.1	300000
6	5	1/1	0.1	300000
7	6	1/1	0.1	300000
8	7	1/1	0.1	300000
Cumulative dose				1266000

patient was kept in hospital during the first and second day of the treatment, with close observation of his vital signs. Resuscitation equipment was kept at the bedside. A routine blood count and chemistry were performed on days 1 and 2 and revealed normal results. A 1-mL vial of PDferon® that contained 3000000 IU was diluted to 1/100000 concentration, and 0.1 mL of the resulting solution was injected subcutaneously.

Desensitization started from 8 am on the first day and dose escalation continued until the sixth dose (14.00 h). The dose was kept the same during the afternoon and injections stopped at 21.00 h to allow for patient rest (Table 1). Desensitization restarted on the next day at the same time, with a 1/100 concentration (Table 2). From day 3 onwards, until the end of the first week, the drug was given at a dose of 1500000 IU/d in the morning, with a 1-h period of observation after the injections. From the second week until the end of the treatment (54 wk), interferon was given at a dosage of 3000000 IU every other day.

The only observed reaction during the treatment was a mild generalized pruritus at the start of day 2, with no accompanying rash or change in vital signs. These symptoms responded to intramuscular injection of diphenhydramine. After completing the treatment course, the patient recovered from HCV and nested RT-PCR revealed no cDNA band, which indicated eradication of HCV (Figure 2). RT-PCR was repeated another two times at 6-wk intervals, with the last one at 18 mo after the end of treatment. All turned out to be negative.

DISCUSSION

Since the patient had advanced liver fibrosis and there was no alternative treatment for hepatitis C, after extensive

discussion with the patient about the possible side effects, as well as alternatives, a decision was made to proceed with desensitization. The protocol was based on previous experience with desensitization to penicillin. This decision was reviewed by another independent gastroenterologist and the patient gave written informed consent.

Interferon products are used worldwide as an effective treatment for chronic hepatitis C. Although several potential antiviral drugs are in the pipeline, interferon is still the only proven effective treatment for this viral disease.

Considering the above facts, when a patient is in great need of antiviral therapy for hepatitis C (advanced fibrosis for instance) and has an allergic reaction to interferon, there are very limited, if any, treatment options available. Although desensitization through gradual exposure to incremental doses is an established option for treatment of allergic reaction to protein and non-protein drugs[10], there are no reports of its use for desensitization to interferon. The main theoretical risk (beside the proven risk of anaphylaxis) with desensitization to protein drugs such as interferon is that it induces neutralizing antibodies, which potentially are directed towards the effective sites of the drug, which renders it ineffective, or at least reduces its therapeutic potential.

This case report describes successful eradication of hepatitis C in a patient with advanced liver disease, and is an indication that desensitization may not necessarily damage the therapeutic efficacy of the drug. Although in this case a regular (non-pegylated) interferon product was used, it may be expected that the same procedure can be applied to pegylated interferon products, since it is the protein component towards which hypersensitivity is usually directed.

Further studies including measurement and characterization of possible contributing antibodies are needed before this method can be suggested as a standard protocol for interferon desensitization.

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CASE REPORT

Composite neuroendocrine and adenomatous carcinoma of the papilla of Vater

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Abstract

Malignant tumors of papilla are usually adenocarcinomas. We present a 67-year-old female who became icteric as result of a malignant tumor infiltrating the papilla of Vater. Histopathological assessment of surgically excised tumor showed both neuroendocrine and adenocarcinomatous features. To our knowledge, this is the seventh report of this rare neoplastic association in the duodenal periampullary region.

Key words: Adenocarcinoid; Composite carcinoma; Carcinoma of Vater papilla; Carcinoid; Gastropancreatic neuroendocrine tumor

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INTRODUCTION

In adults the commonest causes of obstructive jaundice are biliary stone disease, pancreatic cancer or chronic pancreatitis. A less frequent cause is a benign or malignant tumor arising from the papilla of Vater. Malignant tumors of papilla are usually adenocarcinomas. We present a patient who became icteric as a consequence of a malignant tumor infiltrating the papilla of Vater which histologically showed both neuroendocrine and adenocarcinomatous features.

CASE REPORT

A 67-year-old cholecystectomized woman was admitted to our department with symptoms of obstructive jaundice and cholangitis. Severe epigastric pain, fever and jaundice developed 1 d before presentation. The serum bilirubin level was 5.3 mg/dL, white cell blood count was 20.4 × $10^3/\mu$L and serum amylase and lipase activities were elevated to about six times the normal upper limit. Abdominal ultrasonography showed moderate dilatation of common bile duct. The patient underwent endoscopic retrograde cholangiopancreatography (ERCP) revealing the papilla of Vater was suggestive of adenoma i.e., was markedly enlarged and covered with smooth surfaced mucosa. Sphincterotomy was performed and biopsies were taken from the inner part of the ampulla. The histopathological diagnosis from biopsy specimens was carcinoid tumor, staining positive for chromogranin A and synaptophysin. The patient underwent surgical transduodenal excision of the ampulla. The postoperative histopathological examination showed a composite tumor i.e. mixed neuroendocrine carcinoma (NE)-adenocarcinoma neoplasm (Figure 1A and B). Immunocytochemical studies were used to delinate the NE component (chromogranin, synaptophisin, EMA, cytokeratin). The NE-cell component represented about 80% of the tumor's area. NE tumor cells were well-differentiated, lacked significant atypia and showed low grade malignancy (Ki-67 labelling index was 5%). The glandular component presented well- differentiated tubules (grade I). No necrosis was found in either the NE or glandular areas. The tumor focally involved lymphatic vessels and nerves and infiltrated the muscle of the ampulla. The margins of the excised tissue specimen were free of neoplasm and no neoplastic cells were found in periduodenal lymphatic nodes (T1N0M0). The patient

has been put under endoscopic, serological (CA 19-9, CEA, chromogranin A) and ultrasound surveillance. Six months after surgery she is doing well.

DISCUSSION

Gastroenteropancreatic Neuroendocrine Tumors (GEP/NETs) are rare neoplasms originating from the diffuse neuroendocrine system, involving 15 types of highly differentiated ectodermal cells located in the gastrointestinal tract and pancreas. The most common location of GEP/NETs is in the appendix (62% of cases), but they may also be found in small intestine (27%), lung (15%), undefined primary locations with hepatic metastases (12%) and other organs (3%)[1]. GEP/NETs secrete peptides and neuroamines that cause distinct clinical syndromes, including carcinoid syndrome. Many tumors are, however, clinically silent until late presentation associated with mass effects. The current WHO classification categorizes GEP/NETs into: (1) well-differentiated neuroendocrine tumor; (2) low-grade malignant carcinoma; (3) high-grade malignant carcinoma and (4) mixed tumor: adenocarcinoma/neuroendocrine carcinoma[2]. At present 14 terms are used to define tumors with mixed exocrine-endocrine features. Volante *et al*[3] proposed classification based on the extension of each component and structural features of the NE component. Three separate patterns can be distinguished i.e. (1) NE tumors with focal non-NE component occupying less than 30% of the tumor, (2) mixed exocrine-endocrine carcinomas (NE or non-NE cells > 30%) and (3) non-NE carcinoma with NE component (< 30%). The type of tumor influences the prognosis, which improves with increasing contribution of NE component[3]. About 50% of GEP/NETs are carcinoids found by chance during laparotomy or as hepatic metastases.

Carcinoids involving the papilla of Vater are rare lesions, accounting for 0.35% of all gastrointestinal carcinoids. So far, only 110 cases have been reported in the literature, mostly as individual case reports[4]. These tumors are predominantly found on ERCP in patients with obstructive jaundice or acute biliary pancreatitis. In the presented case the tumor histologically was a mixed carcinoid-adenocarcinoma neoplasm (type Ⅳ). The concurrence of carcinoid with adenocarcinoma is an unusual phenomenon in the gastrointestinal tract, that has been reported in the oesophagus, stomach, small intestine, appendix, colon and rectum[5-7]. To our knowledge, this is the seventh report of this rare neoplastic association in the duodenal periampullary region.

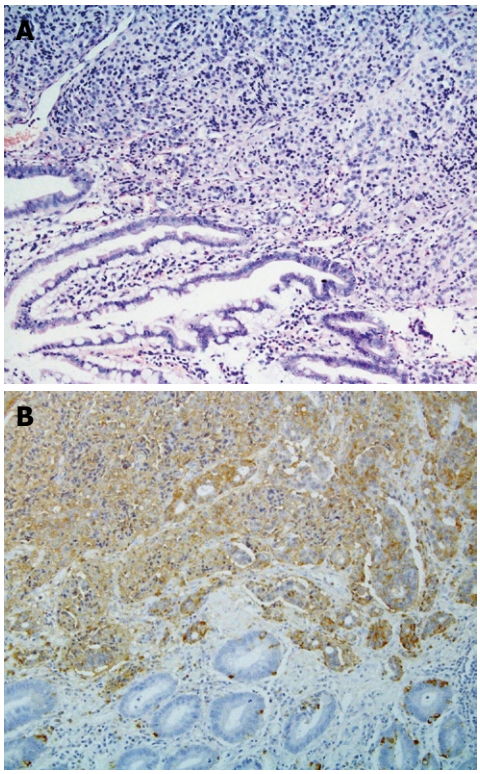

Figure 1 Histopathological examination. A: Mixed carcinoid-adenocarcinoma neoplasm (× 200); B: Chromogranin A positive stain of tumour cells (× 200).

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CASE REPORT

Placement of percutaneous transhepatic biliary stent using a silicone drain with channels

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Key words: Biliary tract; Drainage; Hepaticojejunostomy; Stenosis; Stents

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Yoshida H, Mamada Y, Taniai N, Mineta S, Mizuguchi Y, Kawano Y, Sasaki J, Nakamura Y, Aimoto T, Tajiri T. Placement of percutaneous transhepatic biliary stent using a silicone drain with channels. *World J Gastroenterol* 2009; 15(33): 4201-4203 Available from: URL: http://www.wjgnet.com/1007-9327/15/4201.asp DOI: http://dx.doi.org/10.3748/wjg.15.4201

Abstract

This report describes a method for percutaneous transhepatic biliary stenting with a BLAKE Silicone Drain, and discusses the usefulness of placement of the drain connected to a J-VAC Suction Reservoir for the treatment of stenotic hepaticojejunostomy. Percutaneous transhepatic biliary drainage was performed under ultrasonographic guidance in a patient with stenotic hepaticojejunostomy after hepatectomy for hepatic hilum malignancy. The technique used was as follows. After dilatation of the drainage root, an 11-Fr tube with several side holes was passed through the stenosis of the hepaticojejunostomy. A 10-Fr BLAKE Silicone Drain is flexible, which precludes one-step insertion. One week after insertion of the 11-Fr tube, a 0.035-inch guidewire was inserted into the tube. After removal of the 11-Fr tube, the guidewire was put into the channel of a 10-Fr BLAKE Silicone Drain. The drain was inserted into the jejunal limb through the intrahepatic bile duct and was connected to a J-VAC Suction Reservoir. Low-pressure continued suction was applied. Patients can be discharged after insertion of the 10-Fr BLAKE Silicone Drain connected to the J-VAC Suction Reservoir. Placement of a percutaneous transhepatic biliary stent using a 10-Fr BLAKE Silicone Drain connected to a J-VAC Suction Reservoir is useful for the treatment of stenotic hepaticojejunostomy.

INTRODUCTION

In most operations for hepatic hilum malignancy, the intrahepatic bile duct at the hilum is divided from the hepatic artery, portal vein, and surrounding tissues. Biliary complications, such as leakage or stenosis, are caused occasionally by the presence of several small bilioenteric anastomoses[1]. Stenosis of a bilioenteric anastomosis increases the risk of cholangitis and requires the long-term placement of a stent.

The BLAKE Silicone Drain (Ethicon, NJ, USA) is made of silicone; the entire drain is flexible, and channels along the sides, instead of holes, facilitate drainage. It is always used with a continuous suction device (J-VAC Suction Reservoir; Ethicon), which creates a closed drainage system. Drainage is very efficient: a larger area is in contact with tissue as compared with perforated drains, and fluid is efficiently removed by capillary pressure.

We have reported previously the effectiveness of a BLAKE Silicone Drain connected to a J-VAC Suction Reservoir for the management of pancreatic fistula[2]. We also reported that a 10-Fr BLAKE Silicone Drain has been used as an external stent for a bilioenteric anastomosis during surgery for hepatic hilar malignancy[3].

This report describes a method for percutaneous transhepatic biliary stenting with a BLAKE Silicone Drain, and discusses the usefulness of placement of the drain connected to a J-VAC Suction Reservoir for the treatment of stenotic hepaticojejunostomy.

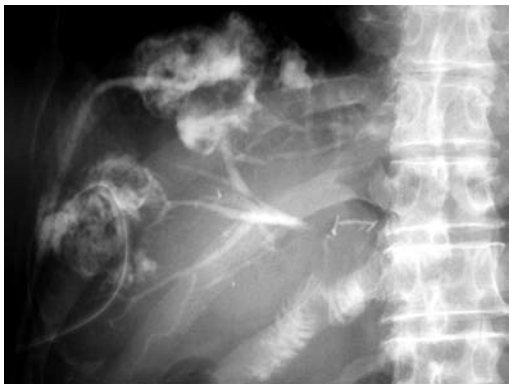

Figure 1 Percutaneous drainage catheters were inserted into each abscess, and pus was discharged. Abscessography demonstrated communications between the dilated intrahepatic bile duct of the anterior branch and each abscess.

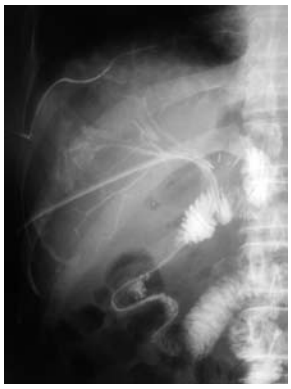

Figure 3 One week after initial insertion of the 11-Fr tube, cholangiography was performed *via* the tube. After insertion of a 0.035-inch guide wire, the 11-Fr tube was switched to a 10-Fr BLAKE Silicone Drain.

CASE REPORT

A 68-year-old man with intrahepatic cholangiocarcinoma invading the hepatic hilum underwent extended left hepatectomy combined with *en bloc* resection of the extrahepatic bile duct and extended lymph node dissection.

Bilioenteric continuity was reestablished by two hepaticojejunostomies of the anterior and posterior branches of the hepatic ducts, performed using a Roux-en-Y jejunal limb. Silicone drains with an internal lumen and side holes, made by cutting a 5-Fr radiopaque tube for pancreatic drainage (Sumitomo Bakelite, Tokyo, Japan), were used as an internal stent for each bilioenteric anastomosis. Two external drainage catheters (19-Fr BLAKE Silicone Drain) were positioned at the cut surface of the liver and connected to the J-VAC Suction Reservoir. Fixation of the greater omentum was performed to avoid delayed gastric emptying[4]. After operation, major bile leakage occurred, but resolved gradually. The drainage catheter was removed on postoperative day 12, and the patient was discharged on postoperative day 18.

Forty days after discharge, the patient was readmitted because of severe pyrexia. Upon admission, computed tomography demonstrated two low-density areas associated with dilatation of the intrahepatic bile ducts in the anterior segment. Hepatic abscesses caused by stenosis of the hepaticojejunostomy were diagnosed.

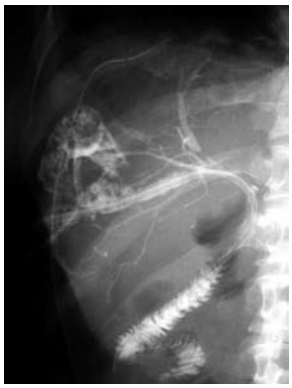

Figure 2 The catheter (11-Fr PTCS tube, Sumitomo Bakelite, Tokyo, Japan) with several side holes was placed in the jejunal limb through the intrahepatic bile duct and passed through the stenosis of the hepaticojejunostomy.

Percutaneous drainage catheters were inserted into each abscess, and pus was discharged. Abscessography demonstrated communications between the dilated intrahepatic bile ducts of the anterior branch and each of the abscesses (Figure 1). Pyrexia improved, and discharge of clear bile from one of the drainage catheters continued. One week after insertion of the drainage catheters, abscessography was repeated. It was difficult to insert the catheter from the abscess into the intrahepatic bile duct. Another percutaneous transhepatic biliary drainage catheter was inserted directly into the intrahepatic bile duct under ultrasonographic guidance. An 11-Fr tube (11 Fr PTCS tube; Sumitomo Bakelite) with several side holes was placed in the jejunal limb through the intrahepatic bile duct, and passed through the stenosis of the hepaticojejunostomy (Figure 2). Bile juice was discharged from the 11-Fr tube, and discharge from the drainage catheters of the abscess decreased. One of the catheters was removed from the abscess because pus discharge stopped. One week after initial insertion of the 11-Fr tube, cholangiography was performed *via* the tube. A 10-Fr BLAKE Silicone Drain is flexible, which made one-step insertion difficult. After insertion of a 0.035-inch guide wire, the 11-Fr tube was switched to a 10-Fr BLAKE Silicone Drain (Figure 3). The drain was connected to a J-VAC Suction Reservoir, and continued low-pressure suction was applied. The remaining catheter was removed from the abscess. The patient was discharged 5 d after switching the 11-Fr tube to a 10-Fr BLAKE Silicone Drain connected to the J-VAC Suction Reservoir. After 4 mo, the patient was readmitted because of liver failure. The portal vein was obstructed by recurrent carcinoma. Three weeks after readmission, the patient died. The 10-Fr Blake Silicone Drain was not occluded.

DISCUSSION

Diagnostic techniques for hepatobiliary disease have improved recently, but advanced hepatic hilum malignancy is still encountered frequently[5-7]. Palliative treatment with a biliary stent is carried out in patients with inoperable malignancy in order to relieve symptoms related to obstructive jaundice[8]. In patients undergoing hepatectomy for advanced hepatic hilum malignancy, aggressive dissection may compromise the ductal blood supply. Peripheral branches of blood vessels,

with small diameters and a poor blood supply, may be damaged along with the bile duct, which increases the risk of biliary leakage[1]. The use of stents has been recommended to decompress the bile duct, reduce the risk of bile leakage, and decrease fibrotic narrowing of the anastomosis during early healing[9-12]. However, the risk of biliary complications such as leakage or stenosis is increased by the presence of small several bilioenteric anastomoses[1].

We performed placement of a percutaneous transhepatic biliary stent using a 10-Fr BLAKE Silicone Drain connected to a J-VAC Suction Reservoir for the treatment of stenotic hepaticojejunostomy. BLAKE Silicone Drains promote efficient drainage: a larger area is in contact with tissue as compared with perforated drains, which facilitates the effective removal of fluid by capillaries. These drains contain no plasticizing agents, which are considered to have deleterious effects in humans. The channels do not interfere with removal, and the drains can be removed easily, safely and securely. These drains also provide a high flow rate when connected to a closed suction device (J-VAC Suction Reservoir). The suction pressure of the J-VAC Suction Reservoir is lower than that with other portable low-pressure continuous suction devices. As a result of low suction pressure, the J-VAC Suction Reservoir does not damage tissue; therefore, we used this drainage system in the lumen of the bile duct and jejunum.

We have evaluated previously the efficacy of a BLAKE Silicone Drain connected to a J-VAC Suction Reservoir for the management of pancreatic fistula. In basic experiments, no collections of fluid were detected around the BLAKE Silicone Drain. When leakage occurred, it did not cause an abdominal abscess, and a "drain canal" linking the anastomosis with the extracorporeal orifice was formed all along the drainage route[2].

In the patient described in this report, 5-Fr silicon drains were used as a stent for the bilioenteric anastomosis. Stenosis of the anastomosis occurred after leakage. We used a 10-Fr BLAKE Silicone Drain as an external stent for bilioenteric anastomosis after major hepatectomy with pancreatoduodenectomy. The drain is placed within the hepaticojejunostomy *via* the stump of the jejunal limb. It is then connected to the J-VAC Suction Reservoir. Bile and pancreatic juice can be drained by a single drain. Even if bilioenteric anastomosis leaks, a 10-Fr intraluminal diameter of the anastomosis is maintained[3].

The treatment of stenotic bilioenteric anastomosis requires the long-term placement of a stent in the anastomosis. The BLAKE Silicone Drain is flexible and contains no plasticizing agents. It has four continuous channels instead of holes along the sides, and prevents obstruction of small biliary branches. The BLAKE Silicone Drain is therefore suited for long-term placement. The input port of the J-VAC Suction Reservoir has an anti-reflux valve, which reduces the risks of the reverse flow of fluid into the body and of retrograde infection. This valve enables the patient to use the reservoir without having to worry about the level of the drainage bag during position changes and ambulation. The J-VAC Suction Reservoir is portable and convenient in size, which enables the patient to carry it around in a specially designed pochette on the shoulder. A BLAKE Silicone Drain connected to a J-VAC Suction Reservoir usually remains free of occlusion for about 6 mo.

In conclusion, placement of a percutaneous transhepatic biliary stent using a 10-Fr BLAKE Silicone Drain connected to a J-VAC Suction Reservoir was useful for the treatment of stenotic hepaticojejunostomy.

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CASE REPORT

Sclerosing epithelioid fibrosarcoma of the liver infiltrating the inferior vena cava

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Abstract

Sclerosing epithelioid fibrosarcoma (SEF) is a rare and distinct variant of fibrosarcoma, composed of epithelioid tumor cells arranged in strands, nests, cords, or sheets embedded within a sclerotic collagenous matrix. We report a 39-year-old man with SEF of the liver, which infiltrated the inferior vena cava (IVC). The SEF of the liver was successfully resected, and the infiltrated IVC was also removed together with the liver tumor. Histopathological examination of the tumor showed typical histopathology of SEF. Immunohistochemically, the tumor was positive for vimentin. Recurrence was noted 7 mo after surgery. After chemotherapy, the recurrent tumor was resected surgically, and histopathological examination showed similar findings to those of the primary tumor. To our knowledge, this is the first report of SEF of the liver with tumor invasion of the IVC.

Key words: Sclerosing epithelioid fibrosarcoma; Liver tumor; Surgery; Inferior vena cava invasion; Vimentin

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INTRODUCTION

Sclerosing epithelioid fibrosarcoma (SEF) is a rare and poorly defined variant of fibrosarcoma, first described by Meis-Kindblom *et al*[1] in 1995[2-4]. It is a mesenchymal neoplasm characterized histopathologically by a predominant population of epithelioid cells arranged in strands, nests, and sheets, embedded in a fibrotic and extensively hyalinized stroma. Although the SEF belongs to the low-grade sarcoma group of neoplasms, approximately 50% of patients with the tumor develop local recurrence and/or metastases[3-5].

Several investigators have reported cases of SEF arising from various sites such as upper extremities, lower extremities, limb girdles, trunk, and the head and neck area, but there have been no reports of SEF of the liver[1-10]. Recently, we experienced a patient with SEF of the liver with invasion of the inferior vena cava (IVC). Although the SEF in the patient was surgically resected, it recurred after surgery. The recurrent SEF was also surgically resected. In addition to the rarity of the SEF of the liver, the SEF displayed a rare extension pattern

of infiltration in the IVC. To our knowledge, there have been no previous reports of such kinds of tumor extension in SEF. In this article, we report the first case of SEF of the liver with invasion of the IVC.

CASE REPORT

A 39-year-old man presented with the chief complaint of general fatigue and abdominal uncomfortableness. Laboratory findings included no thrombocytopenia and liver enzymes above the normal range (alanine transaminase: 44 U/L, lactate dehydrogenase: 267 U/L, alkaline phosphatase: 412 U/L, and γ-glutamyltranspeptid ase: 187 U/L). Hepatitis virus markers and autoantibodies were negative. Tumor markers including protein induced by vitamin K absence or antagonist Ⅱ, α-fetoprotein (AFP), carbohydrate antigen 19-9 (CA19-9), and carcinoembryonic antigen were all within the normal ranges. Serum soluble interleukin-2 receptor level was not elevated. The patient did not consume alcohol and had no history of exposure to radiation. Computed tomography (CT) and magnetic resonance imaging (MRI) demonstrated a huge liver tumor measuring about 70 mm in diameter located in segment 1 based on Couinaud's classification, with invasion of the IVC adjacent to the site adjacent to the right atrium[11] (Figure 1A and B). In addition, another liver tumor measuring 18 mm was found in segment 8 on the CT and MRI. Fluorodeoxyglucose-positron emission tomography/CT showed abnormal accumulations in both tumors.

With a preoperative diagnosis of primary malignant liver tumor of unknown origin with invasion of the IVC and possibly intrahepatic metastasis, laparotomy was performed. The huge tumor, which was located mainly in the liver, adhered to the diaphragm, right lower lobe of the lung, and pericardium. The tumor also adhered to the suprahepatic IVC. By using the total hepatic vascular exclusion technique (THVE), extended left hepatectomy with resection of the caudate lobe of the liver was performed. The diaphragm, the right lower lobe of the lung, and the pericardium were resected. Next, after clamping the suprahepatic IVC below the right atrium and the retrohepatic IVC above the renal veins, the adhered IVC wall was removed together with the liver. The resected IVC wall was replaced with an expanded polytetrafluoroethylene graft. Since the tumor in segment 8 was suspicious of intrahepatic metastasis, partial hepatectomy of segment 8 was also performed. No other liver tumors were found during surgery.

A macroscopic view of the resected tumor was shown in Figure 2. The tumor, measuring 68 mm × 54 mm, was mainly located in segment 1 of the liver. Microscopically, the tumor mainly existed in the liver with invasion into neighboring extrahepatic soft tissue including the wall of the IVC and diaphragm. Moreover, tumor thrombi were identified in the left portal vein and its branches. The tumor consisted of uniformly round or polygonal epithelioid cells with a faintly eosinophilic cytoplasm (Figure 3A and B). The tumor cells were arranged in strands, nests, cords or sheets and embedded in a heavily hyalinized matrix. The microscopic findings of the tumor in segment 8 were similar to those of the primary tumor, and that tumor was diagnosed as an intrahepatic metastasis. Immunohistochemical staining of the main tumor was positive for vimentin (Dakopatts, Glostrup, Denmark), bcl-2 (Dakopatts), and CD99 (Dakopatts), and was negative for AE1/AE3 (Dakopatts), CAM5.2 (Becton Dickinson, San Jose, CA), desmin (Dakopatts), epithelial membrane antigen (EMA) (Dakopatts), CD34 (Nichirei, Tokyo, Japan), S100, α-smooth muscle actin (α-SMA) (Dakopatts), neuron-specific enolase (Dakopatts), CD56 (Dakopatts), leukocyte common antigen (LCA) (Dakopatts), CD30 (Dakopatts), HMB45 (Dakopatts), AFP (Dakopatts), and CA19-9 (Zymed Laboratories Inc., San Francisco, CA) (Figure 3C). The MIB-1 labeling index (Dakopatts) was 30%. Moreover, cytogenetic analysis did not show t (X; 18) chromosomal translocation, which is frequently observed in synovial sarcoma[12,13]. Based on the results of histopathological examination, cytogenetic analysis, and immunohistochemical patterns, the tumor was finally diagnosed as SEF. Although the tumor was associated with invasion to the wall of the IVC and diaphragm, the most part of the tumor existed in segment 1 of the liver. Moreover, tumor thrombi were identified in the right portal vein and its peripheral branches, and intrahepatic metastases, which were frequently seen in the primary liver malignant tumor. Therefore, the SEF probably originated from the liver. The resection margins were free of tumor. The noncancerous area of the liver was histologically normal. The patient had an uneventful postoperative course and was discharged from the hospital 24 d after surgery.

Seven months after surgery, the patient complained of back pain. CT examination showed recurrent SEF in the extrahepatic retroperitoneal tissue (Figure 1C and D). Systemic chemotherapy consisting of adriamycin and ifosfamide was applied. Close follow-up showed arrest of tumor growth and no new metastatic lesions within the first 3 mo. Subsequently, laparotomy was performed for the excision of the recurrent tumor 12 mo after the initial surgery. The recurrent tumor, located in the retroperitoneal tissue and adherent to the remnant liver, also infiltrated the graft IVC. Using the THVE approach, the tumor was resected with the adherent liver tissue and the infiltrated IVC graft. The resected IVC was again replaced with an expanded polytetrafluoroethylene graft. The microscopic findings of the resected tumor were similar to those of the primary SEF. The immunohistochemical staining patterns of the primary tumor and the recurrent tumor were also similar. The patient had an uneventful postoperative course and was discharged from the hospital 30 d after the surgery.

At the last follow-up examination 6 mo after the second surgery, he remains in good condition, with no evidence of recurrence.

DISCUSSION

SEF is a rare tumor characterized histopathologically by a predominant population of epithelioid cells arranged in strands, nests, cords, or sheets, which are

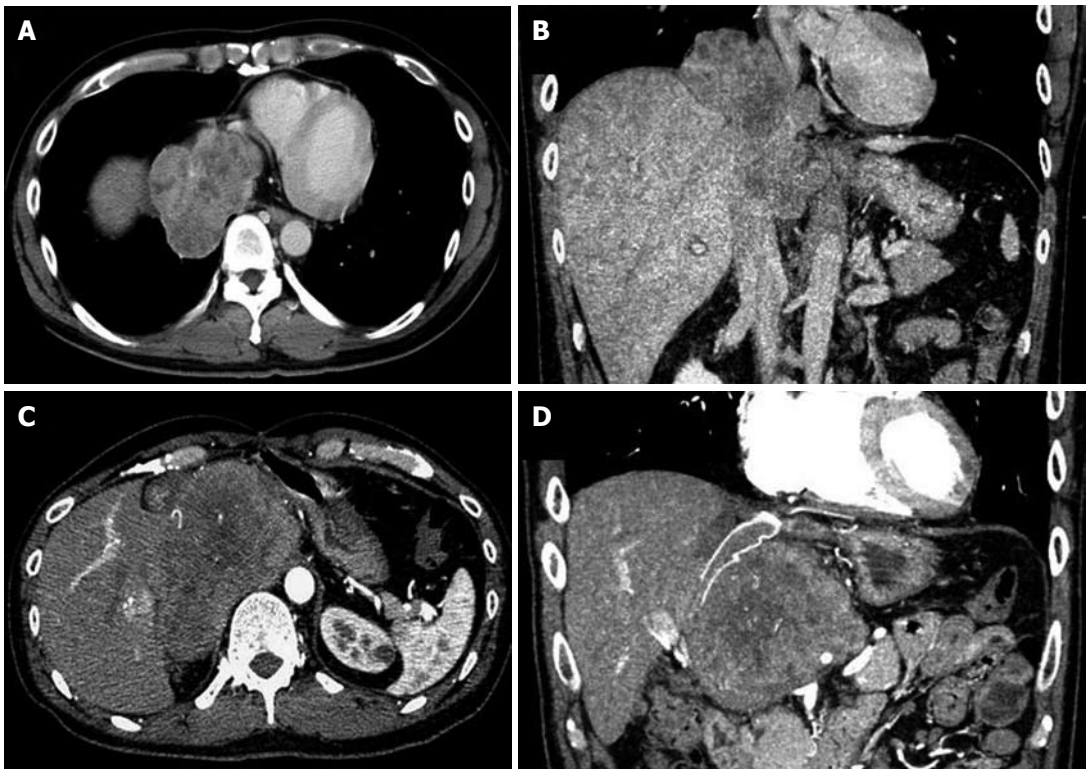

Figure 1 CT of the patient, showing the liver tumor infiltrating the suprahepatic inferior vena cava (A: Axial scan; B: Coronal scan), and showing the recurrent tumor (C: Axial scan; D: Coronal scan).

embedded within a sclerotic collagenous matrix[1-4]. Immunohistochemically, staining for vimentin is positive in all SEF whereas staining for CD34, leukocyte markers, HMB45, CD68, desmin, glial fibrillary acidic protein, and TP53 is negative. Focal and weak immunostaining for EMA, S100 and more rarely for cytokeratins may be seen in a minority of cases.

The histopathological differential diagnosis of SEF generally includes a wide variety of tumors with sclerotic or epithelioid features, and thus immunohistochemical analysis is essential for precise diagnosis of SEF. In the present case, differential diagnosis was challenging for the following reasons. Undifferentiated hepatocellular carcinoma and infiltrating adenocarcinoma should be differentiated from SEF, but immunohistochemical staining for AFP, CAM5.2, and AE1/AE3 was negative in the present case. Synovial sarcoma should also be differentiated from SEF. Cytogenetic identification of t (X: 18), which is found in synovial sarcoma, could differentiate synovial sarcoma from SEF[12,13]. No chromosomal translocation was observed in the present case. Furthermore, smooth muscle neoplasms, such as hyalinized leiomyoma or leiomyosarcoma, frequently resemble SEF histopathologically, but they are characterized by immunohistochemical positivity for α-SMA and desmin. Clear cell sarcoma and malignant peripheral nerve sheath tumors may need to be distinguished from SEF. However, these tumors are positive for S100 immunostaining. Moreover, sclerosing lymphoma and malignant melanoma of the soft part are also on the list of differential diagnoses, but these tumors are usually positive for LCA and HMB45, respectively, and were negative in our case. Epithelioid hemangioendothelioma could be ruled out based on immunonegativity for CD34, and alveolar rhabdomyosarcoma could be excluded by the negative result of desmin and absence of rhabdomyoblasts. Moreover, absence of osteroid formation excluded traosseous osteosarcoma in the present case. Thus, taking into consideration not only the histopathological findings but also the results of cytogenetic analysis and immunohistochemical examinations, the tumor was finally diagnosed as SEF.

In reviewing previous reports, SEFs usually arise in the deep soft tissue and are frequently associated with the adjacent fascia or periosteum. Most SEFs are located in the lower extremities and limb girdles, followed by the trunk, upper extremities, and the head and neck area[1-10].

In this case, although the tumor was associated with invasion to the wall of IVC and diaphragm, the most part of the tumor existed in the liver. Moreover, in this case, tumor thrombi and intrahepatic metastases were concurrently identified. These features might suggest that the tumor had originated in the liver. To our knowledge, there have been no reports of SEF originating from the liver.

The etiology of most malignant soft tissue tumors, including SEF, is generally unknown. A recent report reviewed 90 patients with SEF and found no significant gender difference with a mean patient age of 47 years (range, 14-87 years)[5]. The average tumor size at diagnosis is about 8 cm. Several investigators reported previously

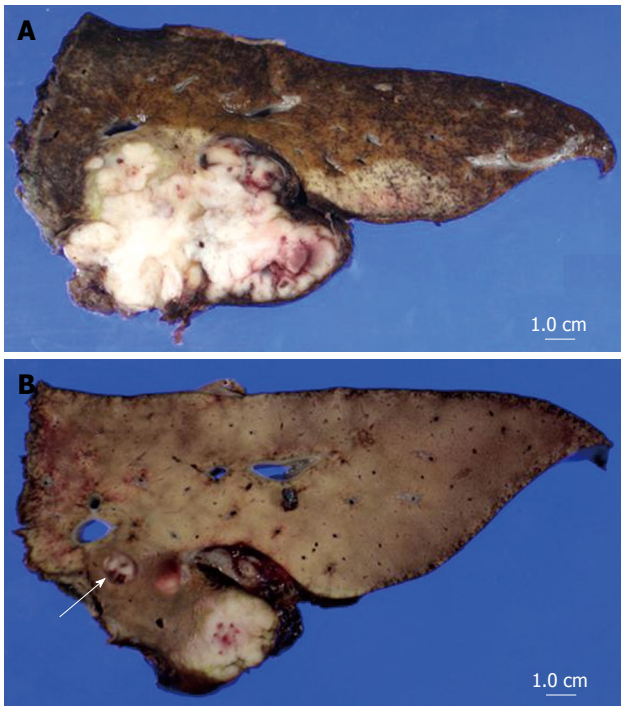

Figure 2 Macroscopic findings of the resected tumor. A: The tumor was directly contiguous to the normal liver parenchyma; B: Tumor thrombus was identified in the left portal vein (arrow).

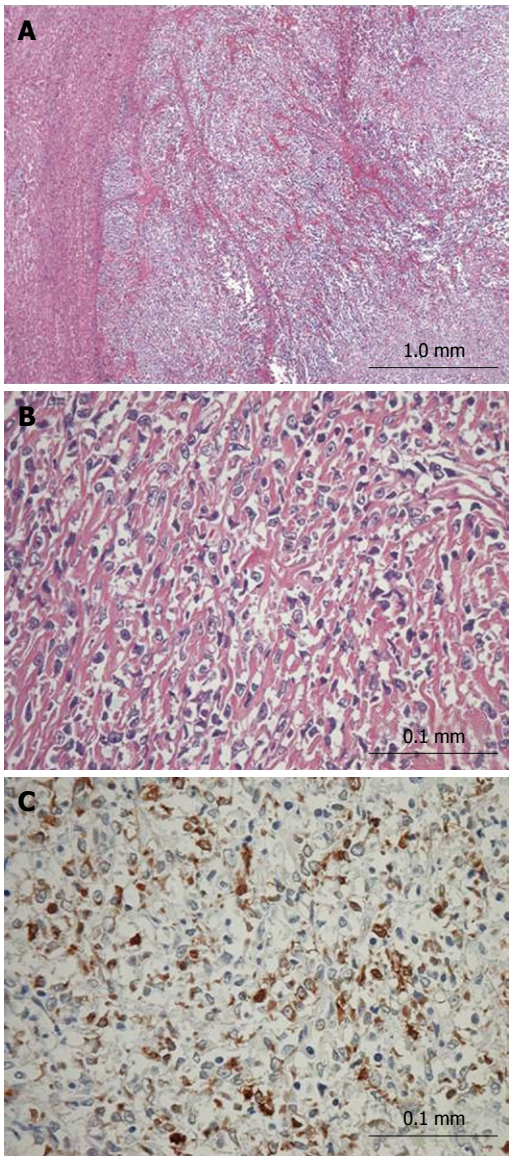

Figure 3 Histopathological findings of the resected liver tumor. A, B: Hematoxylin-eosin staining; C: Immunohistochemical staining for vimentin. A: The tumor (right side) was close to the normal liver parenchymal tissue (left side) (× 4); B: The tumor consisted of uniformly round or polygonal epithelioid cells, which were arranged in strands, nests, cords or sheets and embedded in a heavily hyalinized matrix (× 40); C: Positive immunohistochemical staining for vimentin (× 40).

the association between radiation and occurrence of fibrosarcoma, though our patient had no history of radiation[9,14].

The established treatment for SEF is complete resection. There is no evidence to support the effect of chemotherapy and/or radiotherapy, though these therapies were used in some previous reports[3,10]. As for prognosis, Chow *et al*[4] reported 57 patients with SEF with a local recurrence rate of 48%, metastasis rate of 60%, and mortality rate of 35%. Moreover, in a study of 16 patients with SEF by Antonescu *et al*[3], the local recurrence rate, metastasis rate, and mortality rate were 50%, 86% and 57%, respectively[5]. Distant metastasis is reported to be most common in the lung, followed by bone, soft tissue, brain, and lymph nodes[1-5]. These previous reports of poor prognosis suggest that SEF is a clinicopathologically distinct soft tissue tumor with malignant potential although it is categorized as a low-grade neoplasm in the sarcoma group.

In our case, since no distant metastasis was found preoperatively and since complete resection was thought possible, we selected surgical resection although the tumor had extended at the time of surgery into the IVC, in addition to local intrahepatic metastasis. Indeed, we could completely resect the tumor macroscopically, but the tumor recurred postoperatively. It is quite possible that malignant cells remained at the tumor bed after the resection despite our careful endeavor. Alternatively, considering the high recurrence rate of SEF reported previously, it is also possible that malignant cells might have already exfoliated from the tumor extending to IVC and presumably reached other organs before the resection. Thus, when surgical resection is selected for SEF, perioperative chemotherapy and/or radiotherapy, in addition to macroscopic complete resection, may be necessary to prevent recurrence, though there is no established effective and standardized regimen of chemotherapy and/or radiotherapy. To clarify the effect of perioperative chemoradiotherapy, in addition to surgical resection, further study with a larger number of SEF cases treated perioperatively is needed.

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CASE REPORT

Priapism secondary to penile metastasis of rectal cancer

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Abstract

Metastatic penile carcinoma is rare and usually originates from genitourinary tumors. The presenting symptoms or signs have been described as nonspecific except for priapism. Rectal adenocarcinoma is a very unusual source of metastatic penile carcinoma. We report a case of metastatic penile carcinoma that originated from the rectum. Symptomatic improvement occurred with palliative radiotherapy.

Key words: Penile neoplasms; Neoplasm metastasis; Priapism; Rectal cancer

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INTRODUCTION

Metastatic tumors of the penis are rare despite an abundant blood supply to the penis. Approximately 300 cases have been reported in the clinical literature[1]. Primary sites of origin are the bladder (33%), prostate (30%), colon (17%) and kidney (7%)[2]. Extra-pelvic sites including the lung, pancreas, stomach, esophagus, melanoma and testis have been noted[3-6]. Fewer than 60 cases of penile metastasis from colon cancer have been reported. Penile metastasis has been a part of more widespread disease in about 90% of reported cases[7]. We report a case of a painful penile metastasis as a manifestation of rectal cancer dissemination that was improved by the use of palliative radiotherapy.

CASE REPORT

A 43-year-old man was admitted to our clinic with a penile erection and pain. Two years earlier, the patient had undergone abdominoperineal resection for adenocarcinoma of the rectum, followed by adjuvant chemoradiotherapy with capecitabine. Postoperative pathology revealed a moderately differentiated adenocarcinoma and pathological T3 tumor with metastases in 15 out of 37 regional lymph nodes. Thirteen months earlier, recurrence of rectal cancer was detected in the abdominal para-aortic nodes. Various palliative chemotherapeutic agents were administered for 11 mo and the patient received no further treatment. One month earlier, penile erection and pain developed.

Physical examination showed no visible skin lesions, but multiple palpable hard nodules were present over the penile shaft. Tenderness developed following palpation of the penis.

The penile lesions had multifocal, irregular-shaped, low-density areas as depicted on pelvic computed tomography (CT) images, and these lesions had increased in size and number as compared with previous CT images obtained 3 mo previously (Figure 1). Positron emission tomography (PET) showed low 18F- fluorodeoxyglucose uptake in the lesions (Figure 2). Penile magnetic resonance imaging (MRI) showed low to iso-intensity signals as compared with the surrounding corpus cavernosum on an axial T1-weighted image, low to intermediate signal intensity on an axial T2-weighted image, and the presence of non-enhanced lesions on a gadolinium-enhanced image (Figure 3). Other sites of metastasis were noted in several para-aortic lymph nodes, lung and vertebral body of the L2 spine.

Fine needle aspiration of the nodules was performed and histological examination showed nests of acinar-like cells with cytological atypia, consistent with metastatic adenocarcinoma from primary rectal cancer (Figure 4).

Intravenous morphine infusion and spinal nerve block were given for control of penile pain, but were ineffective. Other treatment options were required but

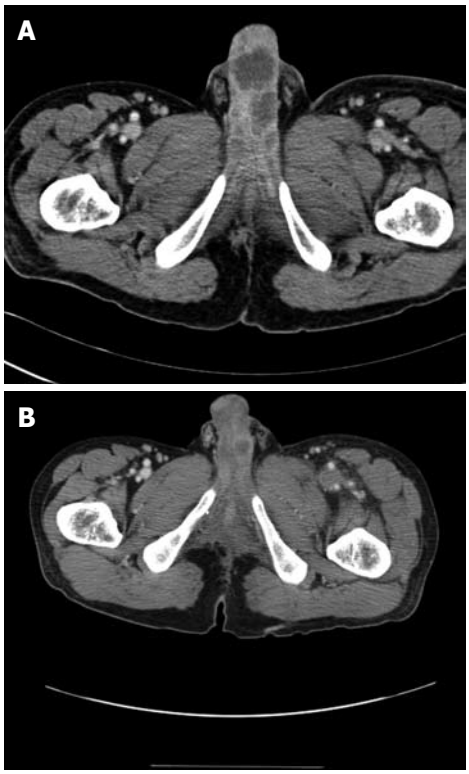

Figure 1 CT image. A: Multifocal irregular shaped low-density areas along the penis; B: Multifocal irregular shaped low density areas are also seen in an CT image obtained 3 mo earlier.

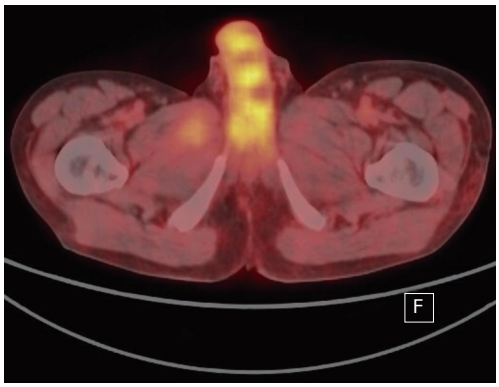

Figure 2 PET image showed low 18F-fluorodeoxyglucose uptake in the lesions.

the patient was in a chemotherapy refractory and systemic disseminated state. As a result of poor performance status, the patient was not allowed to undergo palliative partial or total penectomy. We offered the patient the option of undergoing palliative external beam radiation (2600 cGy, 13 fractions), which was preformed successfully. Penile pain was relieved and the patient was discharged from the hospital.

DISCUSSION

The reason why the penis is a rare site for metastasis despite rich vascularization is not clear. Various mechanisms for penile metastasis have been suggested. These include retrograde venous spread, retrograde lymphatic spread, arterial embolism and local direct extension[3]. Currently,

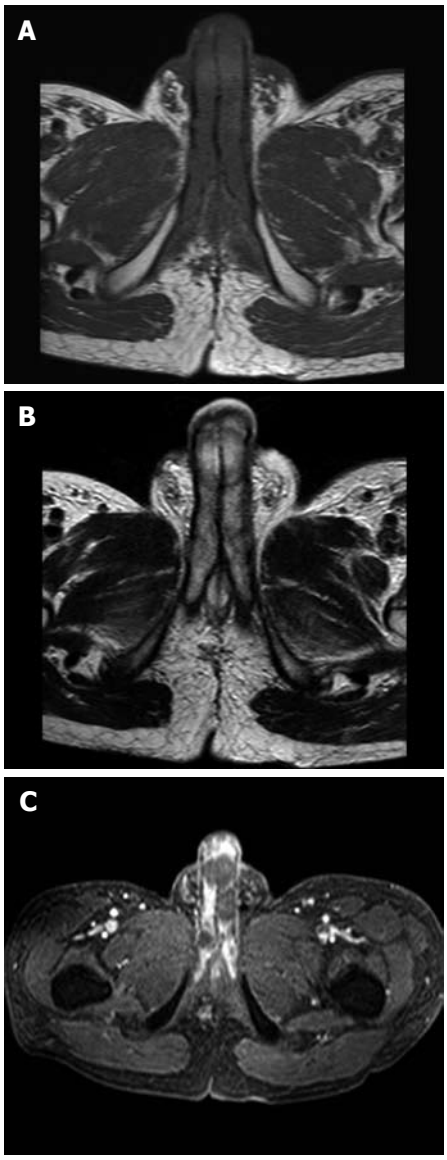

Figure 3 Penile MRI. A: Low to iso signal intensity as compared with the surrounding corpus cavernosum on an axial T1-weighted image; B: Low to intermediate signal intensity on an axial T2-weighted image; C: The presence of non-enhanced lesions on a gadolinium-enhanced image.

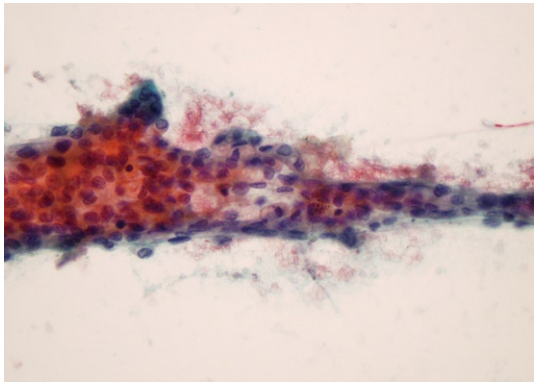

Figure 4 Fine-needle aspiration of penile mass showed nests of acinar-like cells with cytological atypia, consistent with metastatic adenocarcinoma (Papanicolaou stain, × 400).

a route that involves retrograde venous spread from the pudendal into the dorsal venous system of the penis is considered as the most likely mechanism.

Presenting symptoms and signs in order of frequency are malignant priapism (40%), urinary retention, penile nodules, ulceration, perineal pain, edema, generalized swelling, broad infiltrative enlargement, dysuria and hematuria[1]. Penile metastasis must be differentiated from primary penile cancer, chancre, chancroid, non-tumorous priapism, Peyronie's disease, tuberculosis and other inflammatory and suppurative diseases[8]. The corpus cavernosum is usually the site of involvement of metastatic penile carcinoma. The glans penis and corpus spongiosum are involved rarely.

Penile lesions require an excisional or fine-needle aspiration biopsy for pathological confirmation. However, radiological evaluations such as CT, MRI and PET are useful noninvasive methods for the evaluation of lesions[3,9].

Penile metastases typically manifest as multiple discrete masses in the corpus cavernosum. Noninvasive modalities such as CT, MRI and PET are being used increasingly. CT has been used in the evaluation of penile lesions. Due to only single-plane imaging, its use is limited and it may not detect penile metastasis[9]. MRI is a most reliable alternative for diagnosis and assessing the extent of penile metastasis, with its multi-plane imaging and superior soft tissue contrast. These masses have low signal intensity relative to normal corporal tissues in T1-weighted images, and low or high signal intensity in T2-weighted images[9-11]. PET is also useful for detection of clinically silent metastatic sites in hypermetabolic cancer[5,11].

The treatment plan depends on the performance status and primary cancer state of the patient. Treatment modalities include local excision, penectomy, chemotherapy and radiotherapy. Most patients with penile metastasis already have widely disseminated disease, and > 80% of the patients will die within 6 mo, irrespective of the primary tumor and the treatment method, making palliative noninvasive treatment advisable.

In conclusion, we report a case of painful priapism secondary to penile metastasis of rectal cancer, which was improved by palliative radiotherapy. Most patients with metastatic penile carcinoma have developed systemic dissemination and early detection, precise diagnosis and noninvasive treatment are required for improvement of quality of life.

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CASE REPORT

Retroperitoneal desmoplastic small round cell tumor: Pediatric patient treated with multimodal therapy

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Abstract

A desmoplastic small round cell tumor (DSRCT) is a rare, aggressive mesenchymal neoplasm. Although a DSRCT can develop at various sites, the intra-abdominal site is the most common location. These tumors are found most commonly among young adolescents and the prognosis is extremely poor. Multimodal treatment with surgery, chemotherapy and radiotherapy is very important for these rare cases, and this treatment can improve patient survival. In this report, we describe the case of an 8-year-old boy diagnosed with DSRCT located in the retroperitoneal space. The patient has undergone surgical resection and adjuvant chemoradiation therapy, and is currently alive without disease recurrence.

Key words: Retroperitoneum; Desmoplastic small round cell tumor; Multimodal therapy

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INTRODUCTION

A desmoplastic small round cell tumor (DSRCT) is a rare, aggressive mesenchymal neoplasm with an extremely poor prognosis. It was described initially as a distinct clinicopathological entity by Geral and Rossi in 1989[1]. This rare, highly malignant tumor belongs to the primitive tumor family of small round cell tumors (lymphoma, neuroblastoma, alveolar rhabdomyosarcoma, Ewing's sarcoma, neuroectodermal tumor, and DSRCT)[2,3]. Young adolescent males are most commonly affected[4]. Its primary location is in intra-abdominal sites, but it can also be found at other sites such as the kidney and ovary, as well as in the retroperitoneal space[5-7]. In addition, these tumors are associated with a specific reciprocal translocation t (11:22) (p13:q12) that leads to the fusion of the WT1 (Wilms' tumor gene) and EWS (Ewing's sarcoma gene). Patients with this tumor have a very poor prognosis, but multimodal treatment with surgery, chemotherapy and radiotherapy can improve survival[8,9].

We describe here a case of retroperitoneal DSRCT in a boy, who was treated with surgery and adjuvant chemoradiotherapy.

CASE REPORT

An 8-year-old boy with no previous health problems was referred to our hospital with a 2-wk history of a palpable abdominal mass. The family history was non-contributory and there were no complaints except for abdominal pain and indigestion. The physical examination showed an approximately 20-cm palpable abdominal mass predominantly on the right side of the abdomen, with mild tenderness. The laboratory tests showed no significant abnormalities. Computed tomography (CT) showed a septated, very large (21 cm × 17 cm), predominantly cystic, lobulated mass in the right upper quadrant of the abdomen between the liver and the right kidney (Figure 1). The vena cava and the duodenum were deviated to the left side of the abdomen because of the mass effect of the lesion. Upon magnetic resonance imaging (MRI), the lesion had a T1-attenuated high signal suspicious for a high protein content, a predominantly cystic portion with a large solid portion on the medial aspect, and no definite direct invasion into the major vessels. The radiologist suspected an undifferentiated embryonal sarcoma that originated from the liver, or a

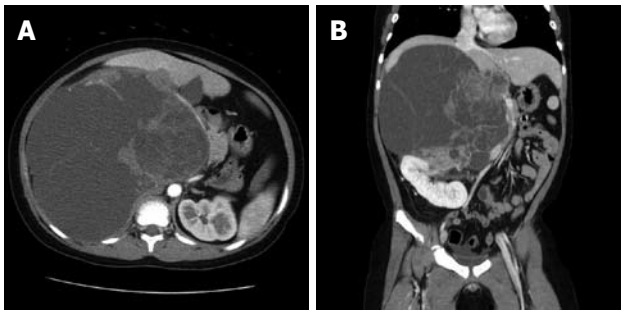

Figure 1 CT (A and B) shows a septated, very large (about 21.8 cm × 16.7 cm), predominantly cystic, lobulated mass in the right upper quadrant between the liver and the right kidney.

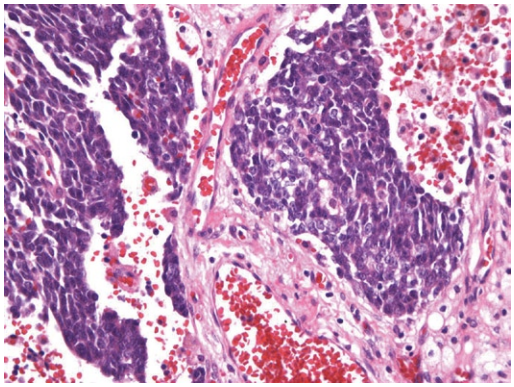

Figure 3 Histopathological findings. Tumor cells consist of small round, hyperchromatic nucleus and scanty cytoplasm (Hematoxylin and eosin stain, × 400).

mesenchymal hamartoma. Surgery was planned for a definitive diagnosis and a proper management.

The operative findings revealed a mass of about 20 cm, irregular in shape, with cystic and solid components. As it was located in the retroperitoneal space, the liver, inferior vena cava, duodenum, right kidney, and intestine were deviated to the left and downward. To obtain a good operative field, we aspirated about 500 mL of brown-colored cystic fluid from the cystic portion of the tumor, and a small portion was excised for frozen sections. Examination of frozen sections revealed that the tumor was highly suggestive of malignancy, and we excised the entire lesion. Since there were severe adhesions to the surrounding tissue and important abdominal vessels such as the inferior vena cava, right renal vessels and celiac trunk, the dissection required a long time and there was a large amount of bleeding. Grossly, there was no evidence of peritoneal seeding or hepatic metastasis. The mass was totally removed without significant problems (Figure 2). The operative time was 500 min, blood loss was about 2 L and eight units of packed red blood cells were transfused. The microscopic examination confirmed a retroperitoneal DSRCT, with the typical appearance of well-defined nests or clusters of small undifferentiated round cells, surrounded by a prominent desmoplastic stroma (Figure 3). Upon immunohistochemical staining, there were positive responses to cytokeratin, vimentin, CD99, and desmin (Figure 4). In addition, there were focal weak positive responses to S-100 and neuron-specific enolase (NSE).

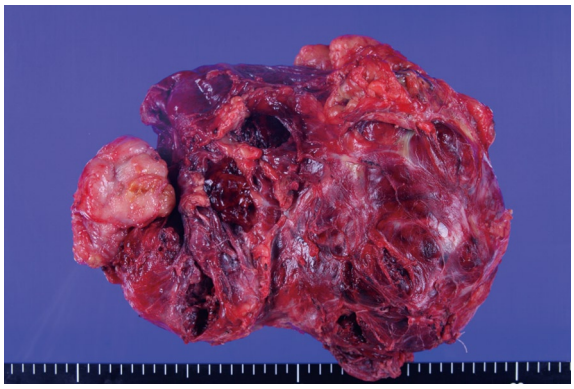

Figure 2 Gross findings after resection show a hemorrhagic and necrotic solid mass with a focal fibrotic, granular cut surface.

After confirmation of the diagnosis, postoperatively, the patient received adjuvant chemotherapy (vincristine, ifosfamide, doxorubicin, etoposide and cyclophosphamide) in combination with consolidating radiotherapy (25 times180 cGy/d, a total dose of 45 Gy). The patient is free of disease recurrence 24 mo after resection and adjuvant chemoradiotherapy.

DISCUSSION

DSRCT is an aggressive malignant tumor with a poor prognosis. Abdominal pain or discomfort is the most common presentation for this rare neoplasm. Its primary location is the intra-abdominal peritoneal cavity, but many other sites have been described[10]. The most common sites of peritoneal involvement have been reported to be the pelvic space, omentum, small bowel mesentery, and the retroperitoneal space. This tumor is typically found in young adolescent men (male:female ratio = 5:1). Usually, it is accompanied by extensive peritoneal seeding at the time of diagnosis[9,10].

Although the findings are nonspecific, CT is the most widely used diagnostic modality for identifying this tumor. It can detect a bulky, lobulated and heterogeneous mass with or without calcification. Sometimes, ascites, adenopathy or liver metastases are found. Upon MRI, T2-weighted imaging shows a heterogeneous hyperintense signal with low or isodense T1-weighted signals. No specific tumor markers have been identified[11].

Histopathologically, the characteristic findings are well-defined nests or clusters of undifferentiated small round cells, surrounded by a prominent desmoplastic stroma. The tumor cells are characterized by small hyperchromic nuclei with scanty cytoplasm. Immunohistochemistry can be positive for AE1/AE3 (88%), desmin (dot pattern, 81%), CD99 (23%), or NSE (84%)[11]. This tumor is associated with a specific reciprocal translocation t (11:22) (p13:q12), which leads to fusion of the WT1 and EWS genes; this rearrangement is detected in almost all cases. The resulting chimeric protein is thought to be a transcriptional activator that fails to suppress tumor cell growth[3].

Although the prognosis is very poor, aggressive surgical tumor resection is the major determinant of patient survival. Lal *et al*[9] recommended surgical resection

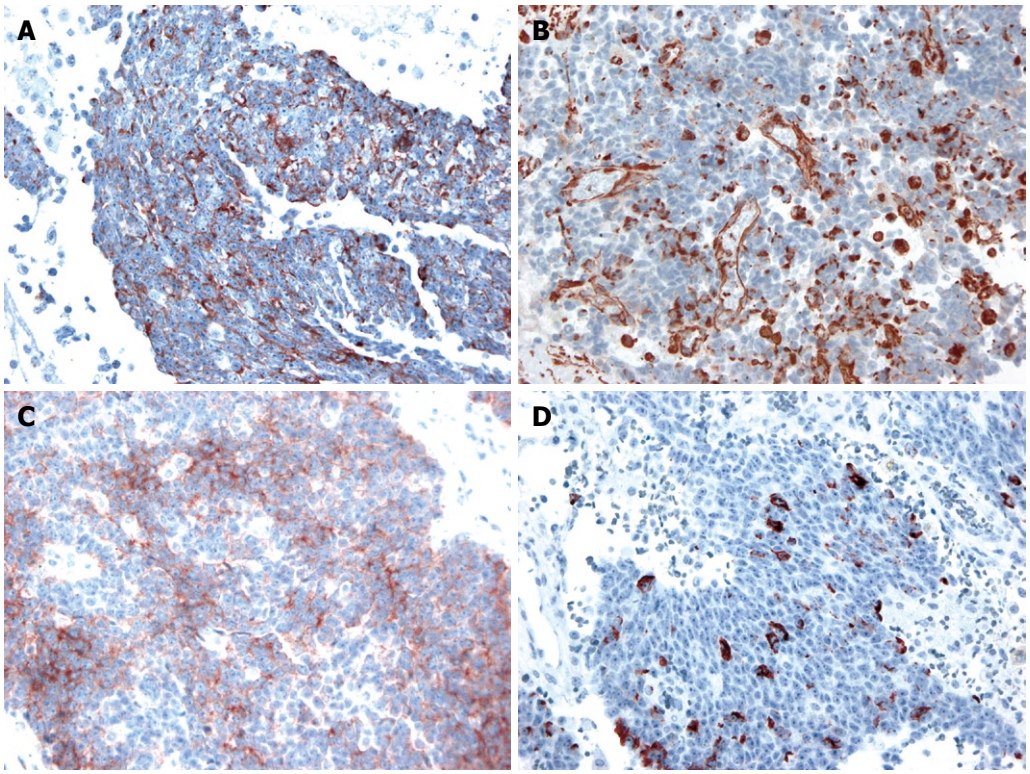

Figure 4 Immunohistochemical staining was positive for cytokeratin (A), vimentin (B), CD99 (C) and desmin (D) (× 400).

of > 90% of the tumor burden. Attempts were not made to achieve microscopically negative resection margins. They used systemic chemotherapy, with the P6 protocol including cyclophosphamide, doxorubicin, vincristine, ifosfamide and etoposide. They reported a good response with this regimen. Adjuvant radiotherapy to the whole abdominopelvic area was also delivered. With this multimodal therapy, including the P6 protocol, > 90% resection, and adjuvant radiotherapy, there was a statistically significant prolongation of patient survival[9]. The presence of nodal invasion and metastasis does not appear to correlate with overall patient survival. The expected 3-year survival was only 27% when multimodal treatment was not used[9]. We used a similar strategy for the treatment of our patient; the patient is free of disease recurrence 2 years after surgery and adjuvant chemoradiotherapy. The platelet-derived growth factor receptor pathway inhibitor SU101 (leflunomide) is being studied in a pediatric phase I trial. The possibility of improved future treatments might help to prolong the survival of patients with DSRCT[12].

In summary, we present a patient with retroperitoneal DRSCT, and expect a longer survival as a result of our aggressive surgical resection and adjuvant chemoradiotherapy.

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CASE REPORT

Enterovesical fistula caused by a bladder squamous cell carcinoma

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Abstract

Enterovesical fistulas are not uncommon in patients with inflammatory or malignant colonic disease, however, fistulas secondary to primary bladder carcinomas are extremely rare. We herein reported a patient presenting with intractable urinary tract infection due to enterovesical fistula formation caused by a squamous cell carcinoma of the urinary bladder. This patient underwent *en bloc* resection of the bladder dome and involved ileum, and recovered uneventfully without urinary complaint. To the best of our knowledge, this is the first case reported in the literature.

Key words: Enterovesical fistula; Squamous cell carcinoma; Urinary bladder; Malignant fistula

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INTRODUCTION

Enterovesical fistulas are not uncommon in patients with inflammatory or malignant colonic disease, however, fistulas secondary to primary bladder carcinomas are extremely rare[1-3]. We herein report a patient presenting with intractable urinary tract infection due to enterovesical fistula formation caused by a squamous cell carcinoma of the urinary bladder. To the best of our knowledge, this is the first case reported in the literature.

CASE REPORT

An 83 year-old male presented with a 4-mo history of recurrent urinary tract infection. He had a history of benign prostate hyperplasia (American Urology Association score 24) that was under medical treatment. In order to correct urine retention and prevent urinary tract infection, photosensitive vaporization of the prostate had been performed. One month after the operation, the patient suffered from urinary tract infection again. The urine culture contained multiple flora of the gastro-intestinal tract including: *Escherichia coli*, *Viridans streptococcus*, *Klebsiella*, *Pneumoniae* and *Enterococcus faecium*. Retrograde urethrography showed leakage of the contrast and cystoscopy disclosed a fistula in the right posterior wall of the bladder with edematous tissue surrounding the fistula (Figure 1). Abdominal and pelvic computed tomography showed a very large neoplasm in the pelvic cavity with suspicion of vesicoileal fistula formation. Under the impression of malignant enterovesical fistula, the patient underwent surgical intervention. At laparotomy, a 10 cm-diameter whitish stony hard tumor was located in the pelvis, involving the distal ileum and urinary bladder, with a frank fistula formation. Resection of the urinary bladder dome and the involved ileum was performed in *en bloc* fashion (Figure 2). The pathology report yielded a moderately differentiated squamous cell carcinoma of the urinary bladder with direct invasion of the terminal ileum (Figure 3). This patient recovered uneventfully and was discharged without urinary tract infection on post-operative day 7.

DISCUSSION

Enterovesical fistula is a rare disease, with an estimated two to three patients per 10 000 hospital admissions, with annular incidence of 0.5 per 100 000[1]. In a literature

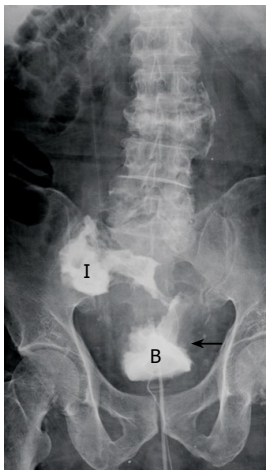

Figure 1 Cystography showed an enterovesical fistula (arrow). B: Urinary bladder; I: Ileum.

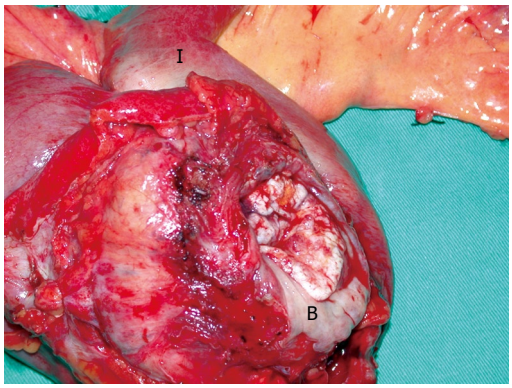

Figure 2 Surgical specimen of urinary bladder dome and ileum resected *en bloc*. B: Urinary bladder; I: Ileum.

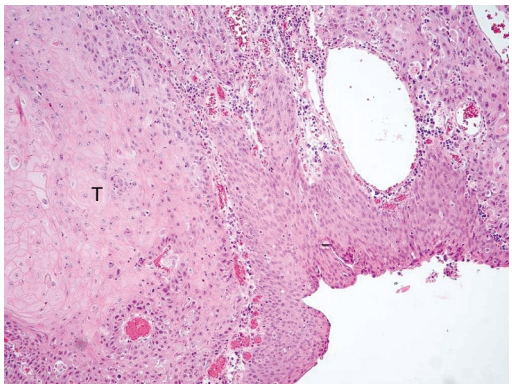

Figure 3 Microscopic examination yielded the diagnosis of squamous cell carcinoma of the urinary bladder. HE staining, original magnification × 40. T: Tumor burden.

review, 20% of vesicoenteral fistulas were rectovesical fistulas and 4%-5% were appendicovesical fistulas. Carson *et al*[2] reported 100 cases of enterovesical fistulas with 51% colonic diverticulitis, 16% colorectal cancer, 5% urinary bladder carcinoma and 16% due to other causes, including secondary to radiation necrosis, cervical cancer, tuberculoma and iatrogenic perforation.

The most common histologic type of bladder cancer leading to enterovesical fistula is transitional cell carcinoma. Primary squamous cell carcinoma of the bladder is a relatively rare tumor. The prevalence of squamous cell carcinoma varies depending on geographic location. It accounts for only 3%-7% of bladder cancers in the United States and 1% in England, but up to 75% in Egypt, where schistosomiasis is endemic[4]. Squamous cell carcinoma are usually related to chronic infection, bladder stones, chronic indwelling catheters or bladder diverticula. To our knowledge, this is the first report of vesicoenteral fistula as a result of bladder squamous cell carcinoma. Only one case of vesicorectal fistula from invasive rectal squamous cell carcinoma cancer had been reported[5]. Almost all squamous cell carcinomas are already advanced and muscle-infiltrative at the time of diagnosis[6]. Squamous cell carcinomas of the bladder have an unfavorable prognosis due to a local advanced stage at the time of presentation.

The most common clinical presentations of enterovesical fistulas are urinary tract infection (100%), pneumaturia (66%), fecaluria (50%), and hematuria (22.6%)[3].

Diagnostic tests include cystoscopy examination, retrograde cystography, and computed tomography. The diagnostic rate using cystoscopy examination is 77%-79%[2,7], while retrograde cystogram and computed tomography showed the fistulous tract in 66.6% and 83.3%, respectively[8,9]. Barium enema or small bowel series have a 20%-35% diagnostic rate to indicate the fistula[4]. Under cystoscopy examination, the fistula may be seen within a hypereremic area at an early stage of the disease, and with cystic mucosal hyperplasia or localized granulation tissue at the late stage[10,11]. Computed tomography has the advantage of showing the fistula tract or gas distinctly in the bladder. In addition, it is useful in delineating the extent of disease involvement, visualizing the anatomic relationship of the adjacent organs and detecting distant metastasis for malignant diseases, which accordingly allows tailoring of the management strategy[7,12,13].

Optimal work-up of a malignant of enterovesical fistula includes detection of the fistula and anatomic extension using the aforementioned diagnostic tests and definitive pathological confirmation, if possible. Except for elderly people in generally poor condition, diverting enterostomy and indwelling urethral catheters are not recommended as permanent treatments for enterovesical fistulae[3].

Whitely and Grabtald proposed that a one-stage *en bloc* resection of the colonic malignancy and involved bladder portion is a reasonable and safe procedure[7,11], avoiding a total cystectomy. Patients must be well-prepared, non-obstructive and without systemic infection before the surgery[1,11,12,14,15]. However, if resection of the bladder is too wide, resulting in a total cystectomy, reconstruction to restore intestinal continuity and provide adequate bladder capacity and continence is pertinent. On the other hand, if the tumor is deemed unresectable and there is a short life expectancy, a palliative surgical procedure with enterostomy, with or without urinary diversion, is suggested[12,16].

The 5-year survival rate for colon cancer with enterovesical fistula is 81% in nodal negative patients (T4N0), compared with 27% in nodal positive patients (T4N1)[17].

Looser *et al*[16] also suggested that colonic cancer with a perforation into the bladder could be treated by resection for a curative purpose, obtaining long-term survival in 50% of the patients[7,16]. Nevertheless, Vidal Sans *et al*[3] reported that surgical morbidity and mortality is relatively high, especially in fistula resulting from malignancy. Despite various methods of treatment for enterovesical fistula, the prognosis for patients with an advanced stage of malignant fistulae is dismal.

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LETTERS TO THE EDITOR

Early aggressive therapy for severe extensive ulcerative colitis

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Abstract

The current ulcerative colitis (UC) treatment algorithm involves a step-up therapeutic strategy, mainly aiming at inducing and maintaining its clinical remission. Although this therapeutic strategy may seem to be cost-efficient and reduce the risk of side effects, recent trials and case reports have shown that top-down therapy using infliximab induces a rapid clinical response, enhances patient quality of life, promotes mucosal healing, reduces surgeries and indirect cost of treatment for patients with severe UC. Moreover, since long-term treatment with infliximab is safe and well tolerated, early aggressive top-down therapeutic strategy may be a more effective approach, at least in a subgroup of severe extensive UC patients.

Key words: Infliximab; Ulcerative colitis; Top-down therapy

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INTRODUCTION

Since ulcerative colitis (UC) cannot be cured by medication,therapeutic strategy is very important for patients with UC. The UC treatment algorithm includes a early aggressive or top-down therapeutic strategy and a sequential or step-up regimen. Recently, the emergency of effective biological therapy in the treatment of UC has led to a clinical debate about "top-down *vs* step-up strategy"which describes two opposite approaches. Since one approach is not suitable for all patients with UC, therapeutic strategy ought to be tailored to the individual patient so as to maximize efficacy while balancing risk. and cost. In this letter the benefits, safety and costs of alternative strategies will be critically appraised.

TO THE EDITOR

I read with great interest the case report recently published in the *World Journal of Gastroenterology* by Cury *et al*[1]. The authors reported a case in which infliximab was safely and effectively administered to a patient with severe and extensive UC. Upon reading this interesting case report, two questions arose in my mind.

First, the white blood cell (WBC) count of 161 000/mm^3 or 161 × 10^9/L reported in the case, which is markedly elevated because the normal WBC concentration is 4000-10 000/mm^3, remains to be elucidated. Although a high WBC count can occur in infections, toxins, acute hemolysis, trauma and malignancies, the leukocytosis described in the case report might be due to leukemoid reaction, leukemia and other myeloproliferative disorders since WBC concentration is over 30 000/mm^3. However, the leukocytosis reported in the case report was not permanent and progressive, and infectious precipitants were ruled out[1]. Moreover, since no endoscopic evidence is available to support toxic megacolon, there might be an error in the WBC count or in a leukemoid reaction in the case report, the cause for which is not clear.

Second, whether top-down therapeutic strategy should be implemented in patients with severe and extensive UC which extends beyond the splenic flexure but not to the cecum. In a recently published consensus, a sequential or step-up therapy, mainly aiming at inducing and maintaining its clinical remission, has been advocated for patients with severe extensive UC which is best defined by Truelove and Witts criteria[2]. The step-up therapeutic strategy may seem to be cost-efficient for the vast majority of UC patients and reduce the risk of side effects. However, this sequential strategy did not induce mucosal healing

and could not achieve the best attainable quality of life until infliximab was administered to the reported patient. In addition, early aggressive therapy with infliximab and azathioprine may reduce the indirect cost of treatment for patients. More recent studies have shown that top-down therapy using infliximab induces a rapid clinical response, has a steroid-sparing effect, enhances patient quality of life, promotes mucosal healing, and reduces hospital stay time and surgeries[3-6]. The reasons why the step-up strategy is advantageous over the top-down are concerned with its side effects and costs of biological agents. However, it was reported that long-term treatment with infliximab is safe and well tolerated and not associated with excess mortality or malignancies[5,7]. Moreover, an 8-wk maintenance treatment schedule with infliximab appears to be a cost-effective treatment option for adult patients suffering from moderate to severe UC[8]. Therefore, the top-down approach is appealing and can result in a modification in the natural course of UC, at least in a subgroup of patients with severe and extensive UC.

Since the top down approach is not suitable for all patients with UC, the future challenge is to identify a subgroup of patients who will develop complicated diseases or are therapy refractory at a later time point and for whom infliximab treatment in the early phase may change the natural history of UC.

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Meetings

Events Calendar 2009

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Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
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Washington, DC
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March 23-26, 2009
Glasgow, Scotland
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Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
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April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
http://www.easl.ch/

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
http://www.ddw.org

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

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Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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