

Angiogenic growth factors in myocardial infarction: a critical appraisal

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Abstract In the recent past, substantial advances have been made in the treatment of myocardial infarction (MI). Despite the impact of these positive developments, MI remains to be a leading cause of morbidity as well as mortality. An interesting hypothesis is that the development of new blood vessels (angiogenesis) or the remodeling of preexisting collaterals may form natural bypasses that could compensate for the occlusion of an epicardial coronary artery. A number of angiogenic factors are proven to be elicited during MI. Exogenous supplementation of these growth factors either in the form of recombinant protein or gene would enhance the collateral vessel formation and thereby improve the outcome after MI. The aim of this review is to describe the nature and potentials of different angiogenic factors, their expression, their efficacy in animal studies, and clinical trials pertaining to MI.

Keywords Angiogenesis · Myocardial infarction · Angiogenic growth factors · Therapeutic angiogenesis · Clinical trials

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Introduction

Extensive research during the last three to four decades has made considerable advances in the diagnosis and management of cardiovascular diseases. Yet, it remains as the most common cause of morbidity and mortality in both the developed and developing nations. American Heart Association [1] reported that >2200 Americans die due to cardiovascular disorders each day, among which a major proportion was <65 years of age. Since three fourths of global deaths occur in low and middle income countries due to coronary heart disease, it poses to be a big disease as well as economic burden in the developing countries [2]. Hence, in light of the above-alarming statistics, research on producing potent therapeutics for use in cardiovascular diseases is getting more focused.

Myocardial infarction (MI) occurs when a coronary artery becomes occluded, resulting in insufficient oxygen supply to the downstream myocardium, thus providing a serious threat to tissue viability. Ischemia is responsible for cardiac muscle damage, including loss of cardiomyocytes [3]. Nature's response to the development of profound tissue ischemia includes the upregulation of angiogenic growth factors and mobilization of circulating cellular elements that together enable development of an accessory vasculature [4]. Angiogenesis refers to the process of formation of new blood vessels from preexisting vascular bed, and it is a tightly regulated process requiring the homeostatic balance of inducers and inhibitors. Angiogenic growth factors exert a fundamental role in blood vessel formation by aiding in various steps, viz., cell proliferation, migration, adhesion, etc. [5]. These growth factors are produced by various cell types and include a diverse range of proteins, viz., vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor

(HGF), platelet derived growth factor (PDGF), platelet derived endothelial cell growth factor (PDEC GF), insulin like growth factor-1 (IGF-1), transforming growth factor (TGF), angiopoietins (Ang), placental growth factor (PIGF), and several others [4]. A number of angiogenic growth factors are known to be expressed during MI. Angiogenesis induced by these growth factors would increase perfusion in the ischemic area and help to salvage the hibernating myocardium [6]. During recent years, a number of experimental studies have suggested that treatment with angiogenic growth factors can stimulate angiogenesis in the infarct myocardium [7–9]. Angiogenic growth factors have been shown to reduce infarct size in animal models of acute MI. This reduction in infarct size is associated with increased vascularity, suggesting that an angiogenic mechanism is operative in the reduction of infarct size [10].

Angiogenesis proves to be a promising strategy for increasing blood flow in patients with ischemic heart disease, especially for individuals who are not candidates for standard revascularization techniques [11]. Therapeutic angiogenesis in MI is an exciting new concept with significant clinical potential. This includes the direct delivery of growth factors into the ischemic target tissues or of genes that encode for synthesis of growth factors by target tissues [10, 11]. Both cell therapy and gene therapy have proved to be effective to promote neovascularization in animal models [12].

Though a vast repertoire of literature is available regarding the therapeutic potentials of various angiogenic growth factors, a comprehensive review on their expression in MI and results on the experimental as well as clinical studies are lacking, which forms the rationale of this review.

The need for exogenous angiogenic growth factors

Angiogenesis and vasculogenesis both develop in response to coronary obstructions and chronic myocardial ischemia in humans. But, this natural compensatory process is often inadequate or the time course is too slow when compared to the development of occlusion [13]. The production of angiogenic cytokine is inadequate or the response to them is attenuated [14]. Angiogenesis is impaired in older when compared to younger animals [15]. Patients with advanced coronary artery disease are often old and they have diabetes, hypercholesterolemia, or other undetermined characteristics that limit upregulation of angiogenic cytokines by ischemia but may nevertheless respond to administration of exogenous angiogenic cytokines. Also, individual differences in cytokine expression may constitute another basis for variations in collateral development [16]. In addition, endothelial dysfunction may reduce endothelial responsiveness to hypoxic stimuli or angiogenic growth factors [17] (Fig. 1).

Routes of administration

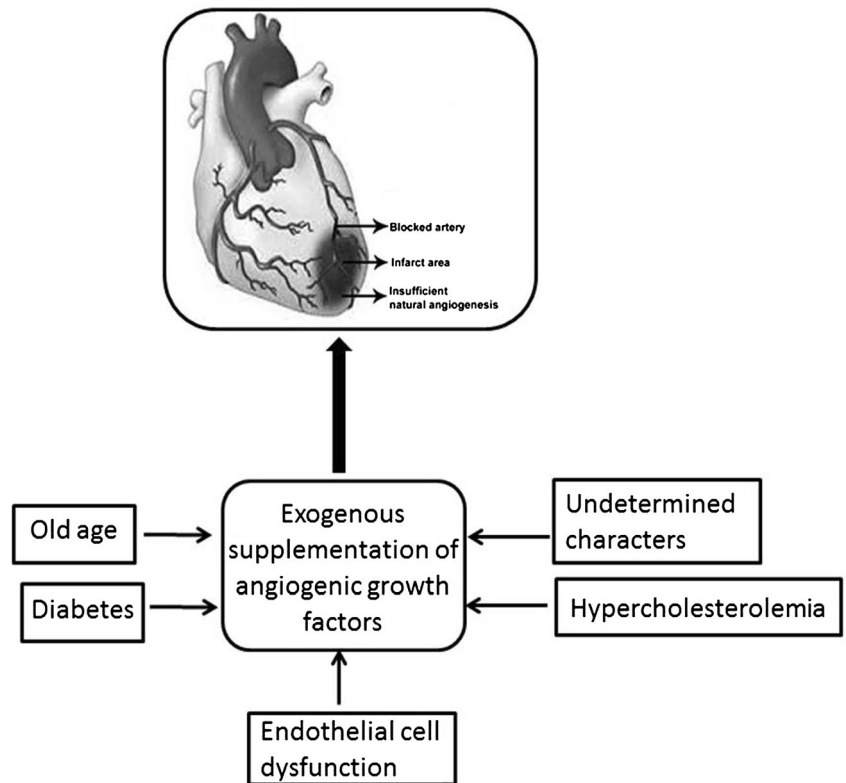
Angiogenic cytokines and genes have been administered by diverse routes (Fig. 2). Injection sites include intravenous, selective pulmonary artery, left atrium, intracoronary, selective intracoronary, transepicaldial intramyocardial or pericardial during bypass surgery or via thoracotomy, transendocardial intramyocardial by electromechanical catheter, and intrapericardial. Because local delivery of recombinant protein or gene is probably ideal, clinical trials have favored the intracoronary (protein or adenovirus) or intramyocardial (naked DNA or adenovirus) route. The transepicaldial route carries the attendant risks of surgery; however, these risks are not an issue if transepicaldial administration is performed as part of a coronary bypass procedure and they may be averted in the future by a catheter-based transendocardial approach [14]. Fujii et al. [18] have reported the successful delivery of VEGF and stem cell factor (SCF) genes into the infarct mice heart by a noninvasive ultrasound-targeted microbubble destruction method.

Vascular endothelial growth factor

VEGF, also known as vascular permeability factor, is a basic 45-kDa heparin-binding glycoprotein. It has been first described as a secreted mitogenic factor specific for endothelial cells in vitro and a proangiogenic molecule in vivo [19]. It is a central regulator of angiogenesis and vasculogenesis. VEGF induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis [20]. VEGF is secreted by intact cells, since the NH₂ terminus is preceded by a signal sequence [21]. Its high affinity binding sites are present on endothelial cells, and VEGF has no mitogenic effect on smooth muscle cells and fibroblasts [22]. This makes VEGF an excellent candidate for studies on endothelial disruption or myocardial ischemia, where rapid endothelialization and/or angiogenesis are desirable, while substances promoting smooth muscle or fibroblast growth may be counterproductive (Fig. 3).

Hypoxia is an important regulator of VEGF expression. Hypoxic regulation of VEGF gene expression is mediated by a family of hypoxia-inducible transcription factors (HIF), including hypoxia-inducible factor-1 (HIF-1)β, HIF-1α, and HIF-2α [23]. Five human VEGF mRNA species encoding VEGF isoforms of 121, 145, 165, 189, and 206 amino acids (VEGF_{121–206}) are produced by alternative splicing of the VEGF mRNA [24]. The VEGF isoforms bind to two tyrosine-kinase receptors, VEGFR-1 (*flt-1*) and VEGFR-2 (*KDR/flk-1*), which are expressed almost exclusively in endothelial cells. Endothelial cells express, in addition, the neuropilin-1 and neuropilin-2 coreceptors, which bind selectively to the 165 amino acid form of VEGF (VEGF₁₆₅) [20].

Fig. 1 The need for exogenous supplementation of angiogenic growth factors in MI



VEGF is upregulated in the myocardium remote to a myocardial infarct and is known to play a role in collateral growth. VEGF may also assist maturation of newly formed vessels by

recruiting smooth muscle cells. In ischemic myocardium, VEGF increases the number of muscular collateral vessels, presumably via stimulation of endothelial release of platelet

Fig. 2 Routes of administration of angiogenic growth factors in MI

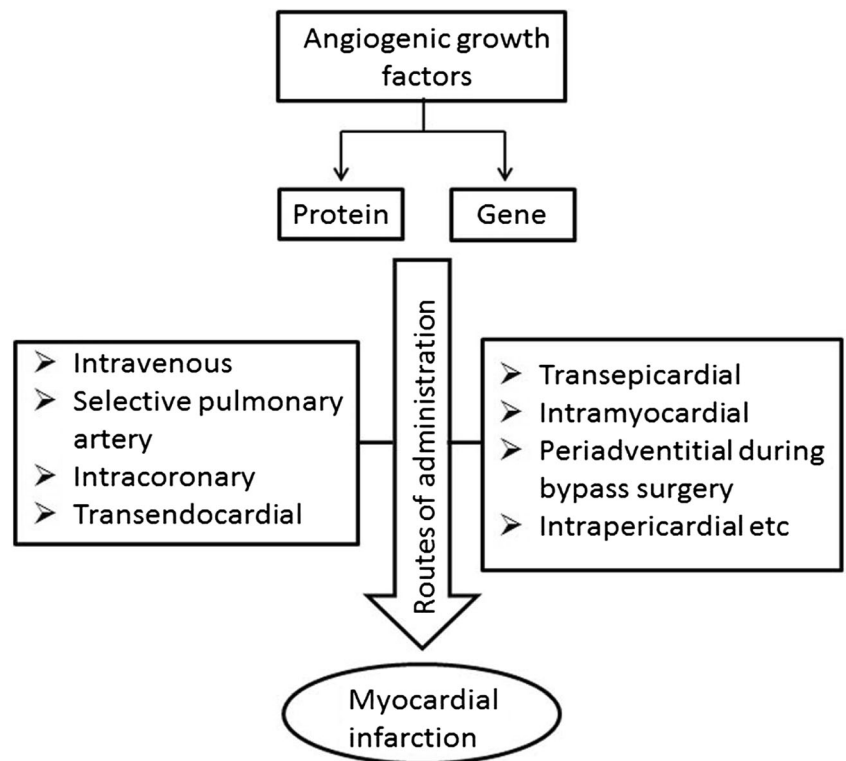
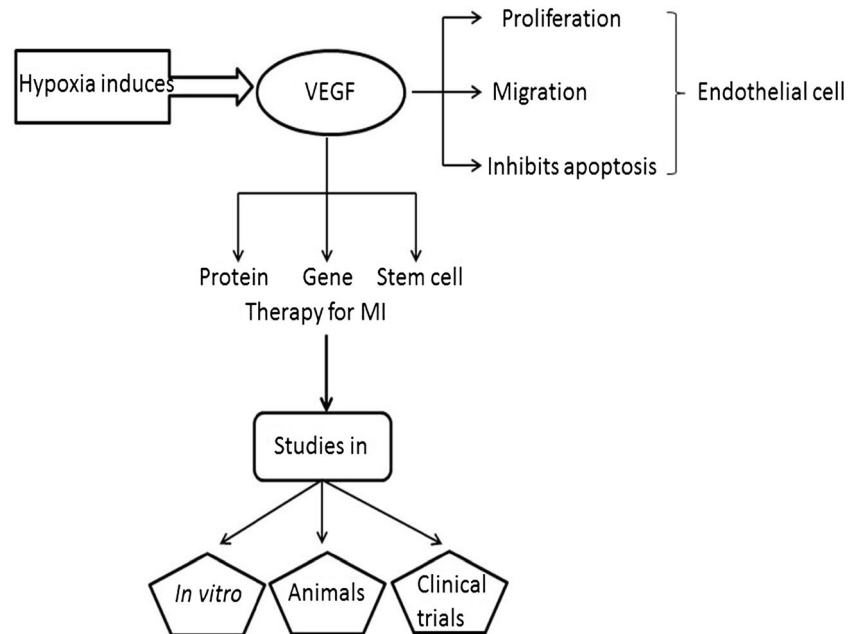


Fig. 3 VEGF in MI

derived endothelial growth factor B (PDGF-B), although a direct effect on mural cells is also possible. VEGF gene transfer or administration of recombinant VEGF to the heart results in improved filling of preformed collaterals as well as in the formation of new capillaries and collaterals [25].

Low doses of recombinant human VEGF, when administered intramyocardially, stimulate angiogenesis in the infarct myocardium [7]. Clinical trials demonstrate the enhancement of myocardial perfusion by VEGF gene transfer [26]. An important additional role of VEGF is the augmentation of circulating endothelial progenitor cells, which has been documented in mice and humans following VEGF gene transfer [27]. Korpisalo et al. [28] report the strategy of combining gene transfer of adenoviral (Ad) VEGF-A and PDGF-B into rabbit hind limb skeletal muscle, resulting in a prolonged angiogenic response. Intramyocardial injection of plasmid VEGF₁₆₅ in canine model of chronic MI has resulted in preservation of left ventricular ejection fraction [29]. Fujii et al. [18] have reported that a noninvasive method (ultrasound-targeted microbubble destruction) of delivery of VEGF and SCF genes to infarct mice myocardium increases vascular density and improves myocardial perfusion and ventricular function. Though VEGF is promising, the results have failed to translate into successful clinical trials in part due to the short half-life of VEGF in circulation. Increasing the dose of VEGF may increase its availability to the target tissue, but harmful side effects remain a concern [30]. Hence, Wang et al. [31] reported that encapsulating and selectively targeting VEGF to the MI border zone significantly improves vascularization and cardiac function after MI. Awada et al. [32] proposed that spatiotemporal

delivery of multiple growth factors involved in angiogenesis, closely mimicking the physiology, would prove to be a better treatment strategy for MI. Accordingly, intramyocardial delivery of VEGF and PDGF-BB improved angiogenesis, cardiac function, and ventricular wall thickness in rat model of MI. Target specificity is yet another limitation associated with VEGF delivery [33]. Yang et al. [34] have overcome this by modifying VEGF which gets localized to the ischemic myocardium by interacting with cardiac troponin I when administered intravenously. Alternatively, repeated low-dose administration of this modified VEGF also had profound benefits on the ischemic myocardium.

Three uncontrolled phase I studies on intravenous VEGF recombinant protein report a mild angina and improved exercise time and improvements in either nuclear or MRI perfusion or LV function [35]. Several phase I and phase II clinical trials have been carried out using VEGF recombinant protein or gene transfer in patients with coronary artery disease for therapeutic myocardial angiogenesis which show some evidence of improvement compared to baseline values or placebo (Tables 1 and 2).

Gao et al. [47] observe that the combined strategy of mesenchymal stem cell transplantation and VEGF gene therapy produce effective myogenesis and host-derived angiogenesis, resulting in the prevention of progressive heart dysfunction after MI. Hagikura et al. [48] have reported that the retrograde transplantation of peripheral blood mononuclear cells expressing VEGF gene efficiently induces angiogenesis and improves left ventricular function in infarct pig hearts. Mesenchymal stem cell-based repair strategies for acute MI are more effective in the presence of HGF or VEGF [49].

Table 1 Clinical trials using VEGF₁₆₅ recombinant protein

Route of administration	Trial phase	Therapeutic target	Follow-up	Outcome	Reference
Intracoronary	I	Angina, viable underperfused myocardium	30 and 60 days	Decreased angina	[36]
Intravenous	I	Angina, viable underperfused myocardium not amenable to revascularization	2 months	Improved SPECT, collaterals	[35]
Intracoronary plus 3 intravenous injections	II (VIVA multicenter study)	Angina, viable underperfused myocardium not amenable to revascularization	60 days, 120 days, 1 year	Trend toward decreased angina	[37]
Intracoronary with intravenous supplement	I (VIVA trial)	CAD not amenable to revascularization	2 months	Decreased angina episodes	[38]

CAD coronary artery disease, VIVA VEGF in ischemia for vascular angiogenesis, SPECT single-positron emission-computed tomography

Fibroblast growth factor

Acidic FGF (FGF-1)

FGF-1, also called acidic FGF, is a 140-amino acid peptide [50]. In its mature form, it is a 16-kDa polypeptide, and like other members of the fibroblast growth factor family, it has a wide spectrum of activity. It has potent mitogenic and chemotactic effects on a variety of cell types, including fibroblasts, endothelial cells, and smooth muscle cells [51]. Hypoxia has been shown to induce release of FGF-1 by macrophages [52].

Despite the apparent therapeutic potential of FGF-1, preliminary in vivo studies with this agent have not been encouraging [53]. FGF-1 and FGF-2 have a 55% amino acid homology [54].

Basic FGF (FGF-2)

Basic fibroblast growth factor (bFGF), also known as FGF-2, is an 18-kDa single chain polypeptide with extensive mitogenic capability. FGFs are produced by and can act directly upon vascular endothelial and smooth muscle cells and

Table 2 Clinical trials using VEGF gene transfer

Route of administration	Trial phase	Therapeutic target	Follow-up	Outcome	Reference
Plasmid, intramyocardial	I	CAD not amenable to revascularization	10 weeks	Improved results on rest and stress nuclear perfusion, increased function on electromechanical mapping	[26]
Plasmid, transendocardial with NOGA catheter	I/II	CAD not amenable to revascularization	12 weeks	Improved results on rest and stress nuclear perfusion	[39]
Plasmid human VEGF-2, intramyocardial via electromechanical catheter in LV	I	CAD not amenable to revascularization	1 year	Improved function on rest nuclear perfusion and electromechanical mapping	[40]
Plasmid human VEGF-2, intramyocardial via electromechanical catheter in LV	I/II multicenter	CAD not amenable to revascularization with type III–IV angina	3 months	Significant reduction in angina class, improvement in exercise time, improvement in perfusion of hypoperfused segments	[41]
Adenovirus VEGF ₁₂₁ , intramyocardial injection during CABG	I	CAD not amenable to revascularization	1 month	Decreased angina, 2 perioperative deaths, improvement in treadmill exercise	[42]
Adenovirus VEGF ₁₆₅ , intracoronary	II	CAD at time of PTCA	6 months	Decreased coronary restenosis, improved myocardial perfusion	[43]
Plasmid (VEGF ₁₆₅), intramyocardial	Euro inject One trial	Severe, stable angina. No option for CAD	3 months	Improved regional wall motion	[44]
Plasmid (VEGF ₁₆₅), intramyocardial-NOGA	II	CMI, NR	3 months	Though there was an improvement, perfusion abnormalities did not normalize	[45]
Plasmid (VEGF ₁₆₅), intramyocardial-NOGA	II	CMI, NR	6–12 months	Improved SPECT, wall motion score	[46]

NOGA is a novel strategy of catheter-based electromechanical assessment of myocardial perfusion (NOGA system, Biosense-Webster, J&J)

CAD coronary artery disease, CMI chronic myocardial ischemia, NR not candidate for revascularization, SPECT single-positron emission-computed tomography, CABG coronary artery bypass grafting, LV left ventricle, PTCA percutaneous transluminal coronary angioplasty

function as angiogenic factors [5]. The single copy gene for human FGF-2 has been localized on chromosome 4 [55]. Human FGF-2 is expressed in four forms: an 18-kDa (155-amino acid) form generated by initiation at the AUG codon and 22-, 22.5-, and 24-kDa forms (196, 201, and 210 amino acids) arising from the CUG codons located upstream of the conventional AUG start site [56].

One of the characteristic properties of fibroblast growth factors is their ability to bind to heparin, a glycosaminoglycan. This property allows them to be classified among the heparin-binding growth factors [57]. Yet, another characteristic feature of FGF is that they need a signal sequence to direct their secretion. Hence, they are not secreted in cultures and they are closely associated with extracellular matrix [58]. The fibroblast growth factor-receptor family consists of four membrane-spanning tyrosine kinases. Each of these receptors gives rise to multiple isoforms as a result of alternative splicing of their mRNAs [59]. Activation of the FGF receptors expressed on endothelial cells, smooth muscle cells, and myoblasts stimulates the proliferation of the respective cell types [60]. FGF-2 can be activated through physical stimuli, such as hypoxia, and through the activity of a variety of other growth factors [52]. Recent studies have suggested that VEGF and FGF-2 may have synergistic effects, both in vitro [61] and in vivo [62]. Since FGF-2 is one of the most potent mitogens and chemotactic factors for the vascular endothelial cell, it has also been considered the prime candidate for inducing angiogenesis in the ischemic heart [63]. Zhao et al. [64] have reported that the enhanced expression of acidic and basic fibroblast growth factors (FGF-1/FGF-2) and FGF receptors (FGFR) at early stages in the infarct rat heart coincides with the angiogenesis, suggesting their involvement in regulating cardiac angiogenesis and repair.

In addition to its effects on angiogenesis and wound healing, bFGF has been shown to be a moderately potent NO-dependent vasodilator, and it has been reported to have direct neuroprotective and myoprotective properties [65].

Therapeutic efficacy of bFGF in MI has been proved by many studies (66–71). Nakajima et al. [66] report that there is an improvement in left ventricular function and increased regional blood flow in the peri-infarct area of rat hearts, when bFGF is subepicardially injected in a controlled manner from biodegradable gelatin hydrogel. Recombinant human bFGF, when administered intramyocardially, stimulates angiogenesis, salvages the myocardium in the border zone, and improves ventricular function after MI in rabbits [7], dogs [67], etc. Garbern et al. [68] have shown that there is a sustained, local delivery of bFGF if it is delivered along with a pH- and temperature-responsive injectable hydrogel. This sustained local delivery improves angiogenesis and cardiac function in a rat model of MI. Liu et al. [69] have developed a novel heparinized bFGF incorporated stent. This stent might keep the myocardial channel open with full luminal endothelialization.

Short half-life of the naked protein is a major therapeutic challenge in clinical trials and hence studies are focusing on sustained release of angiogenic factors. Yang et al. [70] reported that sustained release of bFGF and VEGF was possible with alginate beads. It facilitated vascular growth in a porcine model of cryoinjury, while Chu et al. [71] reported that injection of bFGF coacervate ameliorated ischemic injury caused by MI. Due to the encouraging results in animal models, several clinical trials have been designed to study the effect of bFGF in humans; the results of which are summarized in Table 3.

Hepatocyte growth factor

HGF is another potent multifunctional protein that exerts high proangiogenic activity. It is a novel member of the endothelium-specific growth factors whose mitogenic activity on endothelial cells is very potent. HGF (also called as scatter factor) is a basic heparin-binding glycoprotein consisting of a heavy (58 kDa) and light (31 kDa) subunit. The α and β subunits originate from proteolytic cleavage of a single 92-kD inactive precursor [83, 84]. It is secreted by the stromal cells and it stimulates cell motility and proliferation. In vitro, HGF stimulates vascular endothelial cell migration, proliferation, and organization into capillary-like tubes [85]. It has 38% amino acid sequence identity with the proenzyme plasminogen [84] and is thus related to the blood coagulation family of proteases. It acts through the tyrosine-kinase receptor *c-met*, which is expressed on a variety of cells, including endothelial cells and hematopoietic stem cells [60].

Circulating levels of HGF have been found to be increased in the early phase of MI. An elevation in the levels of serum HGF, within 3 h after onset of chest pain in patients with acute MI, has been observed [86]. Sato et al. [87] report HGF as a new biochemical marker for acute MI. He et al. [88] have noticed that administration of HGF (adenovirus HGF) along with granulocyte colony-stimulating factor induces synergistic effect and enhances myocardial endothelial density, angiogenesis, geometric preservation, and heart function in an ischemic cardiomyopathy model of rats. It has been reported that transfection of human HGF gene into infarct rat myocardium results in improved angiogenesis [8], while Ahmet et al. [89] report the same in ischemic canine myocardium. Saeed et al. [90] have shown that HGF gene (pCK-HGF-X7) ameliorates global function, increases regional perfusion and infarct resorption, and enhances angiogenesis/arteriogenesis in a swine model of myocardial infarction. Xin et al. [91] demonstrate that the combination of HGF and VEGF increases neovascularization in the rat corneal assay greater than either factor alone. Ueda et al. [92] report that the treatment using HGF enhances the survival of the cardiomyocytes subjected to oxidant stress, thus proving HGF's cardioprotective effect.

Table 3 Clinical trials using fibroblast growth factor

Therapy	Route of administration	Trial phase	Therapeutic target	Follow-up	Outcome	Reference
rFGF-1 n	Intramyocardial injection during CABG	I	Multivessel diseases with distal LAD disease	12 week to 3 years	New vessels bypassing distal LAD; increased flow vs placebo; decreased angina and use of antianginal drugs vs placebo; similar angiographic improvement	[72]
rFGF-1	Intramyocardial injection via thoracotomy	I	Severe angina, NR	6 and 12 week	Perfusion of SPECT and exercise capacity improved compared with baseline	[73]
rFGF-2	Periadventitial during CABG	I	NR, viable myocardium	3 months	1 perioperative death, free of angina after surgery, variable changes on nuclear perfusion scan	[74]
rFGF-2	Periadventitial during CABG	I	NR, viable myocardium	3 months	2 deaths during surgery and 3Q wave MI; less angina, improved SPECT in high dose	[75]
rFGF-2	Intraventricular or intracoronary injection	I	CAD, NR, RD	1, 2, and 6 months	Increased exercise tolerance time, improved quality of life, increased LV function, improved perfusion by MRI, decreased angina	[76]
rFGF-2	Intracoronary to left main	I	CAD and angina	28 days	Small short-term increase in coronary artery diameter, no change in exercise time or time to ischemia, hypotension up to 3 days, bradycardia and conduction disturbances, transient proteinuria and thrombocytopenia	[77]
rFGF-2	Intracoronary injection	I	CAD, NR, RD	90 days to 6 months	90-day exercise time and stress nuclear perfusion results did not differ from placebo group; less angina, trend toward improved overall results in older and more symptomatic patients	[78]
FGF-4/adenoviral vector	Intracoronary injection	I/II AGENT	RSA, suitable for CABG/PTI, >1 open vessel	4 and 12 week	Nonsignificant increase in treadmill time compared with placebo at 4 and 12 weeks; favorable trend if baseline treadmill time or adenoviral titer is low	[79]
FGF4-adenoviral vector	Intracoronary injection	I/AGENT	CMI	12 weeks	Increased walking time	[80]
FGF4-adenoviral vector	Intracoronary injection		Stable angina	8 weeks	Improved perfusion	[81]
FGF4-adenoviral vector	Intracoronary	AGENT-3/AGENT-4	chronic angina	12 weeks	Improved exercise tolerance test. No effect on male patients	[82]

AGENT Angiogenic Gene Therapy Trial, CAD coronary artery disease, CMI chronic myocardial ischemia, LAD left anterior descending artery, LV left ventricle, PTT percutaneous transluminal intervention, NR not a candidate for revascularization, CABG coronary artery bypass grafting, RD reversible defect, RSA refractory stable angina, SPECT single-positron emission-computed tomography, MRI magnetic resonance imaging, rFGF-2 recombinant protein FGF-2, rFGF-1 recombinant protein FGF-1

Therapeutic angiogenesis is induced in the ischemic canine heart by the direct injection of plasmid complementary DNA encoding human HGF [93]. Duan et al. [94] report that the treatment of myocardial ischemia with bone marrow-derived mesenchymal stem cells overexpressing HGF restores blood flow and regenerates lost cardiomyocytes. In order to overcome the limitations of the adenoviral vectors used in gene delivery, Konda et al. [95] have used the ultrasound-mediated microbubble destruction method to deliver the HGF gene to the infarct rat heart. This method has resulted in an enhanced angiogenesis and reduced infarct size, and left ventricular remodeling is prevented after MI. Deuse et al. [49] observe that the efficacy of mesenchymal stem cell-based regenerative strategies is maximized on supplementation with HGF protein. Transendocardial delivery of human HGF gene (VM202) in a pig model of chronic myocardial ischemia preserves microcirculatory perfusion and improves wall motion [96]. Lu et al. [97] reported that mesenchymal stem cells (MSCs) transfected with HGF was superior to MSCs transfected with VEGF in improving cardiac function and perfusion in a porcine model of MI, possibly owing to its enhanced antifibrotic effect. Transplantation of umbilical cord-derived MSCs overexpressing HGF in peri-infarct region of mice after MI proved to be more promising in enhancing angiogenesis and proliferation of cardiomyocytes [98]. Though the results from animal studies are promising, more work has to be done before taking up the clinical trials.

Platelet derived growth factor

PDGF is composed of two distinct but highly homologous polypeptide chains, designated the PDGF-A chain and the PDGF-B chain (99–100). The PDGF-A chain and the B chain are encoded by two different genes, and they have a similar size (approximately 24 kb) and structure [101]. PDGF-A and B chains form disulfide-linked homo- and heterodimers. PDGF can therefore be assembled into at least three isoforms with an approximate molecular weight of 28 kDa called PDGF-AA, PDGF-AB, and PDGF-BB [99]. The cellular response to each of these isoforms depends on the presence of specific, high affinity receptors. The two receptor subtypes, viz., PDGFR- α and PDGFR- β [100, 101], belong to the tyrosine-kinase receptor family [102]. Two PDGF receptor subunits are usually necessary for a PDGF ligand dimer to bind and create isoform-specific PDGF receptors [103].

Most of the cells such as monocytes/macrophages, mast cells, lymphocytes, connective tissue cells, pericytes, endothelial cells, and tumor cells normally express PDGF at undetectable or very low levels [104]. Injuries such as perturbations of the extracellular

milieu, growth-regulatory molecules, cytokines, and inflammatory mediators can induce the expression of PDGF [104]. Hypoxia increases expression of PDGF-B chain [105].

PDGF has been originally discovered and isolated from platelets as a major mitogen for mesenchymal cells such as glial cells, fibroblasts, and smooth muscle cells and its generation is tightly regulated [99, 100]. It is stored in platelets [106] and is released upon platelet degranulation, e.g., in response to thrombin and other stimuli [99]. All three isoforms of PDGF can be released from human platelets [106]. In vivo studies have suggested the possibility that PDGF might induce the migration of endothelial cells [107], a prerequisite for angiogenesis.

PDGF-BB stimulates smooth muscle cell recruitment to newly formed vessels and is partially responsible for functionality and maturation [108]. PDGF signaling regulates events critical to fibrous tissue deposition and angiogenesis through interactions with its two PDGF receptors (PDGFR). Activation of both PDGFR- α and PDGFR- β pathways stimulate fibroblast migration, proliferation, and activation [109]. In addition, PDGF-BB/PDGFR- β interactions play a crucial role for investment of developing vessels with pericytes, a key process in vascular maturation [110].

Zhao et al. [111] have reported that increased levels of PDGF-A, PDGF-B, and PDGFR in the infarct rat myocardium are coincident with angiogenesis, inflammatory, and fibrogenic responses. Edelberg et al. [9] state that aging hearts have impaired angiogenic function as a result of depressed PDGF-B production. So, pretreatment by intramyocardial delivery of PDGF-AB promotes angiogenesis and minimizes the extent of myocardial infarction in aging hearts after coronary artery ligation in rats [9]. PDGF-B mediates pericyte proliferation and migration and is thus associated with vessel stabilization [112]. The stabilization of angiogenic vessels through pericyte recruitment is regarded to be essential for the maintenance of blood flow after angiogenic gene therapy of ischemic disease [113]. Korpisalo et al. [28] have observed a combinatorial gene transfer of adenoviral VEGF and adenoviral PDGF-B into rabbit hindlimb skeletal muscle by an intramyocardial injection. The combination therapy results in a prolonged angiogenic response, though the pericyte recruitment to angiogenic vessels is not improved. Hao et al. [114] have employed the same combination in MI-induced rats. Alginate gels of VEGF-A₁₆₅ and PDGF-BB when administered intramyocardially along the border zone of MI result in improved cardiac function and formation of matured blood vessels. Zymek et al. [115] conclusively show from their experiments on reperfused mice infarcts that PDGF signaling critically regulates post-infarction repair.

Platelet derived endothelial cell growth factor

PDECGF has been initially identified as a novel angiogenic factor present in platelets [116]. It is a 45-kDa nonglycosylated single-chain polypeptide that stimulates growth and chemotaxis of endothelial cells in vitro and angiogenesis in vivo [117] and confers resistance to hypoxia-induced apoptosis [118]. It is involved in a wide range of activities including angiogenesis, wound healing, etc. It plays a role in maintaining the integrity of blood vessels and promoting the repair of the endothelium [119].

PDECGF differs from many endothelial mitogens in that it lacks both heparin-binding domains and a secretion peptide. The mechanism of its angiogenic action is still unclear; however, the angiogenic action is dependent on its enzymatic activity [120]. PDECGF is chemotactic for endothelial cells [121]. Usuki et al. [122] reported that human thymidine phosphorylase (dThdPase) is identical with PDECGF, while Miyadera et al. [120] through their mutant studies have proved that dThdPase activity is indispensable to the angiogenic activity of PDECGF/(dThdPase).

Thymidine phosphorylase/PDECGF is involved in pyrimidine nucleoside metabolism, catalyzing the reversible phosphorylation of thymidine, deoxyuridine, and their analogues to their respective bases and 2-deoxyribose-1-phosphate in the presence of inorganic orthophosphate [122]. This degradation product, viz., 2-deoxy-D-ribose, is found to have angiogenic and chemotactic activity [123]. PDECGF is upregulated by both the low oxygen partial pressure and low pH seen in poorly perfused areas [124]. The pattern of thymidine phosphorylase expression in a diseased area often indicates that PDECGF upregulation is due to the hypoxia caused by the disease state [125].

The expression of PDECGF has been reported in a wide range of chronic inflammatory diseases and in solid tumors. Cells known to express PDECGF include mononuclear cells, macrophages, keratinocytes, glial cells, fibroblasts, and epithelial cells within the endothelium of breast, brain, and placenta and within the cells of atherosclerotic plaque [126].

Hemalatha et al. [127] have reported the expression of PDECGF in a rat model of MI, induced by coronary artery ligation (CAL). Macrophages, endothelial cells, fibroblasts, and myocytes, especially at the border region of the lesion, show an enhanced expression for PDECGF. The level of PDECGF expression is enhanced from day 2 after CAL, and a prominent level of expression is observed on day 4 and day 8 after CAL, which coincides with the process of angiogenesis and on day 32 after CAL; the level of expression shows a decrease. Li et al. [119] have reported that transmyocardial laser revascularization-induced angiogenesis is shown to correlate with the expression of PDECGF in infarct myocardium of beagle dogs. The therapeutic effect of PDECGF gene therapy on rabbit severe limb ischemia has already been

demonstrated [128]. Gene transfer of PDECGF markedly inhibits rat vascular smooth muscle cell proliferation and migration in vitro and inhibits neointima formation of balloon-injured rat carotid arteries in vivo [129]. Li et al. [130] have demonstrated that targeting of ischemic myocardium using plasmid vector of PDECGF gene has generated long-term improvement in cardiac function by causing angiogenesis and arteriogenesis and inhibiting apoptosis, but it does not induce neoplasm in the remote organs, and hence, it may be a promising therapy to treat chronic ischemia. The antiapoptotic potential of PDECGF in the presence of hypoxic stress is conclusively demonstrated by Hemalatha et al. [131] with rat aortic endothelial cells.

Erythropoietin

Erythropoietin (EPO) is a low molecular weight (30 kDa) glycoprotein hormone stimulator of erythropoiesis produced in the fetal liver and subsequently in the adult kidney [132]. EPO is the best known hypoxia-regulated gene, and this regulation occurs mainly at the mRNA level and is mediated by HIF-1 [133]. The potential role of EPO in angiogenesis may be considered as a subset of its possible function in improving overall tissue oxygenation and of its antiapoptotic role. HIF-1 is responsible for the transcription of both EPO and VEGF. HIF-1 is a heterodimeric DNA binding complex consisting of α and β subunits [134].

Effects of EPO in the bone marrow are mediated by binding to a specific transmembrane receptor (EPO-R), which is expressed primarily by hematopoietic progenitor cells [135]. The expression of EPO-R is found in a variety of cell lines originating from the cardiovascular system. In vitro, EPO-R is synthesized and is present on the surface of human vascular endothelial cells [136] and administration of EPO prevents apoptosis of endothelial cells subjected to hypoxia through direct modulation of PI3K/Akt phosphorylation [137].

Pretreatment of adult rats with EPO (5000 IU/kg) 24 h preceding ischemia/reperfusion (I/R) increases the functional recovery of isolated hearts during reperfusion [138]. This is accompanied by protection against apoptosis. The involvement of signal transducing pathways is further elucidated by Shi et al. [139] in a rabbit isolated heart model in which the cardioprotective effects of EPO are abolished by inhibitors of two different MAPK (p38 and p42/44). In the same study, potassium channel inhibitors are also shown to block the EPO effects, indicating a role for potassium channels in EPO-mediated preservation of heart function, possibly caused by reduction of calcium overload. Hanlon et al. [140] have shown the importance of protein kinase C (PKC), in EPO-mediated cardioprotection. EPO can also improve the cardiac function by directly modulating the cardiac Na^+/K^+ pump [141] or stimulating the production of atrial natriuretic peptide

in cardiac atrium [142]. In a rabbit model of MI, treatment with EPO at the time of permanent coronary ligation results in a trend toward reduced infarct size and improvement of cardiac contractility and relaxation when measured 3 days later [143]. In addition, EPO administration in I/R model in rabbit hearts is also noticed to be beneficial, resulting in a significant reduction of infarct size, expressed as a percentage of total ischemic area at risk [144]. This protection is associated with the mitigation of myocardial cell apoptosis. Furthermore, in line with the results from *ex vivo* experiments, reduction of infarct size *in vivo* is dependent on the activation of pro-survival pathways PI3K/Akt and MAPK [145]. Hirata et al. [146] have approached the issue of minimal EPO dose still rendering cardioprotection. The EPO treatment in a canine model of I/R is observed to reduce infarct size in a dose-dependent manner, establishing the lowest effective dose of 100 IU/kg. Moon et al. [147] have found that a single dose of EPO (3000 IU/kg) immediately after coronary artery ligation in rats reduces the infarct size (15 to 25%) when compared to ligated animals examined 8 weeks later. This reduction in myocardial damage is accompanied by prevention of LV dilation and improves LV ejection fraction, as measured by repeated echocardiography. It seems that EPO may protect the myocardium also against more severe insults, such as permanent coronary occlusion, and this effect becomes even more pronounced with time. EPO-induced neovascularization in post-MI heart failure in a rat model is mediated through a combination of endothelial progenitor cell (EPC) recruitment from the bone marrow and increases myocardial expression of VEGF [148]. Van der Meer et al. [149] report that an increase in alpha-MHC expression is also associated with improvement in cardiac function. In a murine model of surgically induced MI, EPO-treated animals show significant improvement of survival post-MI with attenuated remodeling, enhanced neovascularization, and diminished apoptotic cells [150]. Hirata et al. [151] show from their experiments on dogs that EPO enhances neovascularization likely via EPC mobilization and improves cardiac dysfunction in the chronic phase. Broberg et al. [152] report that erythropoietin (darbepoetin- α) at moderate doses after MI is not angiogenic but antiapoptotic in MI-induced mice. Bagla et al. [153] reported that erythropoietin administration after MI reduced caspase 3 expression (apoptotic activity) and induced neovascularization around the infarct area in rats and also further higher erythropoietin administration (10,000 U kg⁻¹) did not provide an additional benefit over the standard dose (5000 U kg⁻¹) in myocardial protection.

Recently, two larger placebo-controlled phase II studies with EPO treatment in anemic chronic heart failure (CHF) patients have been conducted that also suggest potential beneficial effects both in terms of quality of life and clinical end points [154]. A possible positive effect of darbepoetin treatment on longer-term infarct healing and/or cardiac

remodeling is demonstrated by a single-center, investigator-initiated, prospective study with 22 non-anemic patients with first acute MI [155].

From the experimental studies, EPO seems to influence two crucial processes during cardiac ischemic injury, first by acutely reducing the infarct size and inhibiting the apoptosis and second by promoting new vessel formation over a longer time frame. Although treatment with EPO is generally well tolerated and safe, it may be associated with adverse effects such as hypertension and thromboembolism. These side effects are related mainly to high-dose chronic EPO treatment, associated with increased hematocrit. Using variants of EPO without hematopoietic effect but retaining tissue protective activity could be useful in a clinical situation in which multiple EPO administrations would be warranted [156]. Ferrario et al. [157] have investigated the effects of short-term high-dose EPO on peripheral blood cells (PBCs) and infarct size in 30 patients with a first uncomplicated acute MI undergoing percutaneous coronary intervention (PCI) in a single-center study and report that the EPO administration increases the levels of circulating CD34+ cells and decreases infarct size. CD34+ cells help to maintain the vascular integrity and also serve as a source of angiogenic factors [158]. Based on the literature analysis, Ali-Hassan Sayegh et al. [159] also concluded that short-term administration of EPO in patients with MI does not result in improved cardiac function. Higher doses might be more effective than low-dose EPO therapy.

Angiopoietins

A further family of growth factors involved in the early processes of angiogenesis and vasculogenesis is the angiopoietins. One isotype, angiopoietin 1 (Ang1), is present in tissues adjacent to blood vessels suggesting a paracrine mode of action, while another, angiopoietin 2 (Ang2), is only found at sites of tissue remodeling [160]. Both angiopoietins, including the two recently discovered angiopoietin-3 (in mouse) and angiopoietin-4 (in humans), have been identified as ligands for the Tie-2/Tek receptor [161]. *In vitro* neither Ang1 nor Ang2 have mitogenic effects mediated via Tie-2. However, Ang1 facilitates endothelial cell sprouting and vascular network maturation [162]. It potentiates the action of VEGF and acts as a survival factor for endothelial cells [25].

Ang2 antagonizes Ang1 by blocking Ang1-induced phosphorylation of Tie-2 [160]. On the other hand, Ang2, in combination with VEGF, promotes neovascularization [162]. Ang2 is upregulated by conditions known to promote angiogenesis, including hypoxia, VEGF, and bFGF, but is reduced by molecules that have been implicated in vessel maturation and stabilization, including Ang1 and TGF- β 1 [25].

Sun et al. [163] have used human angiopoietin-1 (hAng1)-modified MSCs to treat acute MI in rats. The hAng1 gene is

transfected into cultured rat MSCs using an adenoviral vector. Five million hAng-transfected MSCs (MSC_{Ang1}) or green fluorescent protein transfected MSCs (MSC_{GFP}) or PBS only (PBS group) were injected intramyocardially into the inbred Lewis rat hearts immediately after MI. MSC_{Ang1} survive in the infarct myocardium and express hAng1 at both mRNA and protein levels. The measurements of infarct ventricular wall thickness, infarction area, and left ventricular diameter indicate that heart remodeling is inhibited and heart function is improved in both the MSC_{Ang1} and MSC_{GFP} groups. The results indicate that hAng1-modified MSCs improve heart function, followed by angiogenic effects in salvaging ischemic myocardium and reduce cardiac remodeling.

Granulocyte colony stimulating factor

Granulocyte colony stimulating factor (G-CSF) is a potent hematopoietic cytokine that influences the proliferation, survival, maturation, and the functional activation of granulocytes, and it is involved in mobilization of granulocytes, stem, and progenitor cells from bone marrow into blood circulation [164]. The process of mobilization is not fully understood but seems to be mediated through binding of G-CSF to a specific cell surface receptor, the G-CSF receptor, leading to a subsequent digestion of adhesion molecules by enzyme release and through trophic chemokines. Stem cell-derived factor-1 (SDF-1) and its receptor CXCR-4 seem to play a central role [165].

Animal studies have suggested a beneficial effect of G-CSF mobilization of stem cells on left ventricular function after MI, with regeneration of myocardium by inducing myogenesis and vasculogenesis and by diminishing post-infarction remodeling. Orlic et al. [166] have injected mice with recombinant rat stem cell factor and recombinant human G-CSF to mobilize stem cells for 5 days, then ligated the coronary artery, and continued the treatment with stem cell factor and G-CSF for 3 days. Afterwards, the ejection fraction progressively improves significantly as a consequence of the formation of new myocytes with arterioles and capillaries. These results are confirmed in another experiment with G-CSF after reperfused myocardial infarction in rabbits and it shows improvement in left ventricular ejection fraction and reduces remodeling [167]. In contrast, G-CSF fails to improve myocardial function in a rat MI model after non-reperfused MI [168]. Study in mice has indicated that the mechanism by which G-CSF prevents cardiac remodeling after ST elevation myocardial infarction (STEMI) is by inhibiting apoptosis of cardiomyocytes through the Jak2-STAT3 pathway, rather than through mobilization of bone marrow cells [169]. In addition, in a murine model of infarction, G-CSF induces improvement in left ventricular function, enhances arteriogenesis, and increases ICAM-1 expression on endothelial cells [170]. Zhao

et al. [171] reported that G-CSF treatment after myocardial ischemia improves survival by accelerating neovascularization and reducing apoptosis.

Mobilization of stem cells into the peripheral circulation for myocardial regeneration using subcutaneous injections of G-CSF has been tested in both patients with acute MI and patients with chronic myocardial ischemia. G-CSF treatment seems to be safe, and unblinded trials in patients with acute MI are encouraging. However, larger double-blind placebo-controlled trials have not been able to demonstrate the effect of G-CSF treatment. The known complex interaction of stem cells and cytokines for induction of vasculogenesis could be thought of in future clinical trials, to elucidate whether G-CSF mobilization of stem cells might be useful as a new regenerative treatment in patients with ischemic heart disease [172]. Engelmann et al. [173] have investigated the influence of the timing of G-CSF treatment on myocardial function and perfusion in a subgroup study of the G-CSF-ST elevation myocardial infarction clinical trial. Results demonstrate that the timing of G-CSF after MI does not improve myocardial function but myocardial perfusion is increased if the cytokine is given early. Overgaard et al. [174] also state that the timing of G-CSF treatment, between 17 and 65 h after STEMI with primary PCI, does not seem to affect the recovery of left ventricular ejection fraction (LVEF).

Placenta growth factor

The first VEGF-related protein, placenta growth factor, discovered in 1991, owes its name to its predominance in placental tissue. It has been later identified as a member of the VEGF family as the molecule shares 53% of a homologous domain with the platelet derived growth factor-like region of VEGF [175]. Three isoforms arise by means of alternate splicing, PIGF-1/PIGF131, PIGF-2/ PIGF152, and PIGF-3 [176]. These molecules are, like VEGF, dimeric glycoproteins. However, the PIGF expression pattern is limited to the placenta and some form of tumors such as brain tumor and renal cell carcinoma [177, 178]. PIGF homodimers bind VEGFR-1 (Flt-1) but have little effect on angiogenesis *in vitro* [176]. On the other hand, naturally occurring VEGF/PIGF heterodimers, identified in rat glioma cells, are mitogenic; their potency is approximately sevenfold lower than that of the VEGF homodimer [177, 178].

Roncal et al. [179] have analyzed the effect of systemic PIGF delivery on myocardial recovery post-MI in mice. MI is induced by permanent ligation of the left anterior descending coronary artery in mice, followed by systemic injection of a PIGF adenovirus, resulting in elevated circulating levels of PIGF for 4 weeks. Functional and morphological analysis has revealed that PIGF treatment induces cardiomyocyte hypertrophy and

improves cardiac recovery at day 28 post-MI [179]. PIGF stimulates angiogenesis in the infarct border and vessel enlargement in the remote myocardium. In this model, capillary to cardiomyocyte ratios in the remote myocardium are maintained post-MI but PIGF is shown to increase the vascular perfusion area in balance with the cardiomyocyte hypertrophy. Systemic delivery of PIGF improves cardiac performance and promotes adaptive remodeling of the post-MI heart.

Insulin-like growth factor

IGF-I and II, also known as somatomedins [180], are shown to be survival factors to motor neurons [181]. IGF-I and II are single-chain polypeptides usually of 7.5 kDa. These growth factors have an important role in controlling the proliferation, differentiation, and metabolic activity of mesodermal cells. IGFs can attach not only to binding proteins that are thought to regulate their biological action and bioavailability but also to insulin receptors and type 1 or 2 receptors [180]. IGF-1 has been shown to be an angiogenic growth factor as potent as FGF-2 in promoting rabbit corneal angiogenesis [182]. Dobrucki et al. [183] have stated that delivery of human IGF-1 gene by adeno-associated virus (AAV) in to MI-induced rats renders a sustained transduction and improves cardiac function at 4 weeks post-MI. IGF-1 expression enhances α_v integrin activation which is linked to angiogenesis.

Patients with chronic heart failure have high levels of growth hormone associated with low levels of IGF-1 which is secreted in response to growth hormone and mediate most of the effects of growth hormone [184]. Battler et al. [185] have reported that exogenous IGF-II, delivered to the infarct area, ameliorates regional cardiac function in the pig, perhaps by inducing peri-infarct myocyte growth.

Transforming growth factor

Transforming growth factor β ($TGF\beta$) is a locally generated cytokine involved in healing processes and tissue fibrosis, relevant for cardiac remodeling and the development of heart failure after MI. Frantz et al. [186] have studied the effect of $TGF\beta$ inhibition by application of a blocking antibody in mice with MI. Results clearly establish that anti- $TGF\beta$ treatment before or after coronary artery ligation increases mortality and worsens left ventricular remodeling in mice with non-reperfused MI. Pertovaara et al. [187] have revealed that $TGF\beta$ induces an increase in the level of VEGF mRNA and protein expression and thus its angiogenic effect.

Nitric oxide

Nitric oxide (NO) donors are pharmacologically active substances that release NO in vivo or in vitro. Apart from vasodilation, NO has a variety of functions such as the release of prostanoids, inhibition of platelet aggregation, effect on angiogenesis, and production of free radicals [188]. The relationship between NO and angiogenesis has been confirmed repeatedly in infarct myocardium. NO donors and growth factors induce angiogenesis in vivo and cause proliferation of endothelial cells in cell cultures [189]. VEGF acts on endothelial cells through NO synthase activation and cGMP production [190]. NO production is necessary for the growth-promoting effect of VEGF. These findings have considerable implication for the use of NO donors as angiogenesis-promoting factors in ischemic myocardium.

Potential hazards

The risks associated with therapeutic angiogenesis include those specific to the growth factor and those generic promoting angiogenesis. The potential side effects include (i) aberrant vascular proliferation in nontargeted tissues, (ii) increased vascular permeability (iii) induction of the development of functionally abnormal blood vessels, (iv) triggering growth of neoplasms, (v) increase in atherosclerotic plaque mass and instability, (vi) vasodilation and hypotension during short-term administration of FGF and VEGF proteins, (vii) hazards associated with viral vectors such as the induction of immune and inflammatory responses, and (viii) hazards associated with direct myocardial delivery such as myocardial inflammation, fibrosis, and angioma formation [191]. The angiogenic factors are double-edged swords which have the capacity to stimulate the growth of new blood vessels as well, and they have also the potential to cause serious complications. However, these limitations could be overcome by delivering the angiogenic agents to target tissues with enough selectivity, so that nontargeted tissues are not aberrantly stimulated.

Future prospects

Normal vessel development requires the expression and activity of multiple gene products; therefore, it seems unlikely that overexpression of a single gene or delivery of a single growth factor would induce the formation of structurally and functionally normal vessels. Hence, multigene/protein therapy may be important to induce increased perfusion and function in cardiac patients [192]. Also, much is still unknown about the optimal delivery of angiogenic factors, i.e., should it be delivered directly into the peri-ischemic or nonischemic

region from which the collaterals will originate or to both? [193]. Hence, a comprehensive evaluation of potential side effects is needed to determine the optimal dose and delivery strategy for each factor or combination of factors. The above questions could be properly answered by controlled clinical trials. Chen et al. [194] have reported the use of plant-derived angiogenic molecule, viz., Angio-T in ischemic rat hearts, which stimulates the growth of new collateral microvessels and improves heart function. A number of plant-derived compounds are proven to be angiogenic, viz., β -sitosterol derived from *Aloe vera* [195], resveratrol from grapes [196], ginsenoside Rg1 from ginseng [197], etc. Hence, future researches could focus on the use of plant-derived angiogenic compounds for therapeutic angiogenesis in MI, either alone or in combination with other factors, provided the side effects are harmless. Apart from angiogenic factors, Trelles et al. [198] report the cardiomyocyte-specific deletion of the transcription factor recombination signal binding protein for immunoglobulin kappa J region (RBPJ) could also improve the microvasculature of the heart without adversely affecting cardiac structure or function. Singla [199] proposes that stem cell-derived exosomes are antiapoptotic and proangiogenic in nature, and they could play a pivotal role in the repair of ischemic myocardium. Zhaofu and Dongging [200] report that cardiac telocytes present in the interstitium of the heart are also damaged during MI which contributes to poor healing. Transplantation of cardiac telocytes in MI improves myocardial function and decreases infarct size.

Conclusion

A number of angiogenic factors are expressed in response to an occlusion in a coronary artery. But when this effort by nature becomes inadequate, exogenous supplementation with recombinant genes or growth factors to enhance myocardial collateral blood vessel formation looks to be a promising approach for the treatment of MI. Angiogenesis has generated tremendous enthusiasm due to its promise as a novel therapeutic modality for ischemic heart disease, especially for patients who could not benefit from standard revascularization techniques. Each growth factor mentioned above has its own advantages and limitations and yet proves to be potential as a candidate for therapeutic angiogenesis. But still, there are a number of unanswered questions which are to be addressed in this growing field. Well-designed clinical studies are required to delineate the long-term benefits of this therapy on the morbidity and mortality of chronic human ischemic disease. The delivery of angiogenic growth factors appears to be potentially feasible, but more studies are necessary to prove this point.

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Compliance with ethical standards

Conflict of interest The authors T. Hemalatha, T. UmaMaheswari, S. Iswariya, D.N. Gunadharini, and K. Anbukkarasi declare that there are no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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