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REVIEW

Cytomegalovirus in human brain tumors: Role in pathogenesis and potential treatment options

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Abstract

During the last years increasing evidence implies that human cytomegalovirus (CMV) can be attributed to human malignancies arising from numerous tissues. In this perspective, we will review and discuss the potential mechanisms through which CMV infection may contribute to brain tumors by affecting tumor cell initiation, progression and metastasis formation. Recent evidence also suggests that anti-CMV treatment results in impaired tumor growth of CMV positive xenografts in animal models and potentially increased survival in CMV positive glioblastoma patients. Based on these observations and the high tumor promoting capacity of this virus, the classical and novel antiviral therapies against CMV should be revisited as they may represent a great promise for halting tumor progression and lower cancer deaths.

Key words: Cytomegalovirus; Oncovirus; Glioblastoma; Medulloblastoma; Brain tumor

Core tip: Cytomegalovirus (CMV) has recently been detected in several human cancers. These findings have raised several concerns whether this virus is the cause or a passenger during oncogenesis. Here we discuss the pathogenesis behind CMV infection, its potential as an onco- or oncomodulatory-virus and possible modes of medical interventions.

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HUMAN CYTOMEGALOVIRUS

Cytomegalovirus (CMV) is a common virus belonging to the herpesvirus family[1], which infects 70%-100% of the world's population. After a primary infection that is generally mild or asymptomatic in the immunocompetent host, this virus establishes latency and persistence in myeloid lineage cells, and periodic asymptomatic reactivations are believed to occur during life[1] without clinical signs of infection. However, in individuals with a suppressed immune system, CMV may cause life-threatening infections. Despite a good prophylaxis and surveillance program for early detection of reactivation of CMV in transplant patients, these infections remain to be a clinical problem both as an acute infection as well as a cause of long-term complications. Patients who have had CMV infections in the post-transplant period are at higher risk of developing acute and chronic rejection, cardiovascular diseases, bacterial and fungal infections, post-transplant diabetes and some malignancies[2-5]. These conditions are mainly believed to be mediated by indirect effects of CMV[4], as the virus has been difficult to detect in affected organs at time of diagnosis[6]. However, more recently, the use of sensitive techniques for detection of CMV demonstrate that the presence of CMV in kidney grafts is associated with decreased organ function and graft survival[7,8], suggesting that this virus causes direct, in addition to indirect effects, in the graft.

Emerging evidences also suggest that CMV is highly prevalent in patients with breast, colon, and prostate cancer, rhabdomyosarcoma, hepatocellular cancer, salivary gland tumors, neuroblastoma and brain tumors[9-25]. Over 90% of these tumors have been shown to be positive for CMV proteins and nucleic acids as determined by methods including in situ hybridisation, PCR, electron microscopy, DNA and RNA sequencing, immunostaining of tissue specimens, flow cytometry analyses of tumor cells from surgical resections and western blot analysis. Also most neoplastic cells in sentinel lymph nodes of > 90% of breast cancers have been shown to be CMV positive[26] as well as 98% of brain metastases of colon and breast cancers contain CMV proteins and/or nucleic acids[27]. In sharp contrast, CMV proteins are not detected in healthy tissues surrounding CMV positive tumors or metastases. These observations suggest that this virus does not represent an epiphenomenon of CMV positive tumors, but rather that it may directly aid in tumor progression or even in the initiation and cancer development. As CMV proteins are mainly confined to metastatic cells in metastases of breast and colon cancer[26,27], CMV may be maintained within cells that initiate the development of the metastasis. However, the role of CMV in tumor initiation, progression and metastasis formation needs to be further thoroughly examined in depth since some researchers have failed to detect CMV in tumors[28-33]. This causes confusion in the field and voices have been raised that the presence of CMV in tumors is false and based on artefact data. However, stimulation of dendritic cells with glioblastoma tumor lysates leads to expansion of CMV specific T cells in glioblastoma patients and CMV pp65 specific T cells can kill autologous glioblastoma cells *in vitro*. This suggests that CMV epitopes are present in glioblastoma tumors[34,35]. The inconsistency in the detection of CMV in tumors samples between laboratories is most likely due to differences in the sample preparations, control specimens and sensitivity of the methods employed for the detection of CMV nucleic acids or proteins. Therefore studies to search for CMV in tumors should be based on techniques that have been developed and proven to work in tumor specimens.

HUMAN MALIGNANT BRAIN TUMORS; GLIOBLASTOMA AND MEDULLOBLASTOMA

Human brain tumors are diverse neoplasms that currently include more than 120 different clinopathological entities[36]. Glioblastomas and medulloblastomas are the most frequently occurring malignant brain tumors in adults and children, respectively. Gliomas accounts for approximately 75% of all primary brain tumors, whereas medulloblastoma is the most common solid childhood tumor[37,38]. The median age of diagnosis of gliomas is 64 years whereas for medulloblastomas about 99% of the tumors are detected in early childhood or adolescence[36,39].

Gliomas are histopathologically classified as ependynomas, astrocytomas, oligodendrogliomas and mixed oligo-astrocytomas and graded with regard to malignancy as Grade Ⅰ-Ⅳ. Grade Ⅲ (anaplastic astrocytomas) and Ⅳ gliomas [glioblastoma (GBM); glioblastoma multiforme] account for approximately 82% of cases and are considered malignant or high-grade gliomas[39]. The current standard of care for malignant gliomas includes surgical resection, radiation therapy with concomitant and adjuvant chemotherapy[40]. Despite aggressive treatment approaches, the median survival for patients diagnosed with Grade Ⅳ glioblastoma is 16 to 19 mo with 25%-30% of the patients alive 2 years after treatment[41].

Malignant gliomas are heterogenous tumors with different clinical and molecular signatures. Glioblastomas have been divided into four molecular subtypes characterized by abnormalities in *PDGFRA*/*IDH1*, *EGFR*, and *NF1* that represent the proneural, classical and mechencymal subtypes, respectively[42]. In addition, a fourth transcriptional subtype termed neural subtype is characterized by high expression of neural markers like *NEFL*, *GABRA1*, *SYT1*, and *SLC12A5*[42]. Many of the molecular abnormalities overlap between the different glioblastoma subtypes and additional rare mutations and chromosomal aberrations have been described, adding to the heterogeneity of glioblastomas. Cells of origin for glioma are not clearly identified but neural stem cells and oligodendrocyte precursor cells that both derive from a neuroepithelial cell, have been suggested[43].

Medulloblastomas are also a compilation of molecular and clinical diverse tumor types that arise either in the cerebellum or brainstem[44]. These tumors are

highly malignant and current treatment of patients with medulloblastoma consists of surgery, whole brain and spinal cord radiation (in patients above 3 years) and aggressive chemotherapy, sometimes followed by stem cell transplantation[44]. Even though long-term survival among medulloblastoma patients is 60%-70%, the patients frequently experience disease or treatment related complications including developmental, neurological, neuroendocrine, and psychosocial deficits[45]. Although medulloblastomas contain significantly less mutations than adult cancers[46], specific subsets have been identified and aberrant expression of key molecules regulating developmental signaling cascades, oncogenic drivers and mutations in tumor suppressor genes, have been described, such as alterations of the Shh and Wnt signaling pathways, overexpression of MYC, MYCN and activation of growth factor independent 1 family proto-oncogenes, GFI1 and GFI1B by enhancer hijacking[44,47-49]. However, since many medulloblastoma tumors have no apparent mutations of any known cancer gene, it has been suggested that epigenetic changes or other etiological factors including infections also may be responsible for tumor initiation and progression[9,44,46,50].

Medulloblastomas have been linked to disordered mechanisms of normal development and medulloblastoma cells retain many features resembling precursor cells of the embryonic brain. Approximately half of these tumors contain abnormal activation of the developmental signalling cascades, Shh and Wnt[51,52]. Moreover, activation of the PI3K/Akt signalling pathway has been shown to be important for initiation and proliferation of medulloblastoma[53-55]. Molecular analysis have shown that there are four major medulloblastoma subgroups (Wnt, Shh, Group 3 and Group 4[44]). These subgroups are distinct in tumor cell histology and biology and exhibit divergent clinical phenotypes. Different subtypes of medulloblastoma are also believed to have distinct cellular origins. One subtype originates from cerebellar granule neural precursor cells located in the external granular layer of the cerebellum as a result of aberrant Shh signalling[56,57]. A subpopulation of cells from these tumors is positive for the progenitor markers Math1 and CD15[58]. A different medulloblastoma subtype arises outside the cerebellum, likely from cells of the dorsal brainstem and is dependent on Wnt signalling. These tumors contain aberrantly proliferating Zic (+) precursor cells[59]. Finally, evidence of a third medulloblastoma subtype deriving from CD133-positive (Prom1) cerebellar stem cells has also been proposed. These tumors contain elevated Myc expression[60,61].

Many malignant gliomas and medulloblastomas are hence thought to initiate from precursors cells within the CNS by sequential and cumulative genetic alterations or developmental errors[43,49,62]. Rare hereditary syndromes including Cowden, Turcot, Li-Fraumeni, tuberous sclerosis, neurofibromatosis and schwannomatosis have been associated with increased risk of glioma, whereas increased risk of medulloblastomas have been observed in individuals with Turcot, Gorlin and Li-Fraumeni syndromes[62]. However, the etiology behind the vast majority of gliomas and medulloblastomas still remain largely unknown and no fundamental environmental factors, except ionizing radiation that is associated with increased risk for glioma development, has been convincingly demonstrated[37]. On the other hand, it seems to be a strong inverse relationship between atopic diseases and glioma[63]. Both glioblastomas and medulloblastomas express high levels of cyclooxygenase-2 (COX-2) that catalyzes conversion of arachidonic acid to prostaglandins and other eicosanoids with concomitant secretion of proinflammatory prostaglandin E_2 (PGE_2)[64-66]. In gliomas, the level of COX-2 expression is directly correlated to glioma grade and associated with shorter survival in glioblastoma patients[65]. Non-steroidal anti-inflammatory drugs, capable of inhibiting cyclooxygenase, significantly suppress the growth of glioblastoma and medulloblastoma in preclinical models[9,64,66]. Also, short-term use (< 10 years) of anti-inflammatory medication is associated with a protective effect against glioblastoma[67]. Taken together, this suggests that brain tumors are at least partly dependent on an inflammatory microenvironment in order to proliferate and progress.

An inflammatory microenvironment can be induced directly by tumor cells through activation of oncogenes that activate transcriptional programs leading to the production of pro-inflammatory eicosanoids, cytokines and chemokines that attract different immunological cells to the surrounding tumor microenvironment. Inflammation in the tumor microenvironment can also be caused indirectly by viral and microbial infections, autoimmune diseases, and dietary products[68]. Tumor-related inflammation is hence important for tumor cells to sustain a proliferative state, escape apoptosis and enhance angiogenesis, metastasis and suppression of the immune system[68].

CMV'S POTENTIAL ROLE IN BRAIN TUMORS: CMV PROTEINS CONFER BOTH ONCOMODULATORY AND ONCOGENIC FUNCTIONS

In the light of the above description of the phenotypic and molecular diversity of gliomas and medulloblastomas, and their different sites of origin in the brain, it is interesting to note that most of glioblastomas and medulloblastomas appear to be CMV positive. The presence of CMV proteins in medulloblastoma and glioblastoma hence raise questions whether this virus plays an important role in tumor initiation and/or progression of these tumors. CMV is not a typical oncogenic virus, but CMV proteins provide many mechanisms that can promote tumor biology relevant mechanisms. During the evolution, there has been a strong evolutionary pressure on CMV to cope with and survive the attacks by the immune system and to create efficient virus factories. CMV was believed to encode for approximately 180 proteins, of which only about 45 have been estimated to be essential for virus replication[69-72]. A more recent study based on ribosomal

profiling suggests that 751 unique CMV proteins are translated in infected cells[73]; if true, this virus is far more complex than previously appreciated. However, regardless of the exact number of CMV proteins that are encoded by this virus, the vast majority of CMV proteins must confer other functions during the virus life cycle than ensuring replication and formation of new virus particles. Several CMV encoded proteins can under certain circumstances initiate cellular transformation or through other ways aid in tumour development and provide mechanisms representing the cornerstones of hallmarks of cancer[74].

The concept of a role of CMV in cancer is not new. Already in the 1970's, Fred Rapp's group reported the frequent presence of CMV in prostate cancer, and isolated a virus strain from tumors that was oncogenic *in vitro* and in immunodeficient mice[75]. However, in several later studies, CMV failed to transform normal human cells, wherefore this virus was not considered to be oncogenic. The classical view implies that oncoviruses encode gene products that can induce cellular transformation under certain circumstances, *e.g.*, HPV, SV40, EBV, Hepatitis B and adenoviruses. For CMV, the term oncomodulation has instead been proposed to describe the indirect influence of CMV on tumorigenesis (reviewed in[6,76,77]). Oncomodulation is defined as the ability to promote, in an appropriate genetic environment supplied by tumor cells, an oncogenic process characterized by disruptions in intracellular signalling pathways, transcription factors and tumor suppressor proteins. For example, CMV proteins control the cell cycle, induce telomerase activity, inhibits apoptosis, induce angiogenesis and cellular migration and hence provide oncomodulatory mechanisms[76-79]. Furthermore, CMV proteins can promote stemness by blocking cellular differentiation and interact with the DNA damage response pathway to alter the cell cycle (reviewed in[77]). CMV proteins induce expression of oncogenes, control expression of tumor supressors, induce specific chromosomal breaks and p53 mutations, inhibit DNA repair mechanisms, control epigenetic functions and cellular proliferation[80-82], and provide immune evasion strategies[83-85].

Experimental data also suggest that CMV can be oncogenic. A gene region of the CMV genome, the transforming region Ⅱ (mtrⅡ), a 980-bp sequence, was first shown to transform rodent fibroblasts[86-90]. Expression of the CMV proteins IE72 or IE86 together with the adenovirus E1A protein can induce cellular transformation through a "hit and run" mechanism[91]. The CMV IE proteins can bind to p53, Rb and degrade p21, and thereby modulate cell cycle regulation, induce telomerase activity[78] and downregulate tumor suppressor proteins, which may aid in oncogenic transformation[77]. The CMV protein US28, a G coupled chemokine receptor homologue, has several characteristics resembling a viral oncoprotein[92-95]. Expression of US28 in NIH3T3 cells renders them tumorigenic upon injection in nude mice[94,95], which involves induced COX-2 expression and VEGF production[95]. Furthermore, transgenic mice expressing US28 only in intestinal epithelial cells developed intestinal adenomas and adenocarcinomas[92], by inhibiting glycogen synthase 3β (GSK-3β) activity, resulting in an accumulation of β-catenin and increased expression of Wnt target genes involved in the control of cell proliferation[92]. US28 also induces STAT3 phosphorylation through IL-6 production, which correlates with poor survival in GBM patients[93]. Analysis of clinical GBM samples *in situ* showed co-localization of US28 with phosphorylated STAT3, COX-2, VEGF and e-NOS, and US28 can induce cellular migration *in vitro*, which suggests that US28 may contribute to tumour invasiveness and angiogenesis *in vivo*[77,93,96]. CMV protein expression in mucoepidermoid cancer also correlated with activation of known oncogenic pathways such as EGFR, ERK and amphiregulin, and protein expression was related to severity[97]. These experimental data suggest a direct molecular link between the expression of US28 and tumorigenesis. In addition, US28 has also been shown to activate the transcription factor nuclear factor B (NF-Kβ), a critical regulator of immunity, stress responses, apoptosis, cellular differentiation and migration[96].

More recently, the microenvironment at the tumor site and the potential close connection to inflammation has received increasing attention, and there seems to be a close link between inflammation and tumor development. COX-2 and PGE_2 are over-expressed in a number of different cancers and high COX-2 expression is often correlated with poor prognosis[66,98]. CMV infection induces COX-2 and 5-lipoxygenase (5-LO) expression and mediate production of PGE_2 and leukotrienes that are both potent inflammatory mediators[99,100]. PGE_2 also induces cellular proliferation, angiogenesis, inhibition of apoptosis and stimulation of invasion, and can contribute to the generation of a tumour promoting inflammatory microenvironment[98]. Interestingly, we observed a clear association between CMV protein expression and COX-2 expression in medulloblastoma, suggesting that CMV may control COX-2 expression in these tumors[9]. Viruses could also by their sole presence induce an immune response through expression of non-self peptides to T cells and create an inflammatory microenvironment.

Epithelial cells can undergo a transition into mesenchymal cells [epithelial to mesenchymal transition (EMT)], involving a series of events resulting in the loss of cell-to-cell contacts and dramatic remodelling of the cytoskeleton. In addition to its role in normal physiological development, recent data implicates a role for EMT and mesenchymal to epithelial transition (EndoMT) in tumor pathology, particularly in regards to metastatic capacity of epithelial tumors. A major factor that regulates the EMT process is transforming growth factor beta (TGFβ). CMVs ability to induce TGFβ provides a role of this virus to facilitate the EMT process[101]. In support of this hypothesis, CMV infected epithelial cells treated with TGFβ *in vitro*, were shown to undergo morphologic and transcriptional changes similar of EMT; this also occurred in uninfected cells[102]. CMV infected epithelial cells can also activate extracellular latent TGFβ1 through induction of metalloproteinase 2 (MMP-2), which was proposed to be mediated by the CMV proteins CMV IE72 or IE86[102]. Induced MMP-2 activity could also in theory mediate

degradation of the extra cellular matrix[103], which would further aid in the formation of a metastasis. In addition, CMV US28 can interfere with the activity of expression of GSK3β, which is known to phosphorylate and control the stability of key oncogenic transcription factors such as the Smads and Snail that can trigger an EMT program. Furthermore, virus induced COX-2 expression, and activation of Ras/Erk and PI3K/AKT signalling pathways may further induce and maintain a viscous paracrine loop leading to possible cellular invasion into surrounding stroma.

POTENTIAL TREATMENT OPTIONS TARGETING CMV IN BRAIN TUMORS

We found CMV DNA and proteins in 92% of primary medulloblastoma tumors and in 99% of glioblastomas and also detected the virus in eight of eight examined medulloblastoma cell lines, grown in culture for decades[9]. When the medulloblastoma cells were implanted subcutaneously in immunodeficient mice, all tumors were CMV protein positive; a majority of the tumor cells expressed CMV IE proteins[9]. We found CMV proteins in medulloblastoma cells in culture expressing CD133 and CD15, which are proposed markers of medulloblastoma stem cells. The cancer stem cell hypothesis states that only a subpopulation of cancer cells have self-renewing ability and the capacity to give rise to tumours[104]. Such cancer stem cells or tumour initiating cells (TICs) exhibit an immature phenotype, *e.g.*, expression of pluri-/multipotency associated transcription factors[105], a slower proliferation rate and increased resistance to cancer therapy relative to more differentiated cancer cells[106]. In theory, TICs could either be directly infected by CMV as immature cells or represent a dedifferentiated mature cell with a potential ability to affect tumorigenesis and EMT. CMV infected tumor cells undergoing EMT may detach from adjacent cells and potentially enter the circulation *via* the lymphatic system or the blood stream. It is possible that CMV positive tumor cells in primary breast, prostate and colon tumors can undergo EMT to obtain stem cell characteristics, *i.e.*, a potential TIC/ EMT cell will circulate, undergo the reverse process of EndoMT as metastases are developing in lymph nodes or distant organs such as the brain. If CMV resides in TICs, it would explain why all xenografted tumors were virus positive, although only a minority of medulloblastoma tumor cells in culture expressed CMV proteins[9]. If this holds true, drugs targeting CMV infection may not only be beneficial to inhibit primary tumor growth but may also reduce the capacity of the tumour to become invasive as it may selectively kill the TICs. Such scenario implies that most stages of tumor development, primary growth, migration, invasion, intravasation and potentially metastasis may be sensitive to anti-viral drugs making CMV an ideal target for therapeutic intervention.

Hence, regardless if CMV plays a role in the development of tumors, CMV has been detected in brain tumors such as glioblastoma and medulloblastoma and virus infected tumor cells may therefore represent a new target of therapy. In support of this hypothesis, we showed that animals carrying CMV positive human medulloblastoma or neuroblastoma tumors that were treated with anti-CMV drugs had significantly smaller tumors than placebo treated animals[9,107]. A synergistic effect was observed with a COX-2 inhibitor, which resulted in a 72%-97% reduced medulloblastoma tumor growth *in vitro* and *in vivo*[9]. Both COX-1 and COX-2 inhibitors are efficient anti-viral drugs that prevent the replication of CMV[99,108]. Thus, antiviral drugs and COX-2 inhibitors may act synergistically to affect the growth of CMV positive tumors[9]. Interestingly, a number of recent studies demonstrate that Aspirin, a non-selective COX- inhibitor, significantly prevents cancer development and metastases[109-113]. With current data at hand, it cannot be excluded that some of the preventive effect of COX-2 inhibitors involves CMV mediated tumor mechanisms.

Treatment of GBM xenograft tumors with the anti-CMV drug Cidofovir also reduced tumor growth, although a CMV independent mechanism was observed[114]. Importantly, treatment of 50 glioblastoma patients who received anti-CMV treatment as an add-on to standard therapy at Karolinska University Hospital as adjuvant treatment demonstrate a remarkably high survival: the 2 year survival was 70% among 40 patients receiving 6 mo of anti-viral therapy and as high as 90% among patients with continuous treatment (n = 25) compared with 18% in contemporary controls (n = 137); median OS was 56.4 mo compared with 13.5 mo in controls in the latter group ($P < 0.0001$[115]). These observations call for a deeper understanding of CMVs role in cancer and whether this virus is a novel target in anti-cancer therapy. Also, anti-CMV drugs used as an add-on therapy should be further analysed in larger glioblastoma patient populations to give robust statistical data in randomised trials to confirm or dismiss the use of valganciclovir in these patients. The presence of CMV in glioblastoma would also imply that immunotherapy protocols that target CMV epitopes expressed in the tumor can be exploited as cancer therapy[116-118]. Several immunotherapy protocols are currently under evaluation in clinical trials to evaluate different CMV based protocols for glioblastoma patients; these need to also consider the immunosuppressive state of glioblastoma patients.

Several studies indicate that GBM patients exhibit functional impairments of their T cell functions[119,120]. However, polyfunctional CMV specific T cells can be restored by *in vitro* stimulation with CMV antigens and gamma C cytokines. It was recently demonstrated the CMV pp65 specific T cells can kill autologous glioblastoma cells *in vitro*[35], and that immunotherapy using CMV specific T cells was associated with prolonged survival in a single patient[18]. This suggests that adoptive therapy of *in vitro* expanded T cells may be a preferred protocol to be used to overcome the problem of unresponsive T cells *in vivo*, although T cell activation may be possible to overcome by the right stimulus also *in vivo*. Today

two clinical studies are open for enrolment of GBM patients; one is testing genetically modified CMV specific cytotoxic cells for recurrent GBM; a chimeric antigen receptor recognizes human epidermal growth factor receptor 2 coupled to CD28. The other is based on DC vaccination together with a monoclonal antibody against CD25 aimed to inhibit IL-2 signalling. Results from these trials are expected in 2015 and 2016, respectively, and currently results from two other studies that are closed for recruitment are also awaited in the near future. Most likely, future protocols will have to evaluate the effect of immunotherapy in combination with anti-viral therapy for CMV to obtain optimal anti-CMV effects in cancer patients.

CONCLUSION

In summary, emerging data suggest that CMV may play a pathogenic role in cancers of epithelial and neuronal origin. Under such circumstances, anti-viral treatment strategies may provide new options in cancer therapy of CMV positive tumors and metastases to improve patient outcome.

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REVIEW

Internal ribosome entry site-based vectors for combined gene therapy

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Abstract

Gene therapy appears as a promising strategy to treat incurable diseases. In particular, combined gene therapy has shown improved therapeutic efficiency. Internal ribosome entry sites (IRESs), RNA elements naturally present in the 5′ untranslated regions of a few mRNAs, constitute a powerful tool to co-express several genes of interest. IRESs are translational enhancers allowing the translational machinery to start protein synthesis by internal initiation. This feature allowed the design of multi-cistronic vectors expressing several genes from a single mRNA. IRESs exhibit tissue specificity, and drive translation in stress conditions when the global cell translation is blocked, which renders them useful for gene transfer in hypoxic conditions occurring in ischemic diseases and cancer. IRES-based viral and non viral vectors have been used successfully in preclinical and clinical assays of combined gene therapy and resulted in therapeutic benefits for various pathologies including cancers, cardiovascular diseases and degenerative diseases.

Key words: Vector; Gene transfer; Internal ribosome entry site; Gene therapy

Core tip: Combined gene therapy has emerged for a few years as a promising strategy to improve treatments of many diseases including cancer, cardiovascular diseases and degenerative diseases. In this context, internal ribosome entry site (IRES)-based vectors provide a powerful system to co-express several therapeutic genes from the same transcription unit. IRESs are translational enhancers, exhibiting tissue-specificity, and activated by stress. Different IRES-based vectors including plasmids, adeno-associated virus-derived and lentiviral vectors have been used successfully in many preclinical protocols of gene therapy. Moreover the few clinical assays launched with IRES-based multicistronic vectors resulted in therapeutic benefits.

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INTRODUCTION

Combined gene therapy has appeared for a few years as an attractive approach to optimize the therapeutic benefits of gene transfer. In the field of cancer, the first examples of antitumoral cooperative effect have been provided by co-expression of the co-stimulation molecules CD70 and CD80, and of the two anti-angiogenic factors, angiostatin and endostatin, respectively[1-4]. Synergistical effects have also been obtained with co-expression of angiogenic growth factors generating therapeutic angiogenesis in ischemic diseases. This rational has been proven first with co-administration of vascular endothelial growth factor A (VEGFA) and angiopoietin as recombinant proteins as well as by co-administration of two plasmids coding these growth factors[5]. A few years later, combination of recombinant fibroblast growth factor 2 (FGF2) and PDGF-B also improved hindlimb ischemia in rats whereas a bicistronic vector expressing FGF2 and VEGFA efficiently induced vessel formation in a mouse angiogenesis assay[6,7]. These studies launched the concept of combined biotherapy. They also revealed that combined gene therapy is a promising therapeutic approach, allowing long term efficiency of treatments compared to recombinant proteins whose half life is often very short.

Internal ribosome entry sites (IRESs) are translational enhancers naturally present in a series of mRNAs, mediating internal initiation of translation when present between the genes of interest (Figure 1). IRESs thus allow the design of multicistronic expression cassettes resembling bacterial operons, able to drive translation of several genes coded by the same mRNA[8]. We have demonstrated that the use of IRES-based vectors co-expressing two genes of interest allows stable transgene expression with a constant ratio of the proteins of interest, in contrast to the use of two different plasmids expressing each transgene[9]. Actually, a bicistronic IRES-based vector co-expressing FGF2 and Cyr61 has revealed more efficient to generate therapeutic angiogenesis at low doses than the monocistronic vectors expressing large amounts of only one of these angiogenic factors[10]. It must be underlined that the IRES-based vector had no side effects on promotion of tumoral angiogenesis in contrast to the monocistronic ones, a very important feature for increased safety in clinical assays. These observations prompted us to deepen the features of IRESs applicable to vectorology and assess progress made in the field of gene transfer and combined gene therapy clinical assays using IRES-based vectors.

IRESS, TRANSLATIONAL ACTIVATORS FOR COMBINED TRANSGENE EXPRESSION

At a time when it was admitted that initiation of translation in eukaryotes required recognition of the capped mRNA 5' end to recruit ribosomes, translation of the uncapped picornavirus mRNAs from an internal start codon remained a mystery. Indeed, the so-called ribosome scanning mechanism predicted that ribosomes bound to the mRNA 5' end scanned the mRNA molecules until they recognized an AUG codon[11,12] (Figure 1). The event of internal ribosome binding was thought impossible. This puzzle raised by picornaviruses was solved by the discovery of RNA elements, called IRES, present in the 5' untranslated regions of their mRNAs, which allow internal recruitment of ribosomes[13,14]. The dogma of the scanning mechanism was thus broken. In addition, it was quickly extended to cellular mRNAs as the first cellular IRES was discovered three years later in the BiP mRNA, coding for the immunoglobulin chaperone also known as GRP78[15]. This discovery was followed by the finding of several other IRESs in cellular mRNAs, in particular in the mRNAs of angiogenic growth factors such as FGF2, proto-oncogenes such as c-myc, pro and anti-apoptotic proteins such as X chromosome-linked inhibitor-of-apoptosis protein and apoptotic peptidase activating factor 1[16-20]. IRESs were also found in retroviruses, whose mRNAs are capped as cellular mRNAs, leading to the design of IRES-containing retroviral vectors[21,22].

The existence of IRESs in capped cellular mRNAs asked the question of their pathophysiological function[23]. Actually, several reports showed that IRESs from cellular mRNAs are regulated in various physiological processes including cell differentiation, spermatogenesis, neurone plasticity[24-27]. Several IRESs are also activated during cell cycle mitosis[28,29]. Recent reports have also shown that IRESs are aberrantly activated in tumor cells, and are thus involved in dysregulation of gene expression in cancer[30]. Furthermore, cellular IRES activity is stimulated in stress conditions such as apoptosis and hypoxia when cap-dependent translation is blocked[31-36].

IRES-dependent internal initiation of translation reminds the prokaryotic initiation mechanism which can translate polycistronic mRNAs[37,38]. This observation gave the idea that such operons could be created in eukaryotes using IRESs to design expression vectors[39]. A large majority of expression vectors allow co-expression of two genes under the control of two promoters. However such an approach has revealed that one of the genes may be silenced despite of the expression of the other one even though it expresses an antibiotic[40]. This can result from competition between the two promoters or counterselection of the gene of interest in case of toxicity or of cell growth inhibition. In such a context, IRESs have been used to generate transgene co-expression under the control of a single promoter (Figure 2).

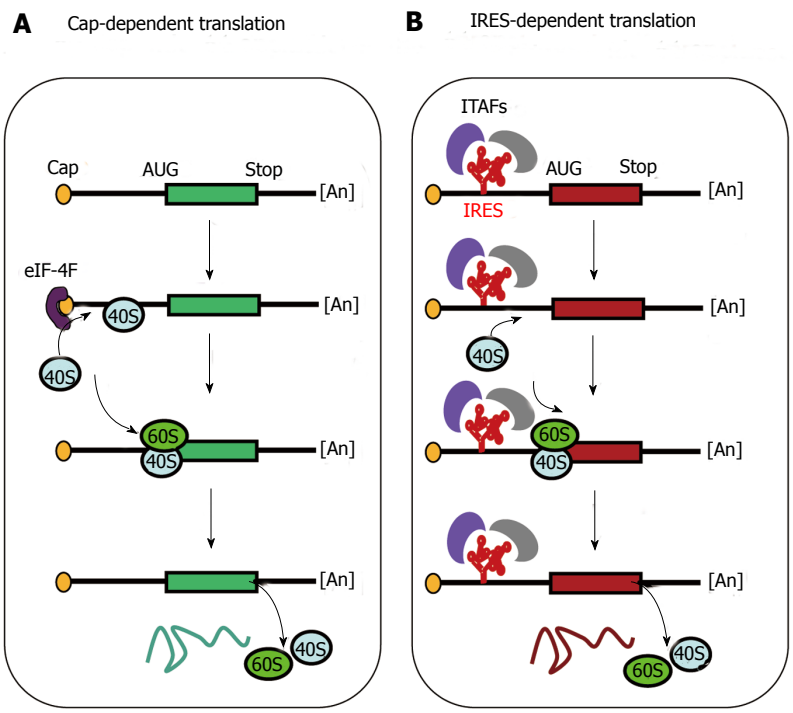

Figure 1 Cap-dependent and internal ribosome entry site-dependent initiation, two alternative mechanisms of translation. A: The so-called cap-dependent ribosome scanning mechanism predicts that ribosome 40S subunit binds to the mRNA 5' end. Ribosome binding requires the initiation factor 4F (eIF-4F, composed of the three proteins eIF-4E, -4A and -4G). Then the mRNA is unwound under the control of the helicases eIF-4A and -4B, allowing the ribosome to scan the mRNA until recognition of an initiation codon (classically AUG)[11,12]; B: When an Internal ribosome entry site (IRES) is present in the mRNA 5' untranslated region, IRES trans-acting factors (ITAFs) allow ribosome 40S internal recruitment, independently of the presence of cap and eIF-4F. The IRES-dependent mechanism occurs in the case of picornavirus uncapped mRNAs as well as for cellular capped mRNAs.

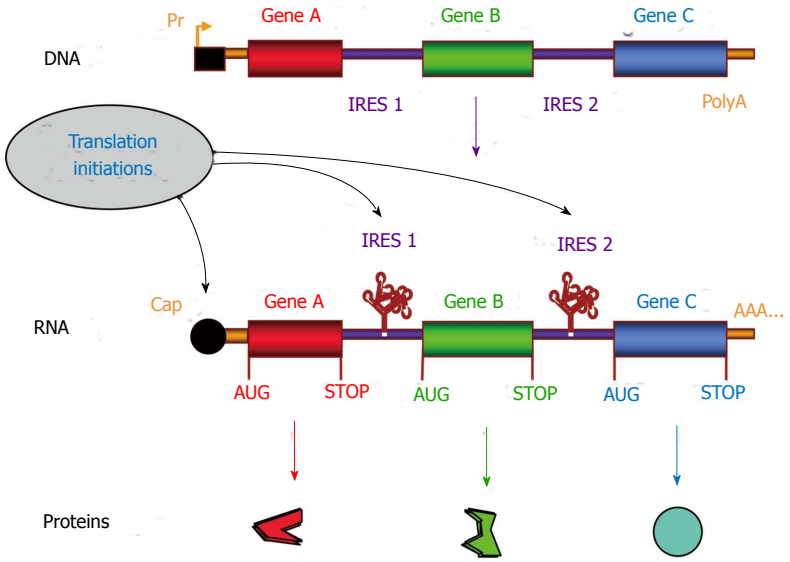

Figure 2 Internal ribosome entry site-based multicistronic vector concept. The internal ribosome entry site (IRES)-based expression cassette contains several genes, separated by IRESs, under the control of the same promoter (Pr). This transcription unit gives rise to a single mRNA coding the different genes. Translation initiation occurs at the 5' end by the cap-dependent mechanism, resulting in translation of the first open reading frame (ORF, Gene *A*). Internal initiations of translation occur at each IRES, resulting in translation of the other ORFs (Genes *B* and *C*). Thus the multicistronic mRNA generates several proteins from a single transcription unit, allowing more stable long term expression and stable transgene ratio[9,48]. For each ORF, initiation (AUG) and termination (STOP) codons are indicated.

The first retroviral tricistronic IRES-based vector appeared in 1992, providing an exciting potential for gene therapy[41]. This vector successfully co-expressed adenosine desaminase with neomycin (NEO) resistance and chloramphenicol acetyltransferase reporter genes, using the two picornavirus IRESs from poliovirus and encephalomyocarditis virus (EMCV), respectively. Two years later, a therapeutic tricistronic vector expressing the two interleukin-12 subunits with NEO validated the concept of IRES-based vectors to co-express two subunits of a protein with an adequate stoechiometry together with a resistance gene[42]. In the following years, bicistronic vectors were used successfully to select cell clones expressing a protein of interest with a resistance gene, preventing the problems generated by the use of two promoters[40,43].

TISSUE-SPECIFICITY OF CELLULAR IRESS

Most IRES-based vectors developed up to now use picornavirus IRESs, based on the strong efficiency of such IRESs in transient transfection, compared to cellular IRESs. It has been observed that cellular IRESs often exhibit a low efficiency in transiently transfected cells. Such a feature may result from the cell and tissue specificity of the cellular IRES activities. Actually, the FGF2 IRES activity varies with the cell type, the lowest being in fibroblasts, and the highest in neuroblastoma and osteosarcoma cells[44]. Similar variations have been observed for other cellular IRESs[45] (Creancier L and Prats AC, unpublished results). The strongest regulation

of cellular IRESs has been shown *in vivo*, in transgenic mice expressing bicistronic dual luciferase constructs containing different IRESs. Clearly, the EMCV IRES was active in most tissues and organs, while the FGF2 IRES was very low in most organs except for testis and brain where its activity increased 200 to 400 times, at least 10 times higher than the EMCV IRES activity[44]. A similar behavior was observed with other cellular IRESs such as c-myc and VEGFA IRESs[31,45].

The tissue-specific features of cellular IRESs are useful to control transgene expression. Thus they can be considered as translational enhancers, if one makes a parallel with transcriptional enhancers upstream of promoters, governing the tissue-specificity of gene expression. The concept of translational tissue-specificity may be applied to gene transfer by coupling tissue-specific IRESs with tissue-specific promoters to create vectors with increased safety. This concept should also remember us that EMCV is not always the best IRES to be used. A recent study reports the failure of expression of the second cistron of a bicistronic adeno-associated virus (AAV) vector using the EMCV IRES, in murine cerebellar Purkinje neurons[46].

The advantage of using a cellular IRES has also been demonstrated for gene transfer into skeletal muscle. The FGF1 IRES is as efficient as the EMCV IRES in mouse muscle after plasmid DNA electrotransfer[47]. Moreover, when this IRES is used in a bicistronic AAV vector, its activity is significantly superior to that of the EMCV IRES in myoblasts and allows a transgene expression 10 times more efficient when this AAV is injected in mouse muscle[48]. Such a difference may be due to the presence of specific FGF1 IRES trans-acting factors (ITAFs) (Ainaoui *et al*, in revision). Alternatively, it can result from the lower ability of the EMCV IRES to maintain a stable long term compared to cellular IRESs, shown in a previous report[9].

On the basis of these different data, it can be recommended to choose the adequate IRES to be used according to the cell type or tissue to be targeted, rather than using systematically the EMCV IRES as presently proposed in all commercial IRES-based vectors.

IRES-MEDIATED GENE EXPRESSION IN STRESS CONDITIONS

In many diseases cells are subjected to different stresses such as hypoxia, apoptosis or ER stress. In stress conditions, translation initiation is inhibited by two ways: blockade the mammalian target of rapamycin pathway which affects ribosome recruitment on the cap, and phosphorylation of eIF2-α which prevents charged initiator Met-tRNA formation. Interestingly, IRES-dependent translation is not affected by these two ways of silencing[35,49,50].

As mentioned above, IRESs are naturally present in messenger RNAs coding for proteins involved in the stress response, especially apoptosis and hypoxia. In particular, an IRES is present in the mRNA of the hypoxia-induced factor 1α (HIF1α), the key of the cell response to hypoxia that induces transcription of all the genes containing a hypoxia responsive element (HRE) in their promoters[51]. This IRES allows HIF mRNA translation to be activated during hypoxia despite of the blockade of global translation[32,52]. Such activation occurs under the control of an ITAF, the pyrimidine tract binding protein, also known as a regulator for various IRESs[52,53].

An important consequence of hypoxia is the stimulation of angiogenesis in order to generate new vessels able to restore the cell supply with oxygen. This process occurs in cancers when cells in the tumor core are oxygen deprived, as well as in ischemic diseases such as heart and lower limb ischemia when tissues are not any more irrigated due to artery occlusion. Strikingly, the major angiogenic factors VEGFA (vascular endothelial growth factor A), FGF1 and FGF2, possess IRESs in their mRNAs[20,47,54-56]. VEGFA expression, transcriptionally induced by HIF1α, is also translationally enhanced *via* the IRES in hypoxic tumors and in ischemic mouse legs[31,32,36]. In contrast to VEGFA, FGF2 is not induced transcriptionally by hypoxia but its synthesis is translationally induced by the IRES-dependent mechanism in ischemic tissues[31,33]. The same phenomenon has been observed for the major lymphangiogenic factor VEGFC, induced by hypoxia at the translational level *via* an IRES, but not at the transcriptional level, in tumors and lymph nodes[36,57]. FGF2 and VEGFC induction is exclusively translational and HIF-independent, revealing that IRESs provide an alternative HIF-independent way of response to hypoxia.

On a biotechnological point of view, the sensitivity of IRESs to hypoxia may be an advantage for several applications. Gene transfer vectors can benefit from this feature as the presence of IRESs allows increased transgene expression in ischemic conditions *in vivo*. Once again, one can see that data from basic research have to be taken into account in the design of optimized expression cassettes. The use of IRES-based vectors seems particularly adequate for gene therapy of ischemic diseases and cancer, as in both cases the transgenes have to be expressed in hypoxic conditions.

BIOMEDICAL APPLICATIONS OF IRESS

IRESs have found biomedical applications for several years. As mentioned above, the first biomedical use of IRESs in an expression vector has been co-expression of subunits of a therapeutic protein with a gene of resistance, as shown for interleukin 12 subunits with a gene of resistance[42]. However this application is limited to therapeutic genes composed of several subunits. In addition, the use of resistance genes is not recommended as it may prevent the use of the vector in a clinical assay.

Another application of IRESs raised during the last decade, resulting form the emerging concept of combined gene therapy. Several studies have validated this concept using a cocktail of two vectors to transfer two genes simultaneously. This has been particularly

Table 1 Preclinical studies of combined gene therapy with co-administration of monocistronic vectors

Pathology	Therapeutic genes	Animal model	Vector type	Ref.
Cancers				
Leukemia, melanoma	Angiostatin + endostatin	Mouse	Retrovirus	Scappaticci *et al*[4], 2001
Ovarian cancer	Angiostatin + endostatin	Mouse	AAV	Ponnazhagan *et al*[3], 2004
Glioblastoma	*VEGF-R1* + angio-endo (Statin AE)	Mouse	SB transposon	Ohlfest *et al*[60], 2005
Pancreatic cancer	*TSP1* + endostatin	Mouse	AAV	Zhang *et al*[61], 2007
Cardiovascular diseases				
Limb ischemia	*VEGFA* + angiopoietin-1	Rabbit	Plasmid	Chae *et al*[5], 2000
Limb ischemia	*VEGFA* + *FGF2*	Mouse	Plasmid	Lee *et al*[59], 2007
Limb ischemia	*VEGFA* + *PDGFB*	Rabbit	AAV	Kupatt *et al*[58], 2010
Heart ischemia	*VEGFA* + *PDGFB*	Pig	AAV	Kupatt *et al*[58], 2010
Rear diseases				
DMD	Microdystrophin + IGF1	Mouse	AAV	Abmayr *et al*[62], 2005

DMD: Duchenne muscular dystrophy; VEGF: Vascular endothelial growth factor; FGF2: Fibroblast growth factor 2; AAV: Adeno-associated virus.

documented in the field of cardiovascular diseases and cancer, with therapeutic benefits obtained in different animal models using different combinations of angiogenic or anti-angiogenic factors[4,5,58-61] (Table 1). Interestingly the combination of VEGFA and PDGFB successfully induced therapeutic angiogenesis both in ischemic leg and in ischemic heart. In the field of rare diseases, two AAV vectors expressing microdystrophin and IGF1 resulted in increased muscle mass and strength, reduced myofiber degeneration and increased protection against contraction-induced injury in *mdx* mice[62]. These different studies were performed either with naked DNA or with recombinant adeno-associated virus vectors.

The use of two different vectors for multiple transgene expression exhibits disadvantages: on the one hand, the ratio of the therapeutic molecules cannot be controlled, leading in the loss of the cooperative effect: expression of one of the vectors often decrease or is silenced earlier than the other one[40]. On the other hand, the cost of two therapeutic vectors in a clinical perspective is higher than a single one. These disadvantages are still more important in case of a cocktail of three or more therapeutic genes.

The concept of IRES-based vectors for combined gene therapy has been validated for combined immunotherapy of cancer using a tricistronic retrovirus expressing the two co-stimulation molecules CD70 and CD80[2] (Table 2). In addition to the EMCV IRES, several cellular or retroviral IRESs were successful in this approach[63]. *In vivo* gene therapy has also been validated for the treatment of ischemic limb in a mouse model, following intramuscular injection and electrotransfer of a plasmid containing the FGF1 IRES for co-expression of FGF2 and Cyr61[10]. This study showed than the two angiogenic factors, although expressed at lower doses from the bicistronic vector than from the monocistronic ones, have a synergistical effect in stimulating therapeutic angiogenesis, rendering the bicistronic construct more efficient. More importantly, due to the lower doses of therapeutic molecules, the bicistronic vector induces no side effects on tumoral angiogenesis, in contrast to one of the monocistronic vectors expressing huge amounts of Cyr61. Thus combined gene therapy using IRES-based vectors is also a safer therapeutic approach.

Additional studies have confirmed the successful use of IRES-based vectors for combined treatment of limb ischemia with VEGFA and FGF4 or bone morphogenetic protein7 (BMP7)[64,65]. Combined gene therapy of cancer was also reported using IRES-based vectors co-expressing IL-12 and CD80, as well as antiangiogenic factors angiostatin and endostatin, or CXCL4I and fibstatin[66-69] (Table 2). Combination of angiostatin and endostatin in an IRES-based vector was also successful to treat age-related macular degeneration in a mouse model[70]. In the field of degenerative diseases, mucopolysaccharidosis type ⅢA has been addressed in presymptomatic MPSⅢA mice by intrastriatal administration of an AAV vector co-expressing N-sulfoglycosamine sulfohydrase (SGSH) with the sulfatase-modifying factor (SUMF1) (Winner *et al*, submitted). This study has resulted in a clinical assay[71] see below). Only one report has obtained better data with two separate AAV vectors to deliver FGF14 and a fluorescent protein into *purkinje* neurons, than with an IRES[46]. This study used the EMCV IRES previously reported to function in neurons[72]. However it must be underlined that the EMCV IRES is not very active in neurons *in vivo*, by comparison with the FGF2 IRES that is at least ten times more active[24,44]. In such a case, one can expect that the choice of the FGF2 IRES would provide better data.

Multigene transfer has also been validated for combinations of three genes. A tricistronic IRES-based lentivector expressing three catecholaminergic proteins, Prosavin, was administrated by bilateral striatal injection for treatment of Parkinson in rats, resulting in important therapeutic benefits[73,74] (Table 2). Moreover, a tricistronic 2A-based lentivector administrated in situ was also efficient in co-expressing Gata4, Mef2c and Tbx5 for postinfarct ventricular functional improvement in rats[75].

It is often mentioned that the IRES-driven translation of the downstream cistrons is lower than the cap-dependent first cistron translation. This issue can easily be addressed by intelligent vector design: First, one can take into account the tissue specificity of the IRES by choosing the most adequate IRES rather than using systematically the EMCV IRES. Most bi- and- tricistronic vectors use this IRES although it is far to be the best one

Table 2 Preclinical studies of combined gene therapy using multicistronic vectors

Pathology	Therapeutic genes	Animal model	IRES	Vector type	Ref.
Cancers					
Fibrosarcoma	CD70 + CD80	Mouse	EMCV	Retrovirus	Couderc *et al*[2], 1998
Melanoma	Angio-endo fusion	Mouse	None (fusion)	Retrovirus	Scappaticci *et al*[79], 2001
Multiple myeloma	IL12 subunits + CD80	Mouse	EMCV + FMDV	Retrovirus	Wen *et al*[69], 2001; Li *et al*[67], 2003
Melanoma	CD70 + CD80	Mouse	EMCV, c-myc, FGF2, HTLV1	Retrovirus	Douin *et al*[63], 2004
Ovarian cancer	Angiostatin + endostatin	Mouse	EMCV	AAV	Isayeva *et al*[66], 2005
Head and neck cancer	Angio-endo fusion	Mouse	None (fusion)	Vaccinia virus	Tysome *et al*[80], 2011
Pancreas cancer	CXCL4L1 + fibstatin	Mouse	FGF1	AAV, Lentivector	Prats *et al*[68], 2013
Cardiovascular diseases					
Limb ischemia	*FGF2 + Cyr 61*	Mouse	FGF1	Plasmid	Rayssac *et al*[10], 2009
Limb ischemia	*VEGFA + BMP7*	Rabbit	EMCV	AAV	Zhang *et al*[65], 2010
Limb ischemia	*VEGFA + FGF4*	Mouse	EMCV	AAV	Jazwa *et al*[64], 2013
Heart ischemia	*Gata4 + Mef2C + Tbx5*	Rat	None (2A element)	Lentivector	Mathison *et al*[75], 2014
Neurodegenerative diseases					
Parkinson	*TH + AADC + CH1*	Rat	EMCV	Lentivector	Azzouz *et al*[73], 2002 Stewart *et al*[74], 2011
AMD	Angiostatin + endostatin	Mouse	EMCV	Lentivector	Kachi *et al*[70], 2009

BMP7: Bone morphogenetic protein 7; Gata4: GATA binding protein 4; Mef2C: Myocyte-specific enhancer factor 2C; Tbx5: T-box transcription factor 5; TH: Tyrosine hydroxylase; AADC: aromatic L-amino acid decarboxylase; CH1: GTP cyclohydrolase-1; AMD: Age-related macular degeneration.

in many tissues such as muscle or brain[24,48]. Second, the IRES efficiency can be improved. It must be noticed that the EMCV IRES activity is very sensitive to the position of the start codon of the gene of interest. This IRES, in contrast to the FGF1 IRES, exhibits no flexibility: the AUG must be positioned just downstream from the IRES. The insertion of a single restriction site between the IRES and the AUG codon is sufficient to inactivate the IRES[76]. The insertion of a spacer between the first gene and the IRES is also susceptible to enhance the IRES activity by preventing IRES structural alterations by RNA sequences located upstream[77]. In addition, mutations of the upstream AUG codons in the EMCV IRES improve its efficiency[78]. Finally, an important parameter is the IRES regulation by microenvironment. In particular, FGF or VEGF IRES activities are more sensitive to hypoxia than the EMCV IRES and may allow a more efficient transgene expression in ischemic diseases.

ALTERNATIVES TO IRESS FOR MULTICISTRONIC VECTORS

IRES-based vectors are not the only approach to co-express several gene products under the control of a single promoter. The first alternative is gene fusion. It has been successfully used to combine endostatin and angiostatin in a treatment of melanoma and of head and neck cancer[79,80]. A second alternative to IRESs is the use of alternative splicing-based vectors. Such an approach had been proposed many years ago using retroviral vectors, using the natural alternative splicing features of retrovirus genome[81,82]. This concept has been developed more recently in the purpose of co-expressing two immunoglobulin chains[83]. The interest of this system is the ability to adapt the ratio of the two transgenes by mutating the splicing sites. However one limit of this attractive system is that splicing site efficiency and consequently the ratio of the two proteins of interest, is influenced by the presence of exon splicing enhancers or silencers in the transgene sequences, preventing the design of vectors with a stable transgene ratio applicable to co-expression of any pair of therapeutic proteins.

A third exciting system of co-expression is provided by the 2A peptides. Such peptides, occurring in many viral genomes, are peptide sequences of about 19 amino-acid residues, which can produce a discontinuity in the translated polypeptide when encoded in a longer open reading frame (ORF)[84]. In contrast to what is currently admitted, 2A peptides do not catalyze a protein cleavage, but they catalyze termination of translation in the absence of a stop codon, followed by reinitiation. They are currently used as a tool to co-express two or more separate proteins from a single ORF[85]. 2A peptides thus constitute an alternative to IRESs, but do not work in all systems. By example, in the study in *purkinje* neurons mentioned above, a 2A peptide was used but did not function, resulting in detection of the longer ORF rather than the two expected proteins[46]. In another report comparing bicistronic constructs expressing Sox9 and EGFP separated by the EMCV IRES or by the FMDV 2A peptide, the authors detected 42% of Sox-EGFP fusion protein, reflecting an inefficient ribosome skipping mechanism[86]. Formation of such fusion proteins often occurs with proteins bearing N-terminal signal sequences[87]. In addition, no information is available about the 2A peptides tissue-specificity or behavior in response to stress, in contrast to IRESs.

CLINICAL APPLICATIONS OF IRES-BASED VECTORS TO GENE THERAPY

All the preclinical studies mentioned above show that

Table 3 Clinical studies of combined gene therapy

Pathology	Therapeutic genes	IRES	Vector type	Outcome	Ref.
Ischemic heart disease	*VEGFA + FGF2*	EMCV	Plasmid	Moderate benefits	Kukula *et al*[90], 2011
Parkinson	*TH + AADC + CH1*	EMCV	Lentivector	Benefits for 15/15 patients	Palfi *et al*[91], 2014
Mucopolysaccharidosis type IIIA	*SGSH + SUMF1*	EMCV	Lentivector	Benefits for 1/4 patients, stabilization for 3/4	Tardieu *et al*[71], 2014

TH: Tyrosine hydroxylase; AADC: Aromatic L-amino acid decarboxylase; CH1: GTP cyclohydrolase-1; SGSH: N-sulfoglycosamine sulfohydrolase; SUMF1: Sulfatase-modifying factor.

IRES-based vectors represent an exciting tool to be used for combined gene therapy. Nowadays, very little clinical trials with such vectors have been reported. The first trial to be cited is the tricistronic IL12-expressing retrovirus, which gave significant decrease of tumor sizes on a few patients with melanoma or head and neck cancer[88,89].

A bicistronic IRES-based vector co-expressing FGF2 and VEGFA has been assessed in a clinical assay of gene therapy on patients with refractory coronary disease[90] (Table 3). The protocol corresponded to intramyocardial transfer of a plasmid expressing the bicistronic cassette. This study showed no improvement in myocardial perfusion, but treated patients exhibited improved exercice tolerance and clinical symptoms. Furthermore the bicistronic gene transfer was safe. This moderate benefit, although encouraging, may be due to the use of a plasmid, which does not provide long term expression in contrast to viral vectors, and also to the choice of the EMCV IRES which is not optimal to drive gene expression in hypoxic conditions[31,36].

Very recently, two gene therapy clinical trials successfully used multi-cistronic IRES-based viral vectors. On the one hand, a gene therapy Ⅰ/Ⅱ phase clinical trial on patients with mucopolysaccharidosis type ⅢA, a severe degenerative disease, has displayed neurocognitive benefits[71]. Four children received intracerebral injections of a bicistronic AAV vector expressing the SGSH and SUMF1 genes separated by the EMCV IRES. Neurocognitive evaluations suggest a cognitive benefit on the youngest patient, where as the other ones are stabilized. Importantly, the treatment was safe and well tolerated after 1 year in all the patients, validating the surgical approach for direct AAV delivery in the brain parenchyma. On the other hand, a phase Ⅰ/Ⅱ assay was performed on 15 patients with Parkinson's disease using Prosavin (see above), a tricistronic lentivector with EMCV IRESs administrated by intrastriatal delivery[91]. A significant improvement of motor scores was recorded in all patients at 6 mo. This is the first-in-man use of a lentiviral-based gene therapy vector for a neurodegenerative disease. These studies validate the clinical use of IRES-based viral vectors.

CONCLUSION

Many reports have shown that combined gene therapy is an attractive approach in animal models. This observation has justified extensive research on optimization of gene transfer vectors able to co-express several proteins. In this context, IRES-based vectors have now been validated in pre-clinical as well as in clinical studies by showing their safety and ability to generate therapeutic benefits.

In addition, the data available on IRES tissue-specificity and activation in response to stress provide promising perspectives of vector improvement, which may result in better efficiency of gene therapy.

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REVIEW

Consolidated and emerging inflammatory markers in coronary artery disease

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Abstract

Coronary artery disease is an event of atherosclerosis characterized by a chronic vascular inflammation. Risk factors like obesity, diabetes mellitus, hypertension, smoking, hypercholesterolemia and positive family history sometimes are not sufficiently adequate to the enhancement of cardiovascular risk assessment. In the past years numerous biomarkers, like C reactive protein, cytokines and adhesion molecules, have been observed to be related to adverse cardiovascular prognosis. Recently, several studies found an association among inflammatory biomarkers and cardiovascular diseases suggesting their utility to identify the risk of an acute ischemic event and the detection of vulnerable plaques. The emerging inflammatory markers are well divided for diagnosis and prognosis and plaque instability of coronary artery disease. Some of them, the lectin-like oxidized low density lipoprotein receptor-1 can be important both in diagnosis and in the evaluation of plaque instability, other are inserted in the above reported classification. The emerging inflammatory markers in acute-phase include amyloid A, fibrinogen and pentraxin 3 while myeloperoxidase, myeloid-related protein 8/14 and pregnancy-associated plasma protein-A are recognize markers of plaque instability. Lastly, some studies demonstrated that circulating miRNAs are involved in coronary artery disease, acute myocardial infarction and heart failure.

Key words: Coronary artery disease; Plaque instability; Inflammation; Acute phase; Biomarkers

Core tip: In this review we want to focus the reader's attention on the differences between inflammatory markers of cardiovascular risk already accepted by the scientific community and the emerging markers in order to encourage the healthcare services to improve laboratory techniques in early diagnosis and more precise evaluation of the risk. Is also important to use a classification according to the stage where the patient is located regarding emerging inflammatory markers for diagnosis, prognosis and plaque instability.

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INTRODUCTION

Atherosclerosis is largely recognized as a chronic inflam-

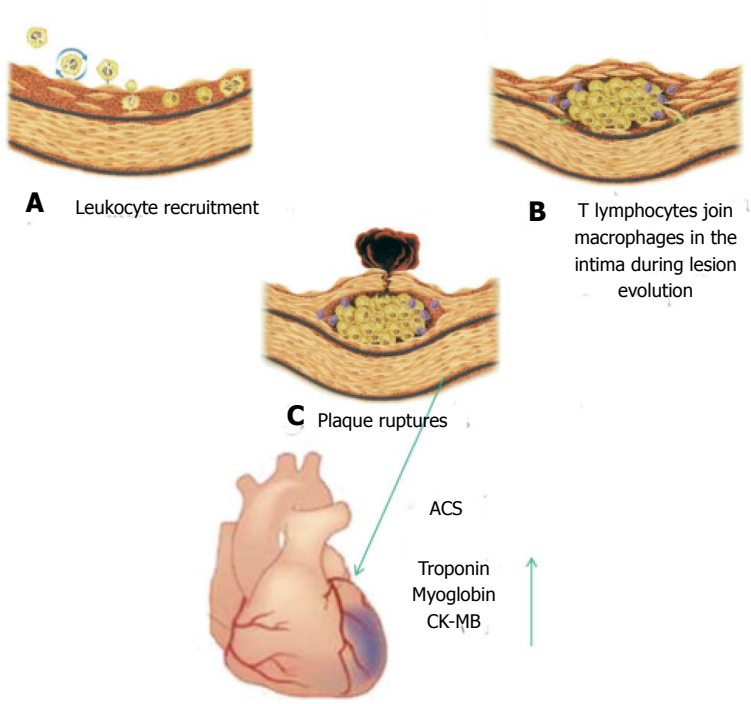

Figure 1 Phases of coronary atherosclerosis. Events implicated in the progression of acute coronary syndrome (ACS).

matory disorder caused by vascular and extravascular factors[1,2] and coronary artery disease (CAD) is its common manifestation. CAD could result in the development of acute coronary syndrome (ACS), which is often associated with breakage of an atherosclerotic plaque and partial or complete thrombosis of the related artery.

In these years a large number of studies permitted a better knowledge of the events implicated in the progression of ACS: here we summarize them (Figure 1). In these processes there is a recruitment of macrophages, that secretes lytic enzymes such as metalloproteinases. The atheroma core is constituted by foam cells and extracellular lipids shrouded by of smooth-muscle cells and collagen matrix. Plaque ruptures release adhesion molecules and soluble factors, such as D-dimers, von Willebrand factor and plasminogen activator inhibitor-1 that have an important role in thrombus formation. In a few hours after thrombus formation, but before the initiation of coronary ischemia, albumin is released. Troponin, myoglobin, and creatine kinase-MB are time-dependent release components associated with myocardial necrosis[3] (Figure 1). The extent of these events influences the circulating troponin level[4]. Therefore it is important to identify the fundamental steps leading to atherosclerotic plaque rupture.

Adequate risk assessment remains the most challenging in individuals classified into low or intermediate risk categories. Inflammation is important in the progression of atherosclerosis and in plaque rupture[1,5]. For this reason, numerous inflammatory markers have been extensively investigated as potential candidates for the enhancement of cardiovascular risk assessment.

Several recent studies have demonstrated the role of inflammation in mediating the stage of CAD, often caused by lipid accumulation.

Moreover the different part of atherogenesis could be related to inflammatory biomarkers that are important for clinical diagnosis, treatment and prognosis of patients with CAD. However, because conventional risk factors do not explain the changes in atherosclerosis, efforts have focused on developing novel biomarkers which identify vulnerable plaques and cardiovascular disease[6,7].

These new laboratory biomarkers should be standardized in variability, sensitivity and specificity from established risk markers. Finally, the cost of the assays has to be acceptable. In this review we analyze the inflammatory markers now considered valid in the stratification of risk for CAD and those emerging, checking if new ones can express something more than the standardized biomarkers.

CONSOLIDATED MARKERS

C-reactive protein

C-reactive protein (CRP), a pentraxin composed of 5 subunits, is an inflammatory marker that may increase in various pathological situations, synthesized mainly in the liver, but it is also produced by leukocytes and adipocytes[8,9].

According considerable evidence, during infection or tissue necrosis circulating CRP may increase 50000 times, but it is also regarded as an independent variable of future cardiovascular events[10].

CRP fosters antigen presentation and phagocytosis attaching to phosphocholine that is usually found in cell membranes and polysaccharides in prokaryotes and fungi and binding to complement C1q complex and factor H[11,12].

Moreover it can attach low density lipoprotein (LDL), and be identify within the plaque[13] where it participates to inflammatory atherogenic processes[14]. CRP is elevated

in patients with acute and chronic coronary syndromes in relation to the composition of the plaque[15,16] and is related to the complications of heart failure[17]. Low plasma levels of CRP indicate a good state of health[18], while increase when the style of life worsens. The MONICA Augsburg Study shows that low quality "Western" diet with low consumption of vegetables, fruit and fiber, extensive use of saturated fat, low physical activity and obesity, are associated with higher CRP levels[19].

Therefore, increment of CRP plasma concentration reflects not good lifestyle choices that lead to a metabolic disequilibrium and inflammation. The study of a large population has revealed that an increase in the levels of CRP (> 3 mg/L, elevated levels) was associated with mortality of 22962 subjects[20].

Ridker *et al*[21] showed that CRP was a better biomarker of cardiovascular diseases than LDL cholesterol. However when measured together, they give better prognostic detail than measured separately[21]. A large prospective study documented a strong association between CRP predictive power and the risks for coronary artery disease[22,23]. Moreover, the Canadian Cardiovascular Society suggested that CRP evaluation in patients at "intermediate risk", could represent a predictive risk of a cardiovascular event from 10% to nearly 20% within the subsequent 10 years[24]. In agreement with this observation, the National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines and the American College of Cardiology Foundation-AHA Task Force on Practice Guidelines affirmed that the evaluation of CRP levels was acceptable for patients at intermediate risk[25,26].

Another study regarding people at intermediate risk for a cardiovascular event showed that the values of CRP and fibrinogen could help to prevent one additional event over a period of 10 years for every 400 to 500 people screened.

Current knowledge, however, suggests that the CRP concentration might reflect the vulnerability of the atheromatous lesion and the prospect of plaque rupture[5,27,28]. The development of high-sensitivity CRP (hs-CRP) assays has been useful to investigate its role in predicting first cardiovascular events.

Cytokines

Interleukin 1 (IL-1), IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor alpha (TNF-α) are the main investigated cytokines among those which predict cardiovascular events involved in vascular inflammation and atherosclerosis[29,30]. IL-1 and IL-6, regulate CRP production *via* direct stimulation of the hepatocytes[31]: IL-6 may increase plaque instability modulating the expression of TNF-alpha, and MCP-1[32]. Elevated IL-6 levels in healthy men correlated with increased risk for future MI independently from hs-CRP[33]. According to some author IL-6 seems to be a marker more sensitive and specific than CRP in vascular inflammation and CRP studies show a weaker association with cardiovascular disease than cytokines[34,35]. In the Fragmin study (FRISC-Ⅱ), IL-6 increment above 5 ng/L was related with a mortality from 6- to 12-mo without a relationship with troponin and hs-CRP[36].

Therefore, IL-6 plasma concentration results as an effective independent index of increased mortality in unstable CAD and characterizes subjects who advantages of an initial invasive strategy. In addition, the intensity of plaque inflammation and its vulnerability seems to be linked with plasma IL-6 levels[36].

Some studies showed that IL-1 could have a regulatory function in the atherosclerotic development suggesting its modulation in vascular smooth muscle cell mitogenesis[37,38], in leukocyte adherence to vascular wall[39,40], in LDL metabolism[41,42], in extracellular matrix proteins[43] and in vascular permeability[44]. Moreover, IL-1 has been found to suppress vascular contractility[45] and induce pro-coagulant activity[46].

Several years ago, increased levels of IL-1α and IL-1β were detected in human atherosclerotic plaque, suggesting their local synthesis[47]. Moreover, IL-1 protein has been detected in macrophages from damaged carotid arteries[48]. In the macrophages IL-1β secretion seems to be induced by the cholesterol crystals present into the plaque[49].

The presence of increased IL-1β plasma concentration in patients affected by unstable angina indicates its important role in the acute stage[50]. However, in mouse models conflicting roles have been reported for IL1β: on the one hand the absence of IL-1β is associated with a reduction of atherosclerotic severity[51], on the other hand, IL-1β inactivation seems to be related to atherosclerotic plaque stability[52].

TNF-α plays a role in myocardial dysfunction and remodeling after acute coronary events[53]. On behalf of this effect, the CARE study showed that TNF-levels increased in recurrent coronary events after a MI compared with controls[54].

The chemokine MCP-1 recruits monocytes into the arterial wall activating these cells to induce endothelial injury[55]. In addition, a positive correlation between MCP-1 levels and the extent of coronary atherosclerosis was found in the coronary circulation of patients with unstable angina[56,57]. Moreover, MCP-1 levels have been found to correlate with older age[58], hypertension[59], hypercholesterolemia[60], and kidney failure[61], while an inverse correlation has been observed with estrogen replacement[62] and HMG-CoA reductase inhibitor therapy[60]. In several studies with small number of subjects, plasma MCP-1 levels were highest among patients with acute coronary syndromes (ACS), intermediate with stable coronary disease, and lowest among healthy control subjects.

IL-10 is an important factor for its anti-atherogenic property. In fact patients with high IL-10 levels had a reduced mortality compared with those that have only elevated CRP[63].

In 158 patients affected by stable CAD, during a 7-year follow-up period, the multivariate analysis of 10 cytokines showed IL-8 as the only independent marker for cardiovascular diseases[64]. In summary, even if the results are still controversial, in our opinion among consolidated

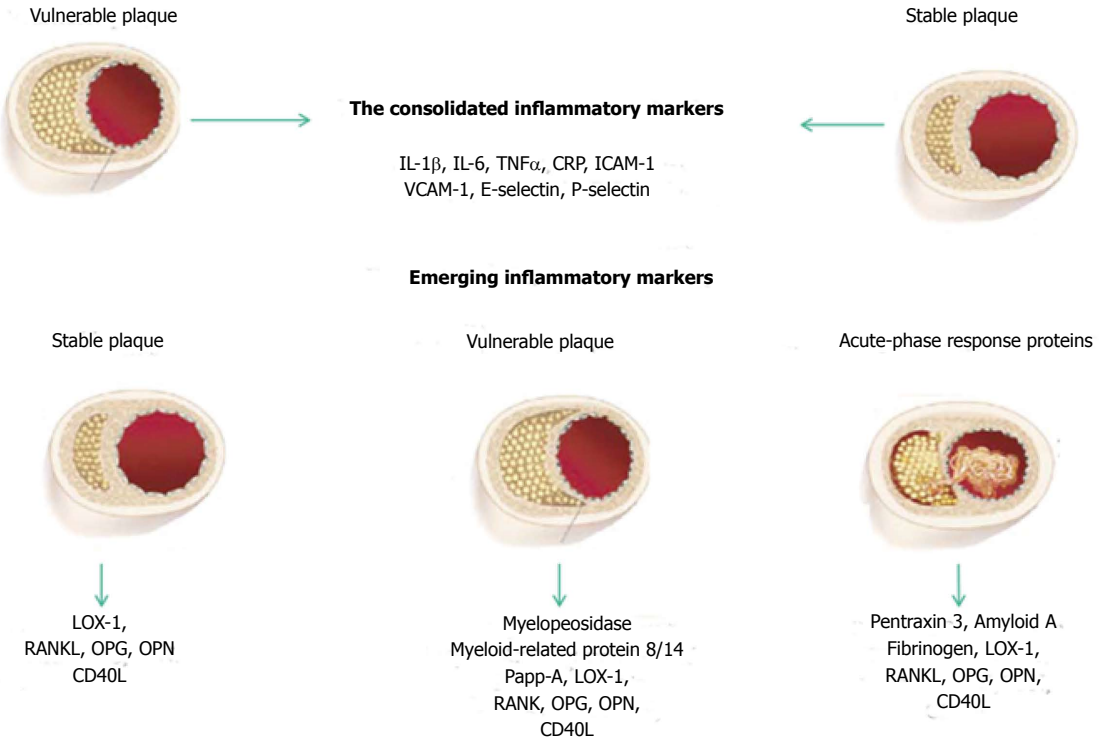

Figure 2 Consolidated and emerging inflammatory markers. A new approach to establish the risk for coronary artery disease. Today new inflammatory markers are studied and classified according to their role in the development of coronary artery disease. TNF-α: Tumor necrosis factor alpha; IL: Interleukin; CRP: C-reactive protein; CAM: Adhesion molecules; OPG: Osteoprotegerin; OPN: Osteopontin. PAPP-A: Pregnancy-associated alpha plasma protein A; LOX-1: Lectin-like oxidized low-density lipoprotein receptor-1.

cytokine, IL-6 represents the best prognostic biomarker in CAD.

Adhesion molecule

Although very broad, adhesion molecules (CAMs) may be regarded as inflammatory markers of cardiovascular risk. Soluble CAMs (ICAM-1, VCAM, P and E selectines) are released from the surface of the cell and reflect cellular activation[65]. CAMs induce the bind between leucocytes, platelets and vascular wall[66]. After the adherence to the endothelium, the leucocytes transmigrate into the arterial wall determining the first phase of atherosclerosis[66].

Several studies reported an association between the increase of plasma CAM concentration and the risk of cardiac events[34,67,68], but their role in CAD prognosis have not been established because their finding are still quite confused.

In patients with stable CAD, CAMs plasma concentrations were measured and informations on cardiovascular events were collected for some years. Among CAMs, only VCAM-1 resulted independently significant with future cardiovascular events[69].

In agreement with this study, other authors observed that the concentrations of sVCAM-1 > 780 ng/mL and CRP > 3 mg/L corresponded to a sensitivity > 90% for predicting future events in patients affected by acutely ACS[70].

On the contrary, other studies did not confirm these findings for sVCAM-1; instead, they suggested that CRP and sICAM-1were useful for identifying the risk of a cardiac event in patients with unstable angina who underwent coronary stenting[71]. Finally, another prospective study showed that only P-selectin and cardiac troponin I, but not the other CAMs, were significantly higher among patients who had a serious cardiac event during the subsequent 3 mo[72].

EMERGING INFLAMMATORY MARERS

The lack of "traditional" risk factors cannot make totally free of the disease and new emerging markers of inflammation have been studied in the effort to identify biomarkers predicting the risk, and at the same time reflecting plaque instability in the early or in the acute phase. On the bases of these studies, we must point out that today is also in use a classification according to the stage where the patient is located (Figure 2).

EMERGING INFLAMMATORY MARERS FOR DIAGNOSIS, PROGNOSIS AND PLAQUE INSTABILITY

Lectin-like oxidized low-density lipoprotein receptor-1

Clinical studies have demonstrated that well-known coronary risk factors, including metabolic diseases, hypertension, obesity and smoking, are associated with oxidative stress. When the LDL are exposed to oxidative stress, they are caught in the vessel and oxidized (ox-LDL). Oxidized LDL promotes the synthesis of a large variety of cytokines and chemokines by the endothelium. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1)

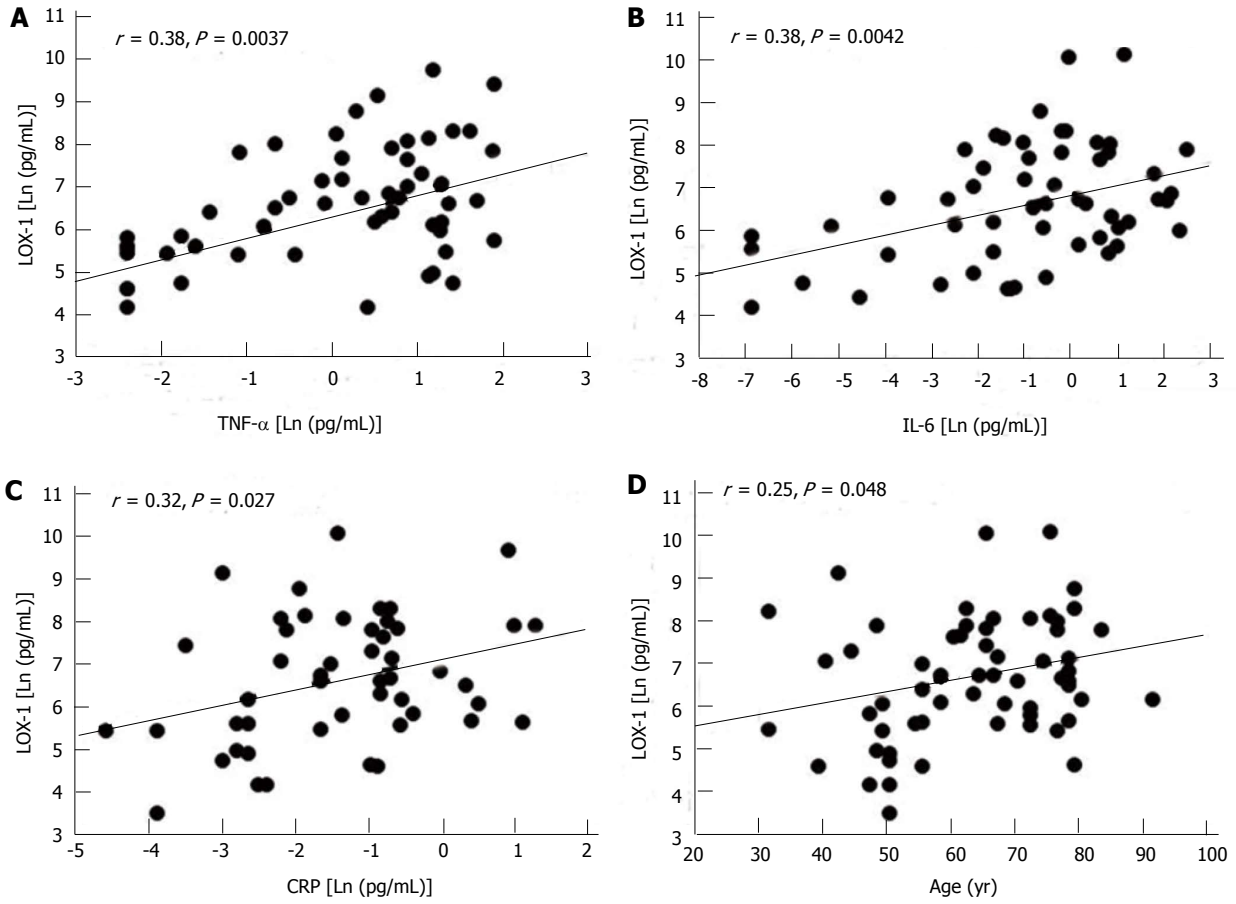

Figure 3 Regression analysis of LOX-1. From Lubrano *et al*[76], 2008. Positive association between circulating levels of LOX-1 and inflammatory markers. TNF-α: Tumor necrosis factor alpha; IL: Interleukin; CRP: C-reactive protein; LOX-1: Lectin-like oxidized low-density lipoprotein receptor-1.

appears to be an important receptor for ox-LDL in endothelial cells[73]. LOX-1 not only allows the passage of oxidized lipids in the cells, but as already described, may cause endothelial dysfunction/apoptosis, inflammation, and the increase smooth muscle cell number favoring the formation of atheroma[74-76].

Moreover LOX-1 increment was observed to be associated with cardiovascular risk factors like hypertension and metabolic disorder. In a population of patients affected by CAD, our previous studies showed a positive relationship between circulating levels of LOX-1 and inflammatory markers: this work suggested also that LOX-1 levels increased with the severity of the disease[76] (Figure 3). Other authors underlined the importance of this novel biochemical marker for the stratification risk of the population and therapeutic strategy for CVD.

Overt cardiovascular disease is typically preceded by a long period of sub-clinical cardiovascular disease and sub-clinical atherosclerosis can be present for decades before the occurrence of a myocardial infarct event. Soluble LOX (sLOX-1) has shown to be informative either early or late in the process disease[77-79].

Recent study observed that the circulating levels of sLOX-1 are very high in acute coronary syndrome and that the plateau value is reached before troponin T, highlighting the instability of the plaque[80].

It has been reported that serum levels of sLOX-1 are also specifically elevated in acute coronary syndrome and the peak value has been reported to rise before troponin T[80]. In conclusion, sLOX-1 levels are related to the prognosis of acute coronary syndrome and reflect the instability of plaque[78].

Nuclear factor-kappa B, osteoprotegerin, osteocalcin, osteopontin, CD40

Receptor activator of nuclear factor Kappa-B ligand (RANKL) is the ligand of the receptor inducer factor-κB (NF-κB) and belongs to the family of cytokines TNF-related. It is synthesized by T cells and stromal/osteoblastic cells and is a strong chemotactic factor for human monocytes[81]. RANKL-stimulated microvascular endothelial cells favor monocyte adhesion and trans endothelial migration thus increasing the recruitment of osteoclast- and osteoblast like cell precursor[81,82].

Osteoprotegerin (OPG) synthesized in osteoblasts is part of the TNF super-family. It binds to RANKL thereby preventing interaction with its transmembrane receptor[83].

RANKL and OPG have been shown to be potentially valuable markers for a better assessment of coronary calcification and cardiovascular risk associated with it. It has been observed that RANKL and OPG may play a key role in maturation and calcification of atherosclerotic

plaque[84,85]. In fact these factors increased in serum of post-infarction of atherosclerotic animal models and of humans with unstable angina[86,87].

Osteocalcin, a protein found in bone and dentin and also synthetized in mononuclear cells, has been related with the severity of aortic calcification[88].

Osteopontin (OPN), an extracellular matrix protein and pro inflammatory cytokine, facilitates the recruitment of monocytes/macrophages through its adhesive domain[89] and promotes the inhibition of vascular calcification In fact it is increased in patients with vascular calcification resulting more like a marker than a mediator of atherosclerosis progression[90].

CD40, a member of TNF family, is also a stimulatory receptor on antigen-presenting cells of the immune system that induces inflammatory processes through the binding of the CD40 ligand (CD40L). Elevated levels of soluble CD40L (sCD40L) have been found in patients with hypercholesterolemia, ACS and cardiovascular disease. sCD40L is also associated with atherosclerosis and plaque instability[91]. In the CAPTURE trial, increased sCD40L (> 5 g/L) was related to a 6-mo mortality or nonfatal MI suggesting that sCD40L may be an independent risk marker of cardiovascular events. Statins, antihypertensive drugs, and antiplatelet agents have been shown to modulate it[92]. Moreover, sCD40L was found to be increased in smokers and positively associated with both total cholesterol and biomarkers of inflammation. However, it was not reported as an independent biomarker for the risk of MI[93].

ACUTE-PHASE RESPONSE PROTEIN

Quantitative and qualitative changes of inflammatory markers are able to identify the acute stage of the disease. The plaque ruptures cause the consequent platelet aggregation and subsequent thrombosis, the final stage in which atherosclerosis leads to acute ischemic syndromes of AMI and sudden death[5].

The literature well documented the association between serum concentrations of acute phase proteins and the onset of coronary heart disease and myocardial infarction[94,95]. The emerging inflammatory markers in the acute-phase include pentraxin 3 (PTX3), amyloid A, and fibrinogen[96-102].

Pentraxin-3

Pentraxins are a superfamily of soluble proteins with cyclic multimeric structure[103]. Among these, PTX3 a protein characterized by a long N-terminal domain, results as an important player in immunity and inflammation[104]. Dendritic cells, macrophages and endothelial cells produce PTX3 in response to IL-1 and TNF[105]. Moreover increased plasma PTX3 levels were observed in patients with cardiovascular disease and resulted also more closely related than CRP levels in acute phase of cardiac damages[106] suggesting that it could be a sensitive and specific prognostic indicator[107].

It is been also hypotheses an association of PTX3 in individuals with stable coronary artery disease and kidney dysfunction. However, an adjustment for the estimated glomerular filtration rate modestly attenuated these associations[108]. By immune histochemical staining PTX3 was strong expressed on the surface of lumen and within the atherosclerotic plaque in humans and animal models[96,97,109]. Moreover, in the same experimental models, soluble PTX3 increased in the early phase after ischemic heart events and PTX3 mRNA and protein expression enhanced in the ischemic area of the heart[110].

In a prospective study of patients with myocardial infarction and ST elevation, PTX3 predicted 3-mo mortality while other markers such as the liver-derived short pentraxin CRP or NT-proBNP, TnT, CK did not[107]. In patients with unstable angina pectoris within the six hours of the chest pain, PTX3 resulted to be more specific for ACS than neutrophil activating peptide-2 and cardiac troponin I (cTnI)[111,112].

Amyloid A

Serum amyloid A (SAA) proteins are a family of apolipoproteins associated with high-density lipoprotein (HDL) and are now considered emerging markers of inflammation. In fact elevated SAA levels are present in coronary artery disease and indicate worse prognosis in CAD. Therefore actions involved to reduce SAA levels could improve the conditions of patients with acute CAD[113].

A study of Kosuge *et al*[99] reported that, in patients with ACS, increased SAA levels were associated with cardiovascular events within 30 d, without any relationship with CRP level. Therefore these data indicated SAA more useful predictor than CRP in these patients.

In the high serum SAA group the left ventricular ejection fraction, measured during follow-up, was significantly lower than in the low serum SAA group and more frequent complications, such as cardiac rupture, carcinogenic shock, subacute thrombosis, and cardiac death, were also present[100].

Furthermore SAA levels were quite well associated with coronary artery disease with a predictive risk for cardiovascular events within 3 years, while this did not happen with hs-CRP[114]. In a substudy of TIMI 11A, elevated SAA levels predicted increased risk of 14-d mortality in patients with ACS[115]. In a Women Ischemia Syndrome Evaluation study, in which women were referred for coronary angiography because of suspected ischemia, elevated SAA values were correlated with angiographic severity of CAD and 3-year risk for cardiovascular events[114]. At the same time, no relationship was observed between SAA levels and recurrent Coronary Events[116].

Fibrinogen

Several studies have indicated fibrinogen as a predictive marker in CAD[117]. Fibrinogen is involved in platelet aggregation, endothelial injury, plasma viscosity and play a central role in the formation of thrombus.

Epidemiological data have shown the important predictive role of fibrinogen in CAD, identifying it as an emerging risk factor because its measurement may improve the estimation of absolute risk obtained by conventional

risk factor for CV[117].

Although it is still discussed the role of fibrinogen as inflammatory markers of risk, many studies indicated an association of hyperfibrinogenemia with atherotrhombosis.

Already in the past, some authors have demonstrated that the risk estimation for CAD could be double when fibrogenemia was also evaluated[118].

Emerging Risk Factors Collaboration showed that, the measurement of fibrinogen level in patients at risk for CAD, could prevent an additional event in the next 10 years for every 400-500 people studied[119].

However, also recent results show that the evaluation of fibrinogen during MI may be useful in identifying patients at high risks for future acute events[102].

BIOMARKERS OF PLAQUE INSTABILITY

The main cause of the acute myocardial infarction (AMI) is the plaque rupture, so that it is important to investigate new markers for early diagnosis of plaque instability.

Due to its sensitivity and specificity, troponin is commonly used in the diagnosis of ACS, even if it provides only indirect details on myocardial necrosis induced by embolization of atherothrombotic material, late event of ACS.

Inflammation is a process that is intensified in plaque instability, so that the markers of inflammation may provide indications of cellular processes related to its formation before it occurs myocardial necrosis[120].

Myeloperoxidase

Myeloperoxidase (MPO) is an enzyme produced by leukocytes that induces the formation of oxygen free radicals and is considered to be one major contributor in the formation and rupture of the plaque[121].

In patients with ACS, MPO produced by neutrophils, is considered a marker of plaque vulnerability as noted by several studies[122, 123].

Yunoki *et al*[124], 2013 observed that the plasma levels of MPO have a significant inverse correlation with levels of paraoxonase-1 bound to HDL, especially, in patients with stable and unstable angina pectoris, suggesting that a mismatch between pro oxidants and anti-oxidants may contribute to the progression of coronary plaque instability[124].

Myeloid-related protein 8/14

Myeloid-related protein 8/14 (MRP8/14), is a heterodimer consisting of two proteins that bind calcium, calgranulin A and B, which play an important role in the signaling pathways of calcium, in cell cycle progression, cell differentiation, and in the interaction between the cytoskeleton and membrane[125]. MRP-8/14, also called calprotectin, is synthesized by activated monocytes and neutrophils, and is a pro-inflammatory protein expressed in atherosclerotic plaques.

High concentrations of MRP8/14 in the systemic circulation may reveal the presence of plaques before necrosis markers suggesting it as a good candidate for the management of ACS unstable.

PAPP-A

PAPP-A is a high-molecular-weight zinc-binding metalloproteinase. PAPP-A was independently associated with recurrent cardiovascular events in patients with ACS. This finding supported the potential usefulness of PAPP-A as a biomarker in patients with ACS[126]. Moreover as described by Mahto *et al*[127] PAPP-A is the reliable marker which can discriminate the cases of MI from unstable angina and controls[127]. Another study has suggested PAPP-A to be a predictor of mortality or myocardial infarction in patients with ACS[128].

Role of microRNAs in CAD

MicroRNAs (miRNAs) are short non-coding RNA molecules that regulate gene expression post-transcriptionally through suppression or degradation of target messenger RNA (mRNA).

MiRNAs were found in the circulating blood and are differently induced in patients with CAD, AMI, and heart failure[129-132].

Of interest is miR-155, which proved to be a new component of inflammatory signal transduction pathways in the pathogenesis of atherosclerosis. In fact the expression of miR-155 is considered to be a prospective marker for predicting the prognosis of CAD since it is found to be expressed mainly in patients with CAD compared to healthy subjects[133].

CONCLUSION

Inflammatory biomarkers appear to have an important prognostic value in patients with cardiovascular disease and may be useful in the diagnosis of apparently healthy subjects without known CAD who cannot be assessed with conventional risk factors.

Inflammatory biomarkers may have prognostic value for future cardiovascular risk among those at high risk or with documented cardiovascular disease. They also may be useful for identifying apparently healthy individuals, without known CAD, who may be at a higher risk than estimated by traditional risk factors.

Although recent data demonstrate that there is a close association between inflammatory biomarkers and coronary artery disease, further studies must be carried out taking into account also some important criteria typically used in the selection of a new biomarker: discrimination, calibration and reclassification, i.e. the ability of a test to discern between those that will face the disease from those that will be free, the assessment of the risk factor predicted and observed, classification in categories of low, intermediate and high risk for CAD[134].

In our opinion the best candidate for this role is LOX-1; it was observed to be associated with cardiovascular risk factors like hypertension and metabolic disorder, showing its positive relationship with inflammatory markers and

its increment with the severity of the disease[76] (Figure 3). Moreover it was elevated in acute coronary syndrome and the peak value has been reported to rise before troponin T reflecting the instability of plaque[80].

In conclusion, the findings observed in a decade showed that LOX-1 could represent an important marker for clinical characterization of coronary artery disease and a target for new drugs to reduce its expression and production.

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Ledipasvir and sofosbuvir: Interferon free therapy for hepatitis C virus genotype 1 infection

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Abstract

Hepatitis C virus (HCV) has infected more than 200 million people around the globe. From 2001-2011, interferon plus ribavirin remained the standard of care for patients with HCV infection. The therapy had a limited response with a number of side effects. Recently, results for phase III trials of ledipasvir and sofosbuvir combination therapy have been announced. In treatment naïve patients, 12 wk of therapy with ledipasvir and sofosbuvir showed a sustained virological response (SVR) rate of 99%. In treatment experienced patients, 12-24 wk of therapy with ledipasvir and sofosbuvir in the absence or presence of ribavirin showed an SVR rate of 94%-99%. In cirrhotic patients the rate of SVR was 86% and 99% for 12 and 24 wk of therapy, respectively. The ledipasvir and sofosbuvir therapy showed very good results in different subgroups of patients regardless of patient's race, alanine aminotransferase levels, sex and host genetic factors. The combination therapy was well tolerated with no emergence of resistant mutants. The most common adverse effects were nausea, headache and fatigue. With the availability of interferon free therapy with minimal adverse effects, it will be easy to decrease the future morbidity and mortality caused by HCV infection.

Key words: Hepatitis C; Interferon; Ledipasvir; Sofosbuvir; Genotype

Core tip: The interferon based therapy for hepatitis C patients has a limited response with a number of adverse effects. The ledipasvir and sofosbuvir combination therapy showed a sustained virological response (SVR) rate of 99% in treatment naïve patients. The rate of SVR was 94%-99% in treatment experienced patients, while in cirrhotic patients the rate of SVR was 86%-99%. The treatment response was not affected by ethnicity or host genetic factors.

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TO THE EDITOR

Hepatitis C virus (HCV) infection is a major health problem around the globe, with more than 200 million people infected worldwide. Although the rate of HCV infection is continuously declining, the rates of HCV associated morbidity and mortality are continuously increasing.

From 2001-2011, interferon and ribavirin therapy remained the standard of care for patients living with HCV. The therapy had a limited response with a number of side effects. The major adverse effects associated with interferon administration were flu like symptoms, cytopenia and depression, whereas ribavirin therapy causes fatigue, anemia, rash and pruritus. The major objective of recent treatment regimens is to eliminate the interferon and ribavirin from the treatment regimen so that the adverse effects of therapy can be reduced and the therapy become available for patients who are ineligible for the interferon and ribavirin therapy.

Sofosbuvir is a nucleoside analogue that can inhibit the HCV polymerase, approved by the Food and Drug Administration for the treatment of patients living with HCV. Ledipasvir is an inhibitor of HCV NS5A protein, showing antiviral activity against HCV genotype 1 infection.

In a phase Ⅱ clinical trial, 100 patients with HCV genotype 1 infection who were treatment naïve or previously treated with protease inhibitors were enrolled at a centre in the United States. The patients were given a fixed-dose combination of sofosbuvir (400 mg) and ledipasvir (90 mg). In cohort A, 60 treatment naïve, non-cirrhotic patients who were given sofosbuvir plus ledipasvir (8 wk), sofosbuvir plus ledipasvir along with ribavirin (8 wk), or sofosbuvir plus ledipasvir (12 wk) showed an SVR rate of 95%, 100%, and 95% respectively. In cohort B, 40 previous non-responders to protease therapy were included. They were given sofosbuvir plus ledipasvir (12 wk) or sofosbuvir plus ledispavir along with ribavirin (12 wk), and the sustained virological response (SVR) rate was 95% and 100%, respectively[1]. The sofosbuvir-ledipasvir combination therapy cured most of patients with HCV genotype 1 infection, irrespective of their treatment history. Further investigations were required to optimize the treatment duration and the role of ribavirin in treatment response.

In a phase Ⅲ clinical trial, 865 previously untreated patients were enrolled and they were randomly divided into four groups. Group 1 received ledipasvir and sofosbuvir for 12 wk and showed an SVR rate of 99%. Group 2 received ledipasvir and sofosbuvir along with ribavirin for 12 wk and showed an SVR rate of 97%. Group 3 received ledipasvir and sofosbuvir for 24 wk and showed an SVR rate of 98%. Group 4 received ledipasvir and sofosbuvir along with ribavirin for 24 wk and showed an SVR rate of 99%. The study concluded that the 12 wk therapy with ledipasvir and sofosbuvir was highly effective for patients living with HCV genotype 1 infection. No additional benefit was observed by the addition of ribavirin or by the extension of therapy to 24 wk[2].

In another phase Ⅲ trial, 440 previously treated patients were enrolled, 20% of whom had cirrhosis. The patients were given ledipasvir and sofosbuvir in the presence or absence of ribavirin from 12 or 24 wk. The rate of SVR achieved was 94%-99%. In patients with cirrhosis the rate of SVR was 86% (ledipasvir-sofosbuvir) and 82% (ledipasvir-sofosbuvir plus ribavirin) with 12 wk of treatment, while the rate of SVR was 99% (with both regimens) in patients having 24 wk of treatment. The study concluded that the single tablet of ledipasvir–sofosbuvir showed a better rate of SVR even in the patients who were not responders to the interferon based therapy[3].

The ledipasvir and sofosbuvir therapy produced very good results in different subgroups of patients regardless of patient's race, alanine aminotransferase levels, sex and host genetic factors. The combination therapy was well tolerated. No S282T variant was observed. The most common adverse effects were nausea, headache and fatigue[2-4].

A total of 1952 patients were enrolled in three different phase III trials of ledipasvir and sofosbuvir, out of which 97% showed SVR[2-4]. Out of the remaining 3%, half of them withdrew consent or were lost to follow-up. Undetectable viral RNA was not achieved in only two patients. The rate of relapse was observed in only 2% after stopping therapy. The rate of relapse was also linked with the treatment duration. The rate of relapse was observed in 5%, 2% and 0.2% of patients who received 8 wk, 12 wk and 24 wk of treatment, respectively[5].

With the availability of oral, short duration, interferon free therapy with minimal adverse effects, the future morbidity and mortality associated with HCV infection will decrease. The major problem with the therapy is its cost. The cost of 12 wk therapy with sofosbuvir alone is $84000 and the addition of ledispavir will further increase the cost[5]. The high cost of the therapy will affect the goal of providing safe and effective treatment for millions of patients living with HCV around the globe.

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