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ABBREVIATION LIST

Ang	Angiopoietin
C-GSF	Colony granulocyte stimulating factor
CHF	Chronic heart failure
cMLCK	Cardiac-specific myosin light-chain kinase
CVD	Cardiovascular diseases
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPC	Endothelial progenitor cell
EPO	Erythropoietin
ErbB	NRG tyrosine kinase receptor
FDA	U.S. Food and drug administration
FGF	Fibroblast growth factor
FGF-1	Acidic fibroblast growth factor
FGF-2	Basic fibroblast growth factor
FGFR	FGF tyrosine kinase receptor
G-CSF	Granulocyte colony-stimulating factor
GF	Growth factor
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia inducible factor-1α
HSPGs	Heparan sulfate proteoglycans
IHD	Ischemic heart disease
LAD	Left anterior descending coronary artery
LVEF	Left ventricle ejection fraction
MCP-1	Monocyte chemoattractant protein-1
MMPs	Matrix metalloproteinases
NO	Nitric oxide
NRG	Neuregulin
PDGF	Platelet-derived growth factor
PDGF	Platelet-derived growth factor
PDGFR	PDGF tyrosine kinase receptor
PEG	Poly(ethylene glycol)
PEO	Poly(ethylene oxide)
Shh	Sonic hedgehog
TGF- β	Transforming growth factor-β
Tie	Ang tyrosine kinase receptor
TNF- α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor
VEGFR	VEGF tyrosine kinase receptor
VSMC	Vascular smooth muscle cell
WHF	World heart federation
WHO	World health organization

1. INTRODUCTION

Cardiovascular diseases (CVD) are, globally considered, the main cause of death in the world. The concept of CVD includes several disorders of the heart and blood vessels, such as ischemia, rheumatic and inflammatory heart disease. Table 1 summarizes the World Health Organization (WHO) data regarding deaths from this cause, published in 2008 [1]. Ischemic heart disease (IHD) is the main problem within CVD and, according to The World Heart Federation (WHF) information, the number of deaths it causes every year is similar in Europe and in South-East Asia, revealing that CVD are a major problem all over the world. Moreover, the WHF report (2008) on the economic impact of diseases shows the high cost of treatment for CVD in developed countries, which in the United States (USA), for example, is as high as €310.23 billion: more than twice the cost of all cancers [2,3].

IHD occurs when a coronary artery narrows, frequently as a result of atherosclerosis, and blood supply in the heart is insufficient, resulting in angina, heart attack, or even sudden death of the patient. When faced with ischemia, the heart tries to make up for the loss of functionality and cardiac remodeling starts. This process is responsible for important alterations in myocyte biology, as well as for myocardial changes, alterations in extracellular matrix (ECM) and in the left ventricular chamber geometry. Briefly, after ischemia, changes at the level of the failing human cardiac myocyte lead to a defect in contractile function. On the other hand, myocardium itself fails as a consequence of myocyte loss through both necrotic and apoptotic cell death, perivascular fibrosis around intramyocardial blood vessels and excessive deposition of fibrillar collagen around myocytes. These changes affect the ventricular chamber geometry, involving the emergence of a larger and a more spherical heart shape. The combination of all these anatomic, functional and biological alterations contributes to progression of the disease [4] as described in Fig. 1.

Current therapies include pharmacological treatments, percutaneous intervention and surgery. However, although these can mitigate the symptoms, they are not able to regenerate the tissue, or to restoring the heart function. Furthermore, for a number of patients, the only alternative is organ transplantation, with all its drawbacks. This has moved researchers and clinicians to explore new approaches. Among others, these have focused on restoring blood flow by inducing angiogenesis by treatment with cells, genes or soluble factors involved in this process.

This review examines proposed options for the treatment of cardiovascular diseases based on the induction of tissue revascularization, particularly focusing on protein-based therapy and the use of controlled drug delivery systems.

2. THERAPEUTIC ANGIOGENESIS

Angiogenesis is the process of formation of new vascular vessels from the existing ones, by sprouting and longitudinal division (intussusception) processes. It also involves incorporation of endothelial progenitors recruited from the bone marrow (postnatal vasculogenesis). The newly formed vessels split and branch into pre-capillary arterioles and capillaries.

Angiogenesis is a crucial phenomenon during embryonic development, but it also occurs in adult tissues under certain physiological circumstances: ovulation, development of the corpus luteum, immune response, inflammation and wound repair. This natural means of giving rise to new vessels is a complex process involving different types of cells, secreted soluble factors (with pro- and anti-angiogenic activities) and extracellular matrix compounds, which operate in a tightly regulated spatial and temporal manner. The outcome (adequate, defective or excessive angiogenesis) depends on the balance between angiogenic activators and inhibitors, and their imbalance may result in pathology because of either excessive or insufficient angiogenesis (Fig. 2). In such cases, several pathologies (brain, cardiac or peripheral ischemia, defective healing in diabetes, etc.) could benefit from therapeutic induction of angiogenesis.

In protein-based therapeutic angiogenesis, one or various exogenous proteins are administered to intervene in the endogenous process at several levels: reducing inflammatory response, controlling ECM renovation, and promoting survival, proliferation, differentiation and migration of cells. The induced therapeutic cardiac environment allows sprouting, branching and maturation of new vessels into arteries and/or veins. In this way, metabolic homeostasis and contractile function would be restored and the recovery of cardiac function could ultimately be achieved.

3. POTENTIAL FACTORS FOR THERAPEUTIC MYOCARDIAL ANGIOGENESIS

Tumor research led to the finding of factors responsible for angiogenesis and their applications as therapy for some ischemic diseases such as myocardial ischemia [5]. Along similar lines, the more recent

knowledge acquired about the factors involved in cardiovascular development during embryogenesis has led researchers to translate these factors to promote cardiac repair in the adult organism. Nowadays it is known that proangiogenic factors expressed in the embryo are newly induced in the adult heart under hypoxia and stress conditions to achieve revascularization when the coronary artery flow is disrupted [6].

Below are described several of the main proangiogenic factors which would be suitable for its use in therapeutic angiogenesis, indicating their signaling pathways, their biological actions and the relationships between them.

3.1. Fibroblast Growth Factor (FGF)

FGF was one of the first angiogenic growth factors related to tumor vascularization to be discovered [7,8]. The FGF family comprises one of the more versatile growth factor signaling systems in vertebrates, acting in a wide variety of biological process. In mice and humans, twenty three FGF ligands and four tyrosine kinase receptors (FGFR), which are subjected to multiple splicing events, have been identified [9]. FGF-1 (acidic FGF) and FGF-2 (basic FGF) are the most extensively studied members and to date, are the only FGFs known that are involved in cardiac repair.

At the myocardium, FGFs are pleiotropic molecules that act on ECs, smooth muscle cell and myoblasts, which express high-affinity FGF receptors. The binding of FGF ligand to FGFR leads to the dimerization and autophosphorilation of the receptor and this event triggers, either directly or through the recruitment of adaptor proteins, the activation of several intracellular signaling pathways that result in different cellular responses involved in angiogenesis and cardiac repair. Among them, several functions have been described, such as the induction of 1) proliferation of ECs, smooth muscle cells and myoblasts [10]; 2) survival of cardiomyocytes, vascular smooth muscle cells (VSMCs) and ECs (reviewed in [11]); 3) cell-cell interactions and physical organization of ECs into tube-like structures [12]; 4) VEGF secretion in endothelial and stromal cells (autocrine mechanism of FGF induced angiogenic response) [13,14]; 5) induction of PDGF receptor expression in VSMCs (contributing to maturation-stabilization of newly formed vessels) [15] and 6) selective upregulation of MCP-1 (monocyte chemoattractant protein-1) on non-endothelial mesenchymal cells (VSMCs and fibroblasts) (contributing to the arteriogenesis driven by immune cells) [16].

3.2. Vascular Endothelial Growth Factor (VEGF)

VEGF was discovered as a factor that induces vascular hyperpermeability and acts as an endothelial cell-specific mitogen [17]. Since then, VEGF has been the protein most widely used to induce angiogenesis both in pre-clinical models and in clinical assays.

In humans, the VEGF family currently comprises members encoded by five genes: VEGF-A (the first identified as VEGF), -B, -C (also called VEGF-2), -D and PlGF (Placental Growth Factor). Due to alternative splicing, multiple isoforms with different biological activities can be produced from each gene. Active VEGFs are mainly homodimers, although VEGF-A and PlGF heterodimers have also been identified. VEGFs present different extracellular distribution and each isoform can bind to co-receptors (neuropilins) or ECM compounds, namely heparin and/or heparan sulfate proteoglycans (HSPGs) [18].

VEGFs are implicated in the vascular development during embryogenesis and in new blood vessel formation in the adult [19]. VEGF-A is the best characterized member and it shows the highest angiogenic potential. Several human VEGF-A isoforms have been identified: VEGF-$A_{145,}$ VEGF-A_{189}, and VEGF-A_{206} which are bound tightly to cell surface; VEGF-A_{121}, a highly diffusible form; VEGF-A_{165a} and VEGF-A_{165b}, which exist as both bound and freely diffusible protein [20].

VEGFs can bind to three receptor tyrosine kinases, known as VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4). Although highly homologous, they exhibit different affinities for the VEGF ligands. VEGFR-1 and VEGFR-2 are expressed predominantly by vascular ECs to participate in vascular angiogenesis while VEGFR-3 in adult is mainly confined to the lymphatic endothelium. VEGFR-1 has a higher affinity for VEGF-A, but it has a much weaker kinase activity and is unable to generate a mitotic response in ECs. In contrast, VEGFR-2 has a lower affinity for VEGF-A but it is able to signal and hence trigger multiple cell responses. VEGFR-1 can also exist in soluble form, binding to VEGF without any signaling, and thus limiting the availability of VEGF-A to VEGFR-2 (reviewed in [21]).

After VEGF ligand binding, VEGFR goes through dimerization and autophosphorilation, triggering the recruitment of cytoplasmic interacting proteins and activation of several signaling molecular pathways involved in a variety of responses in ECs like: 1) permeability [22]; 2) survival [23]; 3) proliferation [24] and 4) migration (reviewed in [18]).

The angiogenic effect of VEGF-A is regulated at different levels (reviewed in [20]). Firstly the expression of VEGF-A can be induced by several stimuli such as HIF-1α (for its part is up-regulated by FGF-2), growth factors (PDGF-BB, FGF-4, Transforming Growth Factor-β or TGF- β) and inflammatory cytokines (Interleukins-1 or 6, Tumor Necrosis Factor-α or TNF- α, etc.). Secondly, the duration and intensity of VEGFR signaling can be modulated by co-receptors such as HSPGs and neuropilins, and also through interaction with adhesion molecules regulated by blood flow. Ultimately transcription of VEGFR-2 is also induced by HIF-1α and TNF-α. Furthermore the interaction between endothelial and smooth muscle cells can also regulate VEGF signal (read below how other factors secreted by these cells affect the VEGF response).

3.3. Angiopoietins (Ang)

This family of growth factors consists of four members of secreted glycoproteins named Ang-1, Ang-2, Ang-3 and Ang-4. The ones which are best known for their involvement in cardiovascular remodeling are Ang-1 and Ang-2. These two members show some differences which could account for the outcome of their signaling. Both bind to tyrosine kinase receptor Tie-2 on ECs with similar affinity, but they act in an opposite way. While the binding of Ang-1 to Tie-2 promotes its autophosphorylation and the subsequent intracellular signaling, Ang-2 acts as a natural antagonist since it binds to Tie-2 without the autophosphorylation event. This may be due to differences in the structure of the domain responsible for receptor binding. Another important feature is that Ang-1 is produced by non-ECs in many tissues and it is incorporated into the ECM, while Ang-2 is accumulated or secreted in a soluble form by ECs in sites of vascular remodeling. This could regulate their availability and biological activity [25]. Moreover, the outcome of angiopoietin signaling depends on the balance between Ang-1 and Ang-2. In fact, during cardiovascular development Ang-1 is expressed early and Ang-2 is detected later [26].

The Ang-1 signaling induces multiple effects on ECs: chemotaxis, tube formation and survival inhibiting endothelial apoptosis through several intracellular pathways. However, there is no evidence of endothelial proliferation in response to Ang-1 [27]. It has also been shown that Ang-1 is able to oppose the permeability action of VEGF-A, inducing the recruitment of pericytes and smooth muscle cells to be incorporated in the vessel wall, besides anti-inflammatory actions. So Ang-1 may have a leading role in vessel maturation and stabilization, regulating cell-cell and cell-matrix interactions [21,28].

On the other hand, the binding of Ang-2 to Tie-2 avoids Ang-1 signaling, leading to vessel destabilization, activation of ECs to respond to angiogenic stimuli (such as VEGF), detachment of pericytes and degradation of ECM. In this way, Ang-2 allows the subsequent sprouting initiated by VEGF. *In vitro* [29] and *in vivo* [30] evidence suggests that under low oxygen tension Ang-2 could act in a biphasic way, initially blocking Ang-1 signaling and allowing ECs stimulation by angiogenic factors and next, contributing to the stabilization and maturation of the newly formed blood vessels. Some studies have shown Ang-2 up regulation and Ang-1 down regulation mediated by hypoxia [31]. Moreover, there is evidence for a coordinated relationship between VEGF and Ang-2 levels. At low levels of VEGF, Ang-2 signaling leads to vascular regression, but in the presence of higher level of VEGF the outcome of Ang-2 signaling is sprouting and vessel formation [32,21].

3.4. Platelet-derived Growth Factor (PDGF)

The first isoform of the PDGF family was discovered in the mid 1970s as a constituent of platelet α-granules with growth promoting activity for fibroblast and smooth muscle cells. Subsequently it has been shown that PDGF is produced in different isoforms by distinct cell types under normal and pathological scenes (during organogenesis, angiogenesis, tissue fibrosis, in tumors, etc). So far, four isoforms of PDGF ligands have been identified: PDGF-A, -B, and more recently -C and –D. These four polypeptides require proteolytic cleavage and dimerization to achieve biological activity. The active homo or heterodimers PDGF-AA, -BB, -AB, -CC and –DD bind to tyrosine-kinase receptors PDGFRs. There are two types of receptors, PDGFR-α and PDGFR-β, which can be expressed in a selective or dual manner depending on the cell type (i.e., PDGFR-β is expressed rather specifically on VSMCs and pericytes, whereas ECs in sprouting vessels express elevated levels of both –α and –β receptors [33]. PDGF-A binds specifically to PDGFR-α whereas PDGF-B can bind to PDGFR-α and PDGFR-β. PDGF-C and PDGF-D bind preferably to PDGFR-β but it seems they also can bind to PDGFR-α on cells expressing both α and β receptors).

The PDGF signal induces over 80 genes, among them matrix and cytoskeleton proteins, growth factors, growth inhibitors, transcription factors involved in cell cycle (c-jun, c-fos, c-myc), etc. One of the physiological functions of PDGF/PDGFR signal is to participate in angiogenesis and vessel stabilization through stimulation of proliferation and migration of vascular ECs, VSMCs, fibroblasts, monocytes and

granulocytes (reviewed in [34]). Several studies have found that administration of PDGF-BB or -AB in combination with FGF-2, leads to an increase in capillary and arteriolar density and vessel stabilization, in models of hind limb ischemia and chronic myocardial infarction in rats [35,36]. Recently, this effect has been attributed specifically to PDGFR-β, but not to PDGFR-α. A possible mechanism suggested for this angiogenic synergy and vascular stability is that FGF-2 induces a strong up regulation of PDFGR on endothelial cells, leading to formation of receptor dimers with persistent activity even after removal of PDGF ligands, that would maintain the angiogenic response [37].

Lately, more interest has been focused on PDGF-C, which presents a wide range of direct and indirect angiogenic effects [38], such as increasing the number and availability of ECs, pericytes and smooth muscle cells and the induction of proliferation of fibroblasts and inflammatory cells, therefore increasing production of angiogenic growth factors, ECM and matrix metalloproteinases that will allow the growth of new vessels and remodeling of arterioles into arteries [39].

3.5. Neuregulin-1 (NRG-1)

To date, NRG-1 is the only neuregulin known to be involved in the development and function of the heart [40]. It presents three distinct isoforms that arise from gene transcription from different promoters: type I, type II and type III. All of them are synthesized as membrane-anchored precursors, and type I and type II NRG-1 are solvable by proteolytic processing signaling to nearby cells in a paracrine manner. On the other hand, mature type III NRGs remains anchored and signals to adjacent cells in a juxtacrine manner [41].

NRG-1 is a member of EGF (Epidermal Growth Factor) family and structurally consists of four main domains. The extracellular EGF-like domain gives rises, by alternative splicing, to α and β isoforms. These isoforms differ in their binding ability, since the β isoforms exhibit 10-100 more activity when binding to receptor.

In spite of the variability, all NRG isoforms perform their biological activity through the tyrosine-kinase ErbB membrane receptors. It appears that during heart development only the type I and type II NRG-1β isoforms have a critical role, but in the adult heart type I NRG-1α is the one predominantly expressed although the NRG-1β isoform continues to be important. NRG-1 ligands appear to be produced on ECs near cardiomyocytes (in the myocardial microvasculature and endocardium) in response to oxidative stress in adult heart [42]. Related to the ErbB receptors, ErbB-2, ErbB-3 and ErbB-4 are critical for heart development, the ErbB3 expression being lost in adult cardiomyocytes [43].

In cardiomyocytes, the NRG-1 ligands bind to the ErbB-4 receptor which dimerizes with ErbB2, leading to multiple cellular responses like the proliferation and survival of neonatal [43] and adult cardiomyocytes [43-45]. Moreover, it has been shown that in pathological conditions, NRG-1 promotes myocardial regeneration and decreases hypertrophy of surrounding infarcted areas [46] by preserving a synchronized beat (through activation of the Src/FAK (Focal Adhesion Kinase) pathway (involved in sarcomeric organization and cell-cell interactions) and upregulation of the cMLCK (a cardiac-specific myosin light-chain kinase that controls muscle contraction and sarcomere organization)) [42,47]). Also, NRG1 is involved in the Ca^{2+} homeostasis (involved in myocyte relaxation [48]), the control of the inotropic response to adrenergic stimulation (due to stress or overload) [49] and indirect paracrine angiogenic effect on ECs, through the release of VEGF-A by other cell types (such as fibroblast) [50].

All of these effects have prompted the potential therapeutic use of NRG-1 in patients with heart disease. Recently, two clinical assays have been carried out in Australia and in China (later referred to in the section 4, [51] and [52]).

3.6. Sonic hedgehog (Shh)

Shh is a lipoprotein that belongs to the Hedgehog (Hh) family of morphogens. The Hh gene was discovered in a developmental study in Drosophila melanogaster [53], with three Hh homologues in vertebrates later being indentified: Desert (Dhh), Indian (Ihh) and Sonic Hedgehog (Shh) [54-56]. Among these, Shh shows the most widespread expression in embryo and in adult tissues with many important functions in the organism, including a crucial role during heart vasculature development (extensively reviewed in [57]) and tissue homeostasis, acting in repair processes after severe injury (tissue regeneration, tissue injury, ischemia and hypoxia, inflammation, etc.) (reviewed in [58]).

Shh is synthesized in the cytoplasm as a precursor protein which undergoes autocleavage and lipidation resulting in the active Shh form (ShhN, about 20 kDa), consisting of the N-terminal signaling domain (Shh-N) with a cholesterol moiety at the carboxy-terminal and palmitoylation at the N terminus. These lipidic modifications of Shh take account of its distribution from the producing cell and it is thought to be involved in several mechanisms affecting the extent of the signaling [59,60]. ShhN could

thereby act either in long-range or in a short-range signaling (by cell-cell contact) resulting in paracrine or autocrine responses. During development, Shh acts mainly as a morphogen by long-range signaling, but in adult tissues the short range signal is most important during repair (reviewed in [61]).

The Shh protein activates several signaling pathways, a canonical one that acts through the Patched receptor that leads to activation and nuclear translocation of Gli transcription factors, which will drive the transcription of several angiogenic genes among others (reviewed in [58]), and a recently described "non-canonical" signaling cascade, which is transcription/translation-independent, and which activates leukotriene metabolism leading to reorganization in the cytoesqueleton to drive the migration towards the Shh-N source [62,63].

Despite its complexity, some investigations in mice have elucidated the critical role of Shh signaling in the maintenance of adult coronary vasculature by promoting angiogenesis and cell survival [64]. Also, during myocardial repair after ischemia, Shh-N seems to be delivered by fibroblasts and acts on endothelium, VSMCs and cardiomyocytes. Like other angiogenic factors, it has been recently shown that hypoxia can trigger HIF-1α-mediated Shh expression, within as little as 1 hour [65], inducing vascular remodelling by nitric oxide (NO) production in ECs [66,67], upregulation of anti-apoptotic molecules in cardiomyocytes [68], release of angiogenic factors (VEGF and Angiopoietins) by cardiac fibroblasts [69] and recruitment of bone marrow derived-EPCs [68]. Regarding the therapeutic potential, Shh protein or gene delivery approaches have shown angiogenesis induction in myocardial ischemia models both in mice and rats [69,68,70]. Also, Shh has been shown to be a critical mediator of erythropoietin-induced cardiac protection [71]. However, the role of endogenous Shh-N is controversial as some data indicate the Hh signal can contribute to injury during myocardial ischemia [72].

4. CLINICAL TRIALS WITH PROTEIN THERAPY

Protein-based therapy has been explored in clinical settings for the promotion of angio- and arteriogenesis in the ischemic myocardium by delivering angiogenic growth factors. The clinical studies with recombinant proteins performed in patients suffering from IHD are listed in Table 2. In most of the trials, patients presented severe coronary artery disease, which could not be treated adequately with conventional revascularization therapies.

The first phase-I clinical trial was performed in 20 patients with three vessel disease [73]. In this study, FGF-1 was intramyocardially injected in patients undergoing coronary artery bypass of the left anterior descending coronary artery (LAD). In this study, safety was proven but, despite an increased capillary density, no evidence of coronary perfusion or ventricular function improvement was determined.

Also, parenteral administration of FGF-2 in humans was first tested in a small placebo-controlled, dose-escalation safety study performed in 25 patients with coronary artery disease and stable angina. In this study, 17 patients received intracoronary infusion of recombinant FGF-2 and 8 patients, placebo infusion. Few side-effects such as mild hypotension, slight transient trombocytopenia and proteinuria were registered but without further complications [74]. In another study, intracoronary infusions of FGF-2 were also well tolerated in another study with 52 patients. In this case, patients were sub-optimal candidates for conventional revascularization. At the two-month follow-up, the patients presented fewer angina symptoms, improved exercise capacity and reduced ischemic territory. Dose-related hypotension was detected and four deaths and four major cardiac events occurred but did not appear to be related to dose or time of administration [75]. Taken together, the results of all phase I studies using FGF-2 suggested that intracoronary delivery of this growth factor was reasonably safe and may produce functionally significant clinical benefits. Next, a multi-center, randomized, double-bind, placebo-controlled phase-II trial (FIRST) with a single intracoronary infusion of recombinant FGF-2 at different doses (0.3, 3 and 30 μg/kg) was performed, but the results were disappointing. Although a significant reduction in clinical angina was detected in the 3 μg/kg group, no significant effect was detected at 180 days in any of the treated groups. In addition, single intracoronary infusion of FGF-2 did not improve exercise tolerance or myocardial perfusion [76].

On the other hand, the results of small phase I trials using intracoronary and intravenous infusions of VEGF-A in patients with coronary artery disease have been encouraging [77-79]. For example, Hendel et al. reported a significant improvement in exercise capacity without any safety issues. Also, the resting nuclear myocardial perfusion scans indicated a VEGF-A treatment effect [78]. However, a randomized, double-blind, placebo-controlled phase II trial of VEGF-A also failed to show differences between the treatment and placebo groups [80]. Another study, The VIVA, compared two doses of VEGF-A to placebo in 178 patients with coronary artery disease. A single intracoronary infusion followed by three separate intravenous infusions was given. Despite the safety and tolerability, the administration regimes revealed that VEGF-A offered no improvement beyond placebo by day 60, although high-dose VEGF-A resulted in better improvement in angina and favorable trends in exercise treadmill test time and angina

frequency, by day 120. Perhaps the most striking contribution of the VIVA trial was to consider that more preclinical data were needed with regard to the time course of angiogenesis and the optimal dose and route of administration to induce effective VEGF-A therapy in the myocardium.

In addition to studies using VEGF-A and FGF proteins, other growth factors known to have a role in tissue repair and angiogenesis have been tested in myocardial clinical settings, including colony granulocyte stimulating factor (C-GSF)[81-84], hepatocyte growth factor (HGF) [85], erythropoietin (EPO) [86,87] and neuregulin. Regarding the latter, two human studies aimed at exploring the safety and efficacy of recombinant NRG-1 in chronic heart failure (CHF) have been recently performed. Jabbour et al. reported sustained haemodynamic effects, as demonstrated by the 12% increase in left ventricle ejection fraction (LVEF) at 12 weeks in patients treated with daily infusion of NRG-1 for 11 days [51]. The Chinese Phase II clinical trial using a short-term administration of rhNRG-1 in CHF patients could result in sustained improvement of cardiac pumping and ventricular anti remodeling compared with baseline, although these changes were not statistically significant between NRG-1 and the placebo groups [52].

In general, although the therapy was safe and well tolerated, statistically significant efficacy was not consistently demonstrated in the clinical trials involving angiogenic growth factors. However, as part of intensive research on protein-based therapy for cardiac repair, further clinical studies are now in progress in patients with coronary artery disease. A new FGF-1 delivery technique is being performed by means of the Myostar® catheter (Cordis Corp., J&J company) in the CardioVascular BioTherapeutics phase II clinical trial (ClinicalTrials.gov Identifier: NCT00117936). Another ongoing phase II study involves the parenteral administration of EPO to evaluate the effect of this growth factor on damage to the heart in patients with acute heart attacks (ClinicalTrials.gov Identifier: NCT00378352).

As a conclusion to these studies, the results of myocardial clinical trials using protein delivery have generally been disappointing and the studies have failed to consistently demonstrate improvements in treated patients as compared with placebo. Many of these trials relied on an intravenous infusion or intracoronary delivery of the recombinant protein. Therefore these negative results have been attributed, at least partially, to the short lived effect and high instability of the protein when injected as a bolus. For example, from pharmacokinetic data collected from the FGF-1 studies in the human heart, it appears that FGF-1, once it exits the heart, is cleared from the circulation in less than three hours [88]. Intravenous administration of VEGF-A is limited by its short *in vivo* half life (~30 min) and overall dose is limited by off-target site toxicity issues [80,89]. In the case of myocardial ischemia, the amount of VEGF-A localized in the ischemic region after systemic administration is minimal and does not persist for more than 1 day [90]. Indeed, the short permanence in the heart of the administered proteins after intracoronary delivery might be an important cause for the missing clinical effect [91].

Local and sustained combined growth factor delivery by controlled release approaches in the heart tissue might be a better strategy to achieve higher efficacy in protein-based therapy for myocardial ischemia. However, many issues remain to be established, such as protein formulation, stability, dosage, routes and safety.

5. CHALLENGES IN PROANGIOGENIC FACTOR DELIVERY

Although protein growth factors that play essential roles in angiogenesis and arteriogenesis have been deeply studied, the suitable manner for making these cytokines available at the target site with a desired dosage and for a determined period of time remains unclear. Also, the ability to efficiently incorporate and release multiple angiogenic factors that mimic the natural microenvironment of the tissue needs to be determined.

5.1. Growth factor dosage and routes of administration

The limited success of the protein-based angiogenic therapy may be related partially to the way of growth factor delivery. As has been shown previously, several delivery routes have been tested in patients including intravenous, intracoronary, intramyocardial and perivascular administration (Fig. 3). Intravenous infusions are appealing because of their practicality, but have a minimal effect in producing angiogenesis [75]. Intracoronary delivery is easily performed with catheter-based techniques but may lead to low protein deposition into the myocardium. Detailed analysis of FGF-2 uptake and retention one hour after its injection showed that only 0.9% and 0.26% of the injected FGF-2 was found in the ischemic myocardium after intracoronary and intravenous administration, respectively. Still, only very low levels of the protein remained in the myocardium 24 hours later (0.05% for intracoronary and 0.04% for intravenous delivery) [93]. Also, intrapericardial administration cannot be used in post-cardiac surgery patients. Therefore, site-specific methods such as intramyocardial delivery are preferred since it includes

the possibility of targeting the desired areas of the myocardium, and has a higher delivery efficiency and prolonged tissue retention. Growth factors can be injected intramyocardially into the border zone of the infarct or the centre of the ischemic area. Alternatively, proteins can be intramyocardially targeted by endocardial injection with a specialized intraventricular catheter. Yet, epicardial zones can be targeted via thoracoscopy without the need for open-chest surgery.

The protein amount retained by the target tissue may be considered to establish a suitable dosage. Previously, the range of effective concentrations used for *in vitro* studies acted as an important guidance. Also, tissue condition (perfused or non-perfused areas) and route of administration may act as critical factors to determine protein concentration at the myocardium [94,95]. Therefore, protein threshold dosage may be established based on previous *in vitro* assays and tissue distribution studies.

5.2. Protein stability

Like protein-based compounds, growth factor molecules are not conventional drugs. A critical issue in protein formulation is the retention of biological activity, as well as the preservation of biological function at pharmacological concentrations for therapeutic effect. Safe, effective and reliable protein formulation requires an in-depth understanding of the properties of the protein, particularly its susceptibility to either chemical or physical instability. During pre-formulation research, protein stability should be assessed using a complementary set of well-established analytical techniques such as SDS-PAGE, circular dichroism, fluorescence, FTIR, dynamic light scattering, size exclusion chromatography, differential scanning calorimetry, etc. [96].

Since protein and peptide drugs are highly susceptible to proteolysis or rapidly cleared from the circulation or from the target site, it has been necessary to control the protein drug delivery. Thus, a critical step is to develop delivery platforms able to protect and release therapeutic proteins effectively. Recent years have witnessed significant progress for improvement and innovation in nano- and microparticles, hydrogels and scaffold manufacture, in order to deliver delicate macromolecules. Indeed, incorporation of therapeutic proteins into polymer devices has been a suitable strategy to protect these special drugs by adding excipients such as buffers, stabilizing sugars and amino acids, surfactants and protein carriers like albumin. These substances are useful in helping to prevent protein adsorption to surfaces, interfacial denaturation and aggregation [97,98].

5.3. Safe angiogenesis

Therapeutic angiogenesis is not free from potential harmful effects. Despite the critical role of different growth factors in the physiological angiogenesis and survival of endothelial cells, there is considerable evidence that some cytokines are important tumor angiogenic factors [99,100]. In general, high doses of recombinant proteins or prolonged exposure to the proteins may cause various side effects including tumor growth, but also hypotension, edema, proteinuria, hemorrhage, diabetic retinopathy, plaque rupture, and angioma formation. Thus, for example, unexpected side effects of FGF-2 therapy have been reported, indicating that protein dosage must be carefully monitored [101]. Careful control of proangiogenic molecules both in dosage and in localization is important to improve the local therapeutic efficiency of the protein and avoid unwanted side effects. Some of the toxic effects have been confirmed in animal models, but the limited results from clinical settings seem to refute some of the aforementioned risks or only show mild and transient effects. A larger number of clinical trials need to be conducted to clarify the possible undesired side effects.

6. CONTROLLED GROWTH FACTOR DELIVERY SYSTEMS

Regarding the issues mentioned above, multiple efforts have been made to overcome these limitations. In general, controlled drug delivery systems have many advantages over bolus or repetitive administration. Patient compliance, drug protection and sustained release are some of the many benefits of incorporating and releasing a therapeutic molecule from an adequate matrix (such as hydrogels, particles, scaffolds, capsules, etc.). Controlled release strategies have demonstrated the importance of maintaining precise concentrations of active GFs over days or weeks and orchestrating the timing of GF release proximal to the site of desired angiogenesis. Also, the matrix may emulate the highly functionalized role of ECM in modulating the stability, activity, release, and spatial localization of GFs [102].

6.1. Polymer-based growth factor delivery systems

Polymers can serve as a matrix for controlled drug delivery as some properties can be modified by changing the monomers ratio and composition, controlling polymerization conditions, or introducing functional groups to the polymers [103]. A number of approaches have been reported on the protein controlled release from polymeric matrices, such as nano- and microparticles, hydrogels, polymer scaffolds and other delivery devices by using natural and synthetic materials. Table 3 summarizes potential and currently used materials in which GFs can be incorporated to stimulate angiogenesis. Important approaches based on targeted GF delivery systems for cardiac repair in animal models of myocardial ischemia are also showed (Table 4).

6.1.1) Hydrogels

Hydrogels are defined as three-dimensional polymer networks swollen by aqueous solvent, which is the major component of the gel system [150]. These systems may comprise an especially appealing class of delivery vehicle, as they can be introduced into the body with minimally invasive procedures and are often highly biocompatible, owing to their high water content [151,119]. However, the localized and sustained release of GFs from conventional hydrogels is difficult because it depends on the cross-linking density and/or the degradation properties of the hydrogels. Consequently, initial burst release and deactivation of the released GFs are generally observed [118,152]. Currently, research efforts are focused on the development of novel approaches that can control the release rate of GFs from carrier gels without changes in the physical and mechanical properties of the hydrogels.

Hydrogels of natural polymers have been used for delivering angiogenic cytokines. Collagen and its derivatives have commonly been used to deliver GFs by hydrogels. Gelatin is a denatured form of collagen that can be isolated from either bovine or porcine skin or bone by the partial hydrolysis of collagen [153]. Intramyocardial administration of FGF-2 loaded gelatine hydrogels induced functionally significant angiogenesis and improved left ventricular function in infarcted myocardium of rats [142] and pigs [154]. Gelatin hydrogels were also used to incorporate other GFs such as angiopoietin-1 [155] and erythropoietin (EPO) for cardiac repair. Regarding the latter, the application of gelatine hydrogel sheets containing EPO reversed left ventricular (LV) remodeling and improved LV function without inducing polycythemia in rat [156] and rabbit [157] chronic myocardial infarct models. These studies demonstrated that post-MI treatment with an EPO-gelatine hydrogel improves LV remodelling and function by activating pro-survival signaling, anti-fibrosis, and angiogenesis without causing any side effect.

Fibrin is one of the major constituents of blood clots, which forms an immediate response to tissue injury, and therefore serves as a natural provisional platform for new cellular ingrowth. Because fibrin lyses slowly and locally, it has been used as a reservoir for GFs. In spite of some positive results with FGF or VEGF-A proteins in fibrin glue, the release kinetics of such preparations are indicative of an uncontrolled burst [120]. On the other hand, the addition of heparin to a fibrin gel has been useful for the sustained release and enhanced activity of angiogenic factors [158].

Angiogenic response was also detected when hyaluronic acid (HA) gels containing both VEGF-A and keratinocyte growth factor (KGF) were subcutaneously implanted into mice [112]. Regarding the myocardial injection of new biomaterials, a HA-based hydrogel was applied into the epicardium of the infarcted area of rats, resulting in a significantly decreased infarct size and apoptotic index [159]. In addition, HA hydrogels with tunable mechanics and gelation behavior have been investigated as a therapeutic material for cardiac repair in an ovine MI model [160].

Alginate-based hydrogels have been used as a localized delivery platform of angiogenic proteins. However, poor bio-resorbability has been reported as a disadvantage [103]. The VEGF bioavailability provided by an injectable alginate hydrogel led to a significant angiogenic response in ischemic hindlimbs [119]. Alginate hydrogels can also be tuned with other natural polymers such as chitosan and dextran becoming temperature/pH sensitive gels [161-163]. Such gels incorporating VEGF-A were stable and protein was released continuously, even after a month, without any initial burst release [118]. Injection of FGF-2 in a temperature-responsive chitosan hydrogel was performed in rat [124] and rabbit [123] models of myocardial infarction resulting in positive cardiac repair.

Poly(ethylene glycol) (PEG), also known as poly(oxyethylene) or poly(ethylene oxide) (PEO), depending on its molecular weight, is one representative material which has been used to prepare synthetic polymer-based hydrogels loaded with angiogenic cytokines. Materials with Mw <100,000 are usually called PEGs, while higher molecular weight polymers are classified as PEOs. Several copolymers of PEG have been developed, such as 2-hydroxyethyl methacrylate, 1-vinyl-2-pyrrolidinone, and polyethylene glycol acrylate (HEMA–VP–PEG). This PEG-based hydrogel was examined as a matrix for the dual release of dexamethasone (DX) and VEGF. In this study, concurrent release of VEGF and DX was determined to be best from either VEGF/DX-loaded hydrogels or VEGF-loaded hydrogels with

embedded PLGA microspheres containing DX [164]. In order to mimic the natural endogenous modulation in the release profile of angiogenic factors, heparin-conjugated polymers have been used in the formulation of hydrogels. Triblock copolymer of PEO and poly(propylene oxide) (PEO-b-PPO-b-PEO, commercially available as Pluronic or Poloxamer) has been used to incorporate FGF-2 into biodegradable Pluronic/heparin composite hydrogels, which induced proliferation of human umbilical vein endothelial cell (HUVEC) in addition to significant neovascularization when implanted into subcutaneous pockets in the dorsal side of Sprague-Dawley rats [165]. Moreover, Yamaguchi et al. reported the assembly, rheological properties, and targeted delivery/erosion profiles of non-covalently associated hydrogel networks produced via the interaction of a low-molecular weight heparin-modified star polymer (PEG-LMWH) and VEGF. The cytokine released from these hydrogels increased proliferation of VEGF-responsive cell lines, suggesting a novel potential mechanism for targeted delivery and erosion via the release of therapeutically important protein cross-links in response to cell surface receptors [134]. In another strategy, VEGF was chemically coupled to PEG peptide hydrogel matrices to induce local angiogenesis by cross-linking matrix metalloproteinase (MMP) substrate peptides, providing retention of the factor in the matrix until its local release, triggered by active MMPs. Thus, the VEGF integrated to PEG peptide hydrogels could behave similarly to those in the natural ECM. When subcutaneously implanted in rats, these VEGF containing matrices, were remodeled into native and vascularised tissue [135]. In another elegant strategy, Wang et al. injected EPO into the rat infarcted myocardium using a supramolecular hydrogel self-assembled between alpha-cyclodextrin and metoxy polyethylene glycol-poly (caprolactone)-(dodecanedioic acid)-poly(caprolactone)-metoxy polyethylene glycol (MPEG-PCL-MPEG) triblock polymer (α-cyclodextrin/MPEG-PCL-MPEG). This hydrogel allowed a sustained release of EPO, which inhibited cell apoptosis and increased neovasculature formation, and subsequently reduced infarct size and improved cardiac function without evidence of polycythaemia [149]. Other synthetic materials used to prepare protein-loaded hydrogels are listed in Table 3.

6.1.2) Polymer scaffolds

Scaffolds are tridimensional matrices with a network architecture, useful to incorporate and release therapeutic proteins. Studies directed towards stimulating vascularization of implanted scaffolds have extensively explored polymeric matrices suitable for the sustained delivery of VEGF [166,167,129,168]. GFs such as VEGF may be incorporated into scaffolds by two approaches. First, lyophilized VEGF is mixed with polymer particles before processing the polymer into a porous scaffold, resulting in a rapid release (days to weeks in duration) of VEGF. The second approach involves pre-encapsulating the factor into polymer microspheres, and then fabricating scaffolds from these particles [169-171,129]. The mechanism of VEGF incorporation into polymer scaffolds can determine the exposure duration and tissue distribution of the protein and, as a consequence, dictate the success of VEGF in therapeutic angiogenesis using scaffold platforms for its delivery. According to previous studies by Ennett et al., VEGF was positioned predominantly adjacent to scaffold pores when incorporated directly, and was rapidly released (40–60% in 5 days). After a small incision on the dorsal side of the rodent, scaffold was subcutaneously implanted into the pocket. On the other hand, pre-encapsulation led to the VEGF being more deeply embedded and resulted in a delayed release [129]. In fact, polymer scaffolds can act to confine microparticles at the defect site and can help maintain structural integrity during healing in addition to being biodegradable and biocompatible [172,106].

Composite scaffolds, constituted by a synthetic biocompatible material, a poly(ether)urethane-polydimethylsiloxane blend, and a biological polymer, the fibrin, were also used to incorporate VEGF and FGF-2. The biological activity of the released GFs was maintained as demonstrated by HUVEC proliferation [173].

Many of the polymer delivery modules used to stimulate vessel ingrowth into scaffolds are able to deliver VEGF for periods greater than 2 weeks, but a disadvantage of these systems is the inability to determine whether VEGF release is complete. To date it has been unequivocally demonstrated that delivery of VEGF from a biopolymer, increases vessel ingrowth into porous scaffolds in a rat model of angiogenesis (implanted in the dorsal paramedian region of the skin), although the long-term stability of the induced neovascularization within scaffolds after VEGF withdrawal has not been intensively investigated yet [167].

6.1.3) Nano- and microparticles

Reports have shown that GFs can promote localized angiogenesis *in vivo* if administered in a nano- or microparticulate depot [174,90,114,127]. Polymeric nano- and microparticles are illustrated in Fig.4.

These particulate delivery systems are considered potential tools to overcome the limitations of intravenous administration of therapeutic proteins. Poly(lactic-*co*-glycolic acid) (PLGA) copolymer is an attractive material to prepare cytokine-loaded particles because of its excellent biocompatibility and high safety profile [175-179]. Most GF delivery strategies using PLGA particles for angiogenesis have been performed in hindlimb ischemia models resulting in an increased blood vessel formation [180-182]. Also, the effect of delivery of PLGA microparticles loaded with VEGF-$A_{165,}$ has been studied in a rat model of cardiac reperfusion–ischemia. An increase in angiogenesis and arteriogenesis was observed in animals treated with VEGF microparticles, besides a positive remodeling of the heart with a significantly greater LV wall thickness [130]. PLGA has been also used to encapsulate heat shock protein 27 (HSP27), which has protective effects in cardiac cells under hypoxic conditions and in ischemia/reperfusion animal models [183]. HSP27 fused with transcriptional activator (TAT) was encapsulated into PLGA particles and the microsphere/alginate hydrogel combination delivery systems maintained protein bioactivity and recovered the proliferation of cardiomyoblasts cultured under hypoxic conditions [184]. A blend of PLGA and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) was used to prepare HGF-loaded composite microspheres with a core-shell structure. This system provided a sustained delivery of HGF with maintained bioactivity for at least 40 days [185]. Other GFs such as EPO [186] and FGF-2 [187] have been encapsulated into PLGA microparticles. In a different approach, VEGF was co-lyophilized with trehalose and rat serum albumin in succinate buffer to yield < 45 μm particles. These microparticles were incorporated into low molecular weight poly(trimethylene carbonate) and induced significant blood vessel formation when injected subcutaneously into the dorsal area of Wistar rats [188]. Recently, d'Angelo et al. have developed a new injectable controlled release device based on polymeric nanoparticles for the delivery of PDGF-BB and FGF-2. Incubation of these nanoparticles with EC culture models confirmed that these GFs were released in a bioactive form [189]. In another study, Tang et al. developed heparin-functionalized chitosan (CS)/poly(γ-glutamic acid) (γ-PGA) nanoparticles (HP-CS/γ-PGA nanoparticles) for multi-functional delivery of FGF-2 and heparin. Sustained release of FGF-2 from the nanoparticles enhanced the proliferation of human foreskin fibroblast cells (HFF) and angiogenic tube formation by HUVECs, suggesting the retaining of bFGF mitogenic activity [190]. Recently, a hyaluronic acid/chitosan polymer combination was also designed to prepare nanoparticles as delivery vehicles for VEGF and PDGF-BB, resulting in entrapment efficiencies of 94% and 54%, respectively [191].

PLGA microparticles have also been combined with other delivery systems in order to optimize the patterns of growth factor controlled release. Alginate gel/PLGA microsphere combination system containing VEGF enhanced the angiogenic response after hind limb ischemia in rats [192] and mice [193]. This combination system also allowed a dual delivery strategy and improved the effects of single factors. Also, sequential release of VEGF and PDGF from alginate hydrogels led to a higher density of α-actin positive in a rat model of myocardial infarction but sequential administration of both free proteins did not achieve this response [117].

A microsphere/scaffold combination strategy has been tested using a porous PLGA scaffold capable of multiple GF delivery. In this approach, mixing particulate polymer and one factor with microspheres containing a pre-encapsulated second factor resulted in dual GF delivery with a distinct release rate for each factor [194,169]. Recently, Saif et al. reported the development of injectable PLGA-based scaffolds releasing single factors or combinations of VEGF, HGF, and angiopoietin-1 with and without concomitant infusion of cord blood–derived vascular progenitors. Dual and triple combinations of scaffold-released GFs were superior to single release. Moreover, combined use of scaffold released GFs and cell therapy improved neovascularization in murine hindlimb ischemia models [195]. As other approach, gelatine microparticles incorporated within the porous network of a scaffold made of poly (propylene fumarate) has been evaluated as a delivery system for the controlled release of VEGF. Although marked burst release was observed, the relative amount of VEGF associated with gelatine achieved an equilibrium value with no strong dependence on its dose. These *in vivo* and *in vitro* release kinetics were characteristic of the specific GF due to the effects of VEGF size, charge, and conformation on its complexation with gelatine [106].

In an elegant strategy, Chung et al. developed a heparin-functionalized nanoparticle–fibrin gel complex containing VEGF, which increased angiographic score and collateral density in a rabbit model of hind limb ischemia [196].

6.2. Lipid-based growth factor delivery systems

Liposomes, solid lipid particles (SLN), and lipid nanocapsules (LNC) are different configurations of lipid-based nanoparticles (reviewed in Zhang and Uludağ 2009), as illustrated in Fig.4. Despite the numerous approaches involving lipid-based formulations for protein delivery [197,198], there are few reports dedicated to these systems as angiogenic proteins carriers for cardiac repair. On the other hand,

the accumulation of liposomes in the areas of experimental myocardial infarction has been demonstrated [199-201]. Scott et al. developed anti-P-selectin-conjugated liposomes for targeted delivery of VEGF to the rat infarcted myocardium, resulting in significant increase in fractional shortening and improved systolic function [145]. In order to face the drawbacks of liposomes regarding clinical applications, particularly their instability and their interaction with high-density lipoproteins in blood, the design and characterization of polymer-supported liposomal systems have been described [202,203,148]. In this context, Oh et al. reported the formation of a temperature-induced gel composed of core/shell nanoparticles for regeneration of ischemic heart. The core was composed of lecithin containing VEGF and the shell was composed of Pluronic-127 (poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer. The inducement of the gel formation took place when Capryol 90 (propylene glycol monocaprylate) was added to an aqueous solution of the core/shell nanoparticles at body temperature. Although a minimum difference in neovascularization was observed between the core/shell nanoparticles and their gel, a comparable improvement in the recovery of heart function was observed with the gel system when applied to a myocardial infarction model in rats [148].

6.3. Other devices for growth factor delivery

Although the GF delivery systems described above are the most versatile and most intensively studied ones, a few other devices have also been utilized for GF delivery. For example, polymeric micelles, dendrimers and inorganic nanoparticles have been tested as delivery platforms. Micellar formulations have been used primarily for antitumor drug delivery in clinical or preclinical trials [204], but they are beginning to be explored for GF delivery. Lee et al. reported FGF-2 entrapment in heparin-conjugated Tetronic®- Poly(ε-caprolactone) polymeric micelles as an injectable vehicle for FGF-2 delivery [205]. Mesoporous silica nanoparticles (MSNs) have attracted attention for their unique structure features, including large surface areas, tunable pore sizes (2–10 nm in diameter), and well-defined surface properties [206]. In addition, MSNs have been approved by the FDA as a new biocompatible material. In a novel strategy, Zhang et al. developed an acid-modified water-in-oil microemulsion to encapsulate FGF-2 within MSNs *in situ.* As a result, high loading efficiency of FGF-2 into MSNs was achieved (around 70%) and the cytotoxicity test indicated that the MSNs are not toxic [207]. Recently, VEGF was conjugated to the surface of gold nanoparticles and this novel approach was studied in a murine ischemic hindlimb model. A 1.7-fold increase in blood perfusion besides increased capillary density was achieved after IV injection of VEGF-conjugated gold nanoparticles via the enhanced permeability and retention (EPR) effect [208]. A new externally-regulated delivery system was developed to explore sequential release of VEGF and sphingosine 1-phosphate (S1P), a GF that stimulates vascular stability. In this strategy, hollow cellulose acetate fibers promoted sequential delivery of factors and cellular recruitment and functional angiogenesis in a murine Matrigel plug model [209].

6.4. Concluding remarks

The different growth-factor delivery systems listed above constitute an important result of intensive efforts to overcome limitations of protein-based therapy for therapeutic angiogenesis. The clearest drawback of GF therapy is the need to maintain bioactivity and therapeutic concentration to induce the desired effect within the required timing. Establishing the protein threshold concentration and its local exposure duration remains to be determined and represents the paramount challenge. The protein stability and pharmacokinetic issues may be solved or attenuated by incorporation of GF in natural or synthetic delivery matrices. However, on the basis of the pre-clinical studies, it is not yet possible to identify the better platform to deliver one or multiple GFs for cardiac repair. Some aspects such as material biocompatibility, protein stability and scale-up may be considered. However, substantial differences between animal models and humans further complicate the scenario. Over the past several years, many growth-factor delivery strategies have been tested in pre-clinical studies. However, little information about clinical settings using protein delivery systems is available. Controlled release of FGF-2 encapsulated in heparin-alginate pellets led to significant angiogenesis with low systemic effects in patients undergoing bypass surgery, but this approach did not alleviate operative risks [210]. Therefore, further clinical trials to evaluate the effects of treatment induced by controlled GF delivery methods may be necessary.

Even though the pathway to reach optimum protein therapy is not free of hindrances, intensive research in rational protein design technology and new biopolymers and nanomaterials for controlled release of proteins will enable significant progress in the efficacy and safety of known and new GFs applied to cardiac repair. Therefore, treatment of IHD with a single protein or, most likely, with a

combination of multiple proteins incorporated into delivery systems may become an effective therapy in the future.

7. FUTURE DIRECTIONS

More than a decade has passed since the first clinical trial employing an angiogenic treatment for IHD was carried out. Since the first clinical studies many questions have emerged. First of all, researchers agree on the need to explore in detail the mechanisms involved in the complex process of angiogenesis. Refined techniques now being perfected such as microarray analysis, proteome and secretome profiling, as well as cell sorting and image analysis will play a major role in achieving this goal [211]. The improvement in our knowledge of the angiogenesis pathways will allow us to find new and better targets. But as our knowledge grows, the difficulty of integrating all the notions involved becomes more evident. To overcome this handicap, which is a result of the interrelation between different factors and pathways, it has been proposed that we should combine quantitative biological experiments and computational models. This systems biology approach can also deal with the individual variability inherent in the ischemic disease population [211].

Angiogenesis is a complex, multi-step process, and various factors are critical at each stage, which indicates that a more effective therapeutic angiogenesis could be achieved by employing multiple growth factor delivery. Once the target has been defined, the issue of what the best therapeutic approach is still remains unclear. However, in the literature there is a certain agreement on the promising role of protein therapy, combined with drug delivery systems. Nevertheless, several questions remain which require solutions before we can move from the bench to the bedside. As a critical starting point, producing and purifying proteins in a large scale manner is a difficult task, particularly as regards the requirements for clinical use, and the economic cost of these processes. The use of bioreactors or high throughput column isolation offers a possible solution [211].

To take a step forward, some authors propose combining both cellular therapies and protein delivery systems. In this case it is important to establish well defined protocols for obtaining and culturing the cells, as well as studying the optimal number of cells to be administered and avoiding incompatibility concerns [212]. In any case, from the clinical trials conducted until now one of the main conclusions has been that it is essential to find the suitable moment to treat the patient, considering the physiological process that follows IHD, and how it might affect the treatment.

Another important issue is the question of evaluating the progression of angiogenic therapy in the heart. A fast growing field in angiogenesis assessment is imaging technology (reviewed in [213]). The improvements accomplished in terms of sensitivity and specificity will result in a better understanding and explanation for the findings of the clinical trials, which are often contradictory. Cardiac magnetic resonance appears to be a very suitable test to assess spatial and temporal changes after angiogenesis therapy. Also, when combined with complementary techniques, this method can provide essential information about physiology, morphology and metabolism [214].

A great effort to obtain beneficial effects in patients as a result of therapeutic angiogenesis has been made over the last 30 years. The number of research areas working in unison to achieve this goal increases as the knowledge does so, and hopefully it will continue this way, until we find the cure to ischemic heart disease. In this context, and despite all the handicaps mentioned above, in our opinion, the drug delivery systems employed to administer and control protein release appear to offer a promising strategy. For cardiac repair purposes, where revascularization and myocardium regeneration are lasting complex process, the encapsulation of GFs into polymeric microparticles shows crucial advantages. As it offers cytokine protection against physical, chemical or enzymatic degradation, it is possible to maintain therapeutic levels over a longer period of time, thus minimizing the dosage and reducing potential adverse effects. Gene or cell therapy shows some limitations including the lack of control over the dose. In contrast, polymeric microparticles make it possible to control the amount of protein administered. Moreover, it is possible to predict the levels of released protein and to alter them by modifications in the raw materials and structure of the particle, to mimic the proper environment in which the tissue can be regenerated. Another important fact is the need for multiple GFs to complete the angiogenic process. It is also possible to combine different GFs loaded into polymeric microparticles with different properties, achieving the most suitable release profile for each GF. In addition, the particle size can be fitted to cardiac administration and modifications in the microparticle surface (such as pegylation) can avoid macrophage uptake of microparticles in the injured heart tissue surrounding. Regarding the cost-effectiveness of the industrial production, various advantages can also be listed: high availability of polymers (including low cost ones), feasible preparation methods, possibility of scaling-up the process, etc. In conclusion, polymeric microparticles seem to be adequate to fulfil most of the requirements that the ideal delivery system must have (reviewed in [215]).

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Figure Captions

Fig. 1 The playground for therapeutic angiogenesis: A) When a coronary occlusion happens, the oxygen local supply decreases dramatically and the tissue responds to hypoxia by inducing transcription of proangiogenic factors, cytokines and matrix metalloproteinases (MMPs). The myocardium attempts to restore oxygen supply and replace the damage tissue. However, often these adaptative responses are not effective and myocardium hypertrophy occurs. Thereafter, there is a permanent injury which would lead to heart failure. B) If a local controlled release of angiogenic factor/s such as FGFs (Fibroblast Growth Factors), VEGF-A (Vascular Endothelial Growth Factor-A), Ang (Angiopoietin), PDGF (Platelet-derived Growth Factor), etc. is carried out following heart injury, the endogenous process of angiogenesis and remodeling would be enhanced over time, allowing effective revascularization, and recovery of myocardial function could ultimately be achieved (EC, endothelial cell; ECM, extracellular matrix; EPC, endothelial progenitor cell)

Fig. 2 Consequences of the imbalance in the angiogenesis process

Fig. 3 Growth factor delivery to the myocardium. Proteins can be targeted to the myocardium by several routes and each one has both merits and drawbacks. Intravenous delivery is a practical strategy, but is not likely to produce functional angiogenesis in the target tissue; also, the downside includes systemic exposure to a growth factor and potential for unwanted effects such as hypotension. Intracoronary delivery can be performed using catheter-based techniques and may be effective when adequate doses are used, regarding the low protein deposition in the myocardium. Intramyocardial delivery may provide better myocardial distribution and retention than intracoronary and intravenous routes and, like perivascular delivery it can be performed either via open chest or via thoracoscopy.

Fig. 4 Schematic representation of different drug delivery approaches used as platforms to deliver GFs.