Expert Opinion

Controlled release systems of angiogenic growth factors for cardiovascular diseases

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Worldwide, there is a growing number of patients with myocardial ischemia and limb ischemia associated with the aging population and an increased prevalence of atherosclerotic diseases. Medical therapy, percutaneous angioplasty and surgical revascularization are the present treatments of choice, but such treatments are not feasible for some patients with severe atherosclerosis. Therapeutic angiogenesis using growth factors or progenitor cells has drawn great attention as a favorable alternative treatment for such patients. This review discusses clinical trials using angiogenic growth factors for myocardial ischemia and limb ischemia, and also introduces a novel controlled release system for growth factors using a gelatin hydrogel.

Keywords: clinical trial, controlled release system, gelatin, growth factor, therapeutic angiogenesis

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1. Introduction

Myocardial ischemia is a leading cause of death and disability. Limb ischemia is a disability affecting many people worldwide. Pharmacotherapy, percutaneous angioplasty and surgical revascularization are established treatments for such tissue ischemia. However, there are some patients who have diffused arterial disease or small/calcified distal arteries that cannot be effectively treated by the existing revascularization techniques or who have co-morbidities which make them poor candidates for invasive procedures. Such patients suffer myocardial damage, resulting ischemic cardiomyopathy or lose a limb because of limb ischemia. The number of these patients is increasing with the aging population because of the increasing prevalence of diseases such as diabetes mellitus and hyperlipidemia. New therapies are required for such severe cases and therapeutic angiogenesis has attracted great attention as a potential future therapy.

There are two kinds of therapeutic angiogenesis. One uses angiogenic cytokines or the genes that encode them, and the other is cell transplantation. Proangiogenic cytokines include angiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), angiopoietin, and platelet-derived growth factor (PDGF), and chemokines such as granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and stromal cell derived factor-1 (SDF-1). In contrast, cell-based therapeutic angiogenesis uses, for example, endothelial progenitor cells, and bone marrow mononuclear cells. This article focuses on randomized, controlled clinical trials using VEGF, FGF and HGF, and also introduces a novel controlled release system of growth factors using a gelatin hydrogel.

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2. Mechanisms of new blood vessel formation

There are three kinds of mechanisms for new blood vessel formation: vasculogenesis, angiogenesis and arteriogenesis. Vasculogenesis is a process by which progenitor cells form blood vessels in a previously avascular area. The mobilization of progenitor cells is stimulated by VEGF, SDF-1, G-CSF, GM-CSF and so on [1]. Angiogenesis is the sprouting of new capillaries from preexisting vessels, mainly in response to hypoxia via activation of hypoxia-inducible factor (HIF)-1α expression. HIF-1α activates VEGF and angiopoietin. Arteriogenesis is the maturation of capillary vessels to arterioles, whereby pre-existing vessels become large, and pericytes and smooth muscles support them. Arteriogenesis is mainly initiated by increased sheer stress, which induces endothelial cells to express monocyte chomotactic protein 1 and other similar substances. These cytokines recruite monocytes and upregulate VEGF, FGF, PDGF-B, transforming growth factor-β_1 [2,3].

3. VEGF, FGF and HGF

VEGFs are the most extensively studied family of angiogenic growth factors, consisting of seven members including VEGF-A (VEGF-1), VEGF-B, VEGF-C (VEGF-2), VEGF-D, VEGF-E, VEGF-F and placental growth factor. There are three types of VEGF receptor: VEGFR-1(Flt-1), VEGFR-2(Flk-1) and VEGFR-3(Flt-4). VEGF-A and -C bind and activate VEGFR-2, which is thought to have a major role in VEGF-mediated endothelial cell proliferation. Therefore, VEGF-A and VEGF-C have often been used in clinical trials. VEGF-A has four isoforms: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ as a result of alternative splicing. $VEGF_{165}$ is the predominant isoform of VEGF-A [4]. $VEGF_{165}$ is much more potent than $VEGF_{121}$. VEGF is an important factor for the mobilization of endothelial progenitor cells from the bone marrow [5].

FGFs are also potent angiogenic growth factors. The FGF family has 22 isoforms [6]. FGF-1, FGF-2 (bFGF) and FGF-4 have been used most commonly in clinical trial for ischemic tissues. FGF-1 and FGF-2 do not contain a signal sequence for secretion, whereas FGF-4 has signal peptides and is efficiently secreted [6]. FGFs stimulate the proliferation of not only endothelial cells, but also smooth muscle cells and myoblasts.

HGF is another potent multifunctional protein that also has angiogenic activity [7]. It activates its receptor, c-met, which is expressed on a variety of cells, including endothelial cells, but also on hematopoietic stem cells.

4. Protein versus gene therapy

4.1 Protein therapy

The level of uptake of growth factor protein into ischemic tissue is relatively low compared with the plasma level. Only 0.26% of systemically administered FGF-2 has been reported to be found in the myocardium [8]. Therefore, if angiogenic growth factor is delivered systemically, high doses of growth factor are needed for effective angiogenesis and it causes systemic side effects such as hypotension and edema with VEGF treatment [9], and hypotension, thrombocytopenia and renal insufficiency with FGF treatment [10]. In addition, angiogenic growth factors such as VEGF and FGF have been reported to be associated with malignant tumors [11,12] and retinal neovascularization [13]. Therefore, in order to obtain the beneficial effects without the systemic side effects, growth factors must be delivered locally. Furthermore, although the growth of blood vessels requires the presence of an angiogenic stimulus, until a steady state of the vessel structure is achieved [14], the half-life of growth factors is very short. In order to maintain a sufficient tissue concentration of growth factors for an extended period, a sustained release system for growth factors is required. One of these systems is gene therapy. An alternative is a drug delivery system such as sustained release systems using biodegradable gelatin hydrogels.

4.2 Gene therapy

There are two kinds of vectors in gene therapy: one is viral and the other is non-viral. The most widely used vectors in clinical trials are adenovirus and plasmid DNA.

Plasmid DNA is a non-viral vector and is safe and easy to produce. However, naked plasmid DNA has a low gene transduction efficiency and requires liposomes or polymer complexes to improve the delivery of plasmid DNA to the cytoplasm.

Adenovirus has a high transduction efficiency, can be produced with high titers and is effective in proliferating and non-proliferating cells. However, adenoviruses do not lead to the stable integration of the transgene into the host genome, but they remain extrachromosomal and cause only transient transgene expression. In addition, circulating antibodies to adenoviruses are common in humans and may elicit an inflammatory response that compromises the incorporation and expression of the gene [15]. Adeno-associated viruses have a lower transduction efficiency than adenoviruses, but have transgene expression lasting up to a year after gene transfer [16]. Lentiviruses have a greater capacity for transgenes and are also capable of transducing both in proliferating and non-proliferating cells. Retroviruses enter cells and their RNA is reverse transcribed to DNA, which is integrated into the host genome. Therefore, retroviral gene transfer can lead to a long-lasting gene transfer effect. However, retroviruses can deliver transgene only to proliferating cells.

There are safety concerns in viral vector therapy, including death, which was reported to occur after adenoviral gene therapy in 1999 [17].

5. Randomized controlled clinical trials for myocardial ischemia

Table 1 summarises the trials discussed in the following subsections.

5.1 Protein therapy

5.1.1 *FGF*

Simons *et al.* [18] performed a trial with a single intracoronary infusion of FGF-2 at 0, 0.3, 3.0 or 30.0 µg/kg in 337 patients who were considered suboptimal candidates for standard surgical or catheter-based revascularization (FIRST [FGF-2 Initiating Revascularization Support Trial]). The majority of the patients had Canadian Cardiovascular Society (CCS) Class 2 or 3 angina (88%). FGF-2 did not improve exercise tolerance or myocardial perfusion in single photon emission computed tomography (SPECT), but it did tend to show a symptomatic improvement at 90 days, but not at 180 days. The symptomatic improvement was most pronounced in the more symptomatic patient subgroups. The ineffectiveness in this trial may be due to the delivery method, because a single intracoronary injection of FGF-2 without a sustained release carrier was reported to show < 1% of FGF-2 to still remain in the heart at 1 h [19]. According to a subgroup analysis, another reason for the ineffectiveness may be due to a lower severity of symptoms in the patients included in this study.

5.1.2 *VEGF*

Henry *et al.* [20] investigated $VEGF_{165}$ in 178 patients with stable exertional angina who were unsuitable for standard revascularization (VIVA trial [VEGF in Ischemia for Vascular Angiogenesis]). The majority of the patients were in CCS 2 or 3. They were randomized to receive placebo, low-dose $VEGF_{165}$ (17 ng/kg/min), or high-dose $VEGF_{165}$ (50 ng/kg/min) by intracoronary infusion on day 0, followed by intravenous infusions on days 3, 6 and 9. The change in exercise treadmill test (ETT) time, angina class and quality of life were not significantly different between the groups at day 60. High-dose $VEGF_{165}$ resulted in a significant improvement in angina class and non-significant trends in ETT time and angina frequency compared with placebo at day 120. However, there was no significant improvement in myocardial perfusion in SPECT. The negative result of this study may be due to the route of administration and protein therapy without sustained release systems.

5.2 Gene therapy

Many clinical trials using gene therapy, especially in combination with an adenovirus, have been studied because the delivery of growth factors as proteins via the intravenous or intracoronary route was insufficient to provide clinically effective angiogenesis.

5.2.1 *FGF-4*

There are also clinical studies using FGF gene therapy. Grines *et al.* [21] reported a double-blind randomized trial of intracoronary injection of adenovirus encoding FGF-4. Incremental doses of 3×10^8 to 3.3×10^{10} AdFGF-4 particles or placebo were administered to 79 patients in CCS Class 2 or 3 (AGENT trial [Angiogenic Gene Therapy trial]). The patients who received FGF-4 tended to have greater improvements in ETT at 4 weeks. A subgroup analysis, which included only patients with baseline ETTs of 10 min or less showed the significant improvement in FGF-4 treated patients.

Grines *et al.* [22] reported another randomized, double-blind, placebo-controlled trial (AGENT 2) of the intracoronary injection of 10^{10} adenoviral particles containing FGF-4 in 52 patients in CCS Class 2 – 4 and who were unsuitable for revascularization. Although the change in reversible perfusion defect size between Ad5FGF-4 and placebo was not significant, a significant difference was observed when a single outlier was excluded. The outlier had poor compliance with antianginal medication during the pre-treatment period.

AGENT 3 and AGENT 4 were large Phase III clinical trials of the intracoronary injection of 10^9 and 10^{10} adenoviral particles encoding FGF-4 [23,24]. AGENT 3 was conducted for patients with CCS Class 2 to 4 angina with optimal antianginal therapy exclusively in the United States. AGENT 4 was to be conducted in Europe, and North and South America and include patients meeting the criteria for inclusion in AGENT 3, but who were poor candidates for revascularization therapy. An interim review of the data from AGENT 3 indicated no safety concerns. However, differences in ETT were unlikely to reach significance. Therefore, further recruitment in the trials was stopped. However, a subgroup analysis of pooled AGENT 3 and 4 data showed that there were significant improvements in ETT time, time to 1 mm ST-segment depression, time to angina and CCS Class at both 12 weeks and 6 months in a female population. The placebo effect was large in men, but the placebo effect in women was negligible, but the reason for this is unknown.

As a result of a subgroup analysis of AGENT 3 and 4, AWARE (Angiogenesis in Women With Angina Pectoris Who Are Not Candidates for Revascularization) trial of intracoronary AdFGF-4 injection in female patients has been started [101].

5.2.2 *$VEGF_{165}$*

Hedman *et al.* [25] reported a study of $VEGF_{165}$ gene therapy after standard percutaneous transluminal coronary angioplasty for 103 patients in CCS 2 or 3 (KAT [Kuopio Angiogenesis Trial]). Thirty seven patients received an intracoronary injection of VEGF adenovirus, 28 patients received VEGF plasmid liposome, and 38 control patients received Ringer's lactate. VEGF treatment did not affect the incidence of

Table 1. Randomized, controlled clinical trials for myocardial ischemia.

Trial	Growth factor	Protein/gene vector	Dose	Administration	N	Baseline CCS	Follow-up (months)	Result	Ref.
FIRST	FGF-2	Protein	0.3, 3.0, 30.0 μg/kg	IC	337	2 – 4	6	Trends toward symptomatic improvement at 3 months (but not 6 months)	Simons *et al.* (2002) [18]
VIVA	$VEGF_{165}$	Protein	17, 50 ng/kg/min	IC + IV ×3	178	1 – 4	2	Improved CCS class at day 120 (but not at day 60). Better trends in ETT	Henry *et al.* (2003) [20]
AGENT	FGF – 4	Adenovirus	3.3×10^8 to 3.3×10^{10}	IC	79	2 – 3	3	Tendency to show an improvement in ETT	Grines *et al.* (2002) [21]
AGENT 2	FGF-4	Adenovirus	10^{10}	IC	52	2 – 4	2	Tendency to show an improved myocardial perfusion	Grines *et al* (2003) [22]
AGENT 3	FGF-4	Adenovirus	10^9, 10^{10}	IC	416	2 – 4	3	Terminated because of ineffectiveness	Henry *et al.* (2007) [24]
AGENT 4	FGF-4	Adenovirus	10^9, 10^{10}	IC	116	2 – 4	3	Terminated because of ineffectiveness	Henry *et al.* (2007) [24]
AWARE	FGF-4	Adenovirus		IC	300	3 – 4	12	Ongoing	Unpublished [101]
KAT	$VEGF_{165}$	Adenovirus and plasmid liposome	2×10^{10} adenovirus, 2 mg plasmid	IC after PTCA	103	2 – 3	6	Improved perfusion in Ad-VEGF group. No difference in CCS class, working ability, nitrate use	Hedman *et al.* (2003) [25]
Euroinject one trial	$VEGF_{165}$	Plasmid	0.5 mg	IM via catheter	80	3 – 4	3	No improvement in perfusion. Increased regional wall motion	Kastrup *et al.* (2005) [26]
REVASC	$VEGF_{121}$	Adenovirus	4×10^{10}	IM via thoracotomy	67	2 – 4	6	Improved ETT and CCS class. No change in SPECT	Stewart *et al.* (2006) [27]
NORTHERN	$VEGF_{121}$	Adenovirus		IM via catheter	120 (planned)	3 – 4	3	Ongoing	Unpublished [102]
NOVA	$VEGF_{121}$	Adenovirus		IM via catheter	129	2 – 4	6	Terminated	Unpublished [103]
GENASIS	VEGF-2	Plasmid		IM via catheter	295	3 – 4	3	Terminated because of low efficacy and high rate of tamponade events	Unpublished [104,105]

CABG: Coronary artery bypass grafting; CCS: Canadian cardiovascular society; EF: Ejection fraction; ETT: Exercise treadmill test; FGF: Fibroblast growth factor; IM: Intramyocardial; IC: Intracoronary; IV: Intravenous; PTCA: Percutaneous transluminal coronary angioplasty; SPECT: Single photon emission computed tomography; VEGF: Vascular endothelial growth factor.

postangioplasty restenosis at 6 months. A significant improvement was seen in myocardial perfusion in the VEGF-Adv-treated patients, but there was only a tendency towards improvement in the other groups. No statistically significant differences were observed between the study groups in CCS classification, working ability or in the need of nitrates. There was no statistical analysis comparing the groups regarding perfusion. In addition, this study also showed an improvement in perfusion without improvement in angina, as well as in the working ability.

Kastrup *et al.* [26] reported a trial of catheter-based intramyocardial injection of 0.5 mg of plasmid DNA encoding $VEGF_{165}$ in 80 no-option patients with CCS 3 or 4 (The Euroinject One Trial). VEGF gene transfer did not significantly improve stress-induced myocardial perfusion abnormalities compared with placebo. However, it was demonstrated that regional wall motion improved with NOGA® (Cordis Corp.) mapping and ventriculography. No ph$VEGF_{165}$-related adverse events were observed. However, NOGA procedure-related adverse events occurred in five patients: one cardiac tamponade, one myocardial infarction, one third-degree atrioventricular block, one temporary loss of vision and one sepsis. An explanation might be that perfusion increased as the regional wall motion increased. In addition, the spatial resolution of SPECT may not have been able to detect any difference in the subtle change of perfusion. MRI or positron emission tomography could detect such subtle changes. The technique and catheter of NOGA should, therefore, be modified and sophisticated.

5.2.3 *$VEGF_{121}$*

Stewart *et al.* [27] reported a randomized, controlled Phase II trial of the gene transfer of $VEGF_{121}$ for 67 patients with CCS Class 2 – 4 and with coronary artery disease not suitable for revascularization (REVASC study [Randomized Evaluation of VEGF for Angiogenesis in Severe Coronary Disease]). Thirty-two patients received 30 direct intramuscular injections of 4×10^{10} particle units of replication-defective adenovirus containing the $VEGF_{121}$ gene in the free wall of the left ventricle via a mini-thoracotomy, and 35 control patients received optimal medical therapy. The exercise time to 1 mm ST-segment depression significantly increased in the $VEGF_{121}$ group at 26 weeks, but not at 12 weeks. Angina symptoms significantly improved after VEGF gene transfer at 12 and 26 weeks. No significant changes in SPECT were seen in the $VEGF_{121}$-treated patients at 12 and 26 weeks. The protocol was not blinded because treatment in the control group was maximal medical therapy, and improvements could have been due to a placebo effect or inflammation resulting from the thoracotomy or vector administration.

There are two other trials from which the results have not been reported. The NORTHERN Trial (NOGA Angiogenesis Revascularization Therapy) is an ongoing multi-center, double-blind, placebo-controlled trial to assess the efficacy of intramyocardial NOGA-mediated delivery of adenovirus encoding $VEGF_{121}$ in patients with CCS Class 3 or 4 angina symptoms, who are also not considered to be ideal candidates for conventional revascularization [102]. The NOVA (NOGA Delivery of VEGF for Angina) trial is a multi-center, randomized, double-blind, placebo-controlled study of percutaneous intramyocardial injection of Ad$VEGF_{121}$ in 'no-option' patients with Class 2 – 4 stable angina. The NOVA trial was prematurely terminated [103].

5.2.4 *VEGF-2*

The GENASIS (Genetic Angiogenic Stimulation Investigational Study) trial is a randomized, double-blind, dose-ranging, placebo-controlled Phase IIb clinical trial of catheter-mediated intramyocardial injection of naked plasmid VEGF-2, which originally planned to enrol 404 patients with Class 3 or 4 angina who are not suitable candidates for traditional revascularization procedures. However, the GENASIS trial was terminated because of low efficacy and a high rate of cardiac tamponade events (1.36% in 295 patients). There was no statistically significant improvement in ETT [104,105]. This trial also demonstrated that the present technique and the device used catheter-mediated intramyocardial injection is not be sufficiently safe and, therefore, these treatment modalities need to be improved.

5.3 Combined therapy with G-CSF (non-randomized study)

Many randomized controlled Phase II or III trials with growth factors have not reported sufficiently positive results. The combination of growth factor therapy and cell transplantation may be a way to improve the outcomes. There is one clinical investigation of bone marrow stem cell mobilisation with a combination therapy of VEGF and G-CSF.

Ripa *et al.* assessed the effects of combined treatment with $VEGF_{165}$ plasmid and G-CSF in patients with severe chronic ischemic heart disease [28]. The G-CSF group received intramyocardial injections of $VEGF_{165}$ plasmid, followed 1 week later by G-CSF (10 mg/kg/day for 6 days). The VEGF group received intramyocardial injections of $VEGF_{165}$ plasmid only, and control group received intramyocardial injections of placebo. There was no improvement in myocardial perfusion in SPECT and also no improvement in symptoms in the G-CSF group compared with the other groups, but $CD34^+$ stem cells increased almost 10-fold in the G-CSF group compared with the control groups ($p < 0.0001$). The reason for the ineffectiveness of this trial may be due to the presumption that the homing of stem cells by G-CSF treatment was not sufficient. Combination with cell transplantation or with therapies that strongly induce the homing of stem cells would, therefore, be more effective. As a result, further investigations are needed.

6. Randomized controlled clinical trial for limb ischemia

Table 2 summarizes trials discussed in the following sub-sections.

6.1 Protein therapy: FGF-2

Lederman *et al.* [29] reported a Phase II trial of intra-arterial infusion of FGF-2 to 190 patients with intermittent claudication (the TRAFFIC study). Sixty-three patients received two intra-arterial infusions of placebo, 66 patients received an intra-arterial infusion of 30 μg/kg FGF-2 on day 1 and placebo on day 30, and 61 patients received an infusion of 30 μg/kg FGF-2 on day 1 and day 30. The single dose of FGF-2 treatment resulted in a significant increase in peak walking time at 90 days, but not 180 days. Repeat infusion at 30 days did not improve on the single infusion. Change of ankle-brachial index (ABI) was small but significant in both the single-dose and double-dose groups at 90 days, but not at 180 days, compared with placebo.

6.2 Gene therapy

For limb ischemia, as well as myocardial ischemia, because protein therapies have not shown satisfactory results, most trials have investigated genes, adenoviruses and plasmids.

6.2.1 $VEGF_{165}$

Makinen *et al.* [30] reported a randomized, placebo-controlled, double-blinded Phase II study of local catheter-mediated VEGF gene therapy after percutaneous transluminal angioplasty for 54 patients. Eighteen patients received 2×10^{10} plaque-forming units of VEGF–adenovirus, 17 patients received 2 mg of VEGF–plasmid/liposome, and 19 control patients received Ringer's lactate at the angioplasty site. Follow-up digital subtraction angiography revealed increased vascularity distally to the gene transfer site in the VEGF-treated groups. However, improvements in Rutherford class and ABI were similar between the treatment groups and control group. The ineffectiveness of this trial may be due to the intra-arterial administration, rather than an intramuscular injection. In addition, this trial also includes both critical limb ischemia and claudication patients, thus making it difficult to evaluate the result.

Kusumanto *et al.* performed a trial of intramascular injection of 2000 μg naked $VEGF_{165}$ plasmid in 54 diabetic patients with critical limb ischemia [31]. The amputation rate was not significantly different between the groups, whereas the percentage of patients with a > 15% increase in ABI or toe-to-brachial index was significantly higher, and the percentage of patients with a > 60% decrease in skin ulcer surface was also higher in VEGF-treated group.

6.2.2 $VEGF_{121}$

Rajagopalan *et al.* [32] studied a Phase II trial of adenovirus-mediated $VEGF_{121}$ therapy for 105 patients who had unilateral exercise-limiting intermittent claudication (RAVE [Rubeosis Anti-VEGF] trial). A dose of 4×10^9 and 4×10^{10} particle units of adenovirus-mediated $VEGF_{121}$ was injected once intramuscularly. The change in peak walk time, ABI, claudication onset time, and quality-of-life were all similar between the therapy group and the placebo group at 12 and 26 weeks. The ineffectiveness may have been due to an inappropriate, dose because $VEGF_{121}$ is much less potent than $VEGF_{165}$.

6.2.3 FGF-1

The TALISMAN (Therapeutic Angiogenesis Leg Ischemia Study for the Management of Arteriopathy and Non-Healing Ulcer) was a double-blind, placebo-controlled study of intramuscular injection of plasmid encoding FGF-1 in 114 critical limb ischemia patients not eligible for revascularization [106]. The percentage of patients with complete healing of ≥ 1 ulcer on the treated leg at week 26 did not differ significantly between the two groups. However, amputations were significantly reduced at week 52 in the FGF group, compared with the placebo group. This was the first trial to reduce amputation, but the other endpoints did not show any improvement.

6.2.4 HGF

The HGF-STAT trial was a randomized, double-blind, placebo-controlled trial of the intramuscular injection of HGF gene plasmid for the treatment of critical limb ischemia [33]. The foot transcutaneous partial pressure of oxygen ($TcPO_2$) in the high-dose group showed a statistically significant increase, compared with the placebo group. In addition, the ischemic ulcer improvement was not significant in the HGF-treated groups [107].

7. Experience of the authors of this review

7.1 Growth factor controlled release systems using a biodegradable gelatin hydrogel

The safety of dealing with genetic material has not yet been established and it is impossible to control the quantity and duration of gene expression. Furthermore, growth factor protein therapies do not achieve the desired results due to their short half-life. Therefore, the present authors have developed a controlled release system using a biodegradable hydrogel composed of gelatin. As the gelatin hydrogel is degraded, growth factor is gradually released (**Figure 1**) over a few weeks *in situ* without increasing the systemic concentration (**Figure 2**). The release period can be controlled by modifying the water content of the hydrogel (**Figure 2**).

Gelatin has been extensively used for pharmaceutical and medical purposes, and the biosafety of gelatin has been proven through long clinical use. Another unique advantage of gelatin as a drug carrier is its electrical nature, which can be changed by the collagen processing method. If the growth factor to be released has positively charged sites on the

Table 2. Randomized, controlled clinical trial for limb ischemia.

Trial	Growth factor	Protein/gene vector	Dose	Administration	N	Claudication or critical limb ischemia	Follow- up (months)	Result	Ref.
TRAFFIC	FGF-2	Protein	30 µg/kg	IA	190	Claudication	3	Improved walking time	Lederman *et al.* (2002) [29]
	$VEGF_{165}$	Adenovirus or plasmid	2×10^{10} adenovirus, 2 mg plasmid	IA	54	74% claudication, 26% CLI	3	Increased vascularity in DSA	Makinen *et al.* (2003) [30]
	$VEGF_{165}$	Plasmid	2 mg	IM	54	CLI	3	Improvement in hemodynamic and skin ulcers. Pain decreased	Kusumanto *et al.* (2006) [31]
RAVE	$VEGF_{121}$	Adenovirus	4×10^9, 4×10^{10}	IM	105	Claudication	6	No improvement	Rajagopalan *et al.* (2003) [32]
TALISMAN	FGF-1	Plasmid	4 mg × 4 times	IM × 4	107	CLI	6	Amputations were significantly reduced	Unpublished [106]
HGF-STAT	HGF	Plasmid	1.2, 8, 12 mg	IM	48	CLI	6	The foot $TcPO_2$ in the high-dose group increased	Unpublished [33,107]

ABI: Ankle brachial index; CLI: Critical limb ischemia; DSA: Digital subtraction angiography; IA: Intra-arterial; IM: Intramuscular; MR: Magnetic resonance; $TcPO_2$: Transcutaneous partial pressure of oxygen.

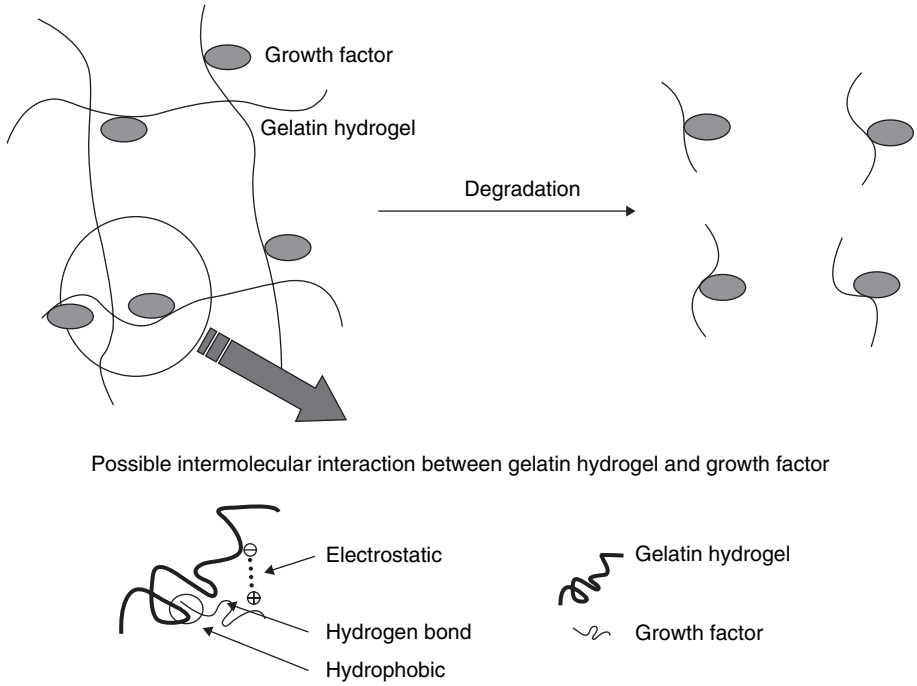

Figure 1. Growth factor release from a gelatin hydrogel.
Reprinted from [35].

portion of its surface that interacts with acidic polysaccharides present in the extracellular matrix, an acidic negatively charged gelatin is preferable as the carrier material. Animal studies have revealed that hydrogels made of gelatin were degraded in the body. Gelatin hydrogels can also be formulated into different shapes, such as disks, tubes, sheets, granules and microspheres.

The process for the manufacture of this hydrogel has been previously described [34,35]. In short, various amounts of glutaraldehyde were added to aqueous gelatin solutions and the cross-linking reaction was allowed to proceed at 4ºC for various time periods. Following the crosslinking reaction, the glutaraldehyde-crosslinked hydrogels were immersed in an aqueous solution of glycine at 37ºC for 1 h, to block residual aldehyde groups of glutaraldehyde, and then were rinsed with water. The prepared hydrogels were freeze-dried and sterilized using ethylene oxide gas. Then the gelatin hydrogel was impregnated with growth factors.

There are other growth factor controlled release systems. One of them uses alginate/heparin-agarose pellets [36], which was used in the Phase I trial of the local perivascular delivery of FGF-2, in addition to coronary artery bypass surgery [37]. Because alginate is a poorly biodegradable polysaccharide, and microsphere erosion is mainly achieved through the natural leaching out of calcium, it may be difficult to control carrier degradation and subsequent FGF-2 release. In contrast, gelatin is completely degraded in the body, thereby avoiding inflammatory and pharmacological responses *in vivo*. Moreover, in the present authors' controlled release system, the release period and shapes can be changed, and many other growth factors, such as HGF, PDGF-BB, BMP-2, can be delivered.

7.2 An animal study for ischemic heart disease

7.2.1 The optimal delivery method for the heart

The optimal delivery method of FGF-2 to the rat ischemic heart has been studied [38]. Myocardial infarction (MI) was created by ligation of the left anterior descending artery (LAD) in Lewis rats. Four weeks later, FGF-2 was administered by four different methods: intravenous injection of free 100 µg FGF-2 (group IV), intracoronary injection of free 100 µg FGF-2 (group IC), intramyocardial injection of free 100 µg FGF-2 into the infarct and periinfarct area (group IM), and intramyocardial injection of 100 µg FGF-2-incorporated gelatin hydrogel (group IMG). The FGF-2 was labeled with ^{125}I radioiodination. The FGF remaining in the heart 72 h after administration was < 0.1% in group IV and group IC, and 1.8% in group IM. However, 32% of the FGF-2 remained after 72 h in group IMG. No FGF-2 was detected in the blood in all groups at any sampling points. Therefore, the intramyocardial injection of FGF-2-incorporated gelatin hydrogel microspheres was the optimal method of FGF-2 administration.

In the clinical setting, gelatin hydrogel sheets or the direct intramyocardial injection of gelatin hydrogel microspheres via a thoracotomy could be applied. However, a thoracotomy is too invasive for some patients. Intramyocardial injection

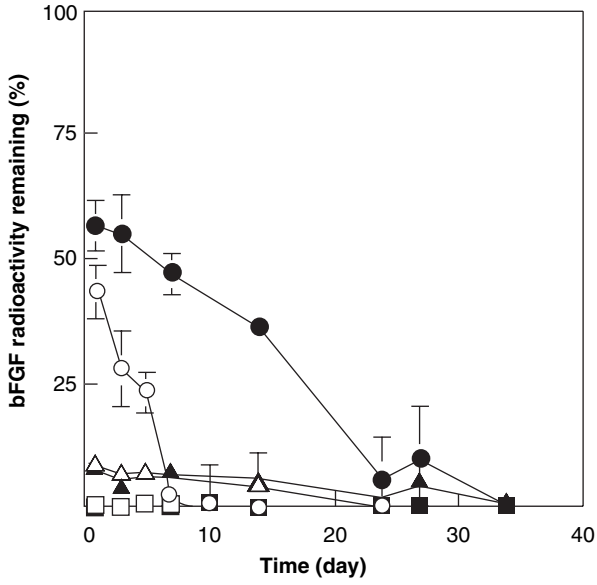

Figure 2. The time course of the radioactivity remaining of ^{125}I-labeled FGF-2-incorporated gelatin hydrogels with water contents of 98.8% (open marks) and 96.9% (closed marks) after implantation into the back subcutis of mice: in hydrogels (○●), around hydrogels (Δ▲), and in the blood (□■). The release period of bFGF is altered by modifying the water content of the hydrogel. There is no increase in the serum concentration of bFGF.

Rerpinted from TABATA Y, NAGANO A, IKADA Y: Biodegradation of hydrogel carrier incorporating fibroblast growth factor. *Tissue Eng*. (1999) **5**(2):127-138.

by either catheter or port-access delivery might, therefore, be the administration route of choice.

7.2.2 *Intramyocardial injection of controlled-release FGF-2 for myocardial infarction*

This controlled-release system of FGF-2 was studied in a pig myocardial infarction model [38]. The pigs were randomized into two groups, 4 weeks after ligation of the LAD. The control group received gelatin hydrogel microspheres containing saline, the FGF group received an intramyocardial injection of controlled-release 200 μg FGF-2 microspheres in the periinfarcted area of the left ventricular wall. Four weeks after the treatment, the FGF group had a smaller left ventricular diastolic diameter (48.7 ± 5.3, 56.7 ± 5.2 mm; FGF, control group, respectively; $p < 0.01$), higher endsystolic elastance (2.96 ± 1.2, 1.06 ± 0.3 mmHg/ml; $p < 0.01$), and higher vascular density (61.5 ± 18.3, 153.0 ± 29.0/mm^2; $p < 0.01$). In addition, the infarcted left ventricular walls tended to expand less and be thicker in the FGF group.

In conclusion, biodegradable hydrogel microspheres with FGF-2 have been reported to improve left ventricular function while inhibiting left ventricular remodeling by angiogenesis in pigs with chronic myocardial infarction.

7.2.3 *Combination therapy of FGF-2 and omentum for ischemic heart disease: 'Bio-CABG'*

In 1936, O'Shaughnessy introduced a technique called omentopexy, in which the greater omentum was brought through the left diaphragm and wrapped around the ischemic heart [39]. This procedure required an extended time period for graft–coronary communication to mature sufficiently. Therefore, a new strategy for the revascularization of severely diseased and tiny coronary arteries was developed, which involved: i) the use of a healthy large-bore donor artery (i.e., gastroepiploic artery [GEA]); and ii) stimulation of angiogenesis from this donor artery with simultaneous administration of FGF-2 to the ischemic myocardium.

In one study, acute MI was created by ligating the major branch of the circumflex artery in rabbits, which were subsequently divided into four groups [40]: no treatment (control group); the infracted area was wrapped with the omentum (GEA group); a gelatin hydrogel sheet incorporating 100 μg of FGF-2 was placed over the infracted area (FGF group); the infracted area was covered with a FGF-2 sheet followed by omental wrapping (Bio-CABG group). The FGF-2 sheets were fixed to the surface of the heart by stitching with 5-0 polypropylene sutures.

Angiography showed rich communication between the GEA and the coronary artery in the Bio-CABG group. The Bio-CABG group also showed a better fractional area change in echocardiography (Bio-CABG group, 65 ± 7%; FGF group, 46 ± 4%, $p = 0.0005$; GEA group, 51 ± 7%, $p = 0.002$; control group, 46 ± 1%, $p = 0.0004$; p values were compared with Bio-CABG group), as well as reduced infarction size (Bio-CABG group, 10 ± 3%; FGF group, 16 ± 5%, $p < 0.05$; GEA group, 19 ± 7%, $p < 0.01$; control group, 23 ± 2%, $p < 0.01$; p values were compared with Bio-CABG group) and increased artrioles from histology at 4 weeks (Bio-CABG group, 23 ± 5/mm^2; FGF group, 14 ± 3/mm^2, $p < 0.001$; GEA group, 10 ± 1/mm^2, $p = 0.001$; control group, 4 ± 2/mm^2, $p = 0.04$; p values were compared with Bio-CABG group).

This method was also studied for chronic myocardial ischemia in rabbits [41]. Cine MRI analysis showed a greater percentage wall thickening (Bio-CABG group, 49.2 ± 4.5%; FGF group, 41.2 ± 3.8%, $p < 0.05$; control group, 32.1 ± 2.5%, $p < 0.01$; p values were compared with Bio-CABG group) and a colored microsphere assay showed higher perfusion in the left circumflex region in the Bio-CABG group than in the other groups (Bio-CABG group, 2.83 ± 0.38ml/min/g; FGF group, 2.25 ± 0.51 ml/min/g, $p < 0.05$; control group, 0.25 ± 0.51 ml/min/g, $p < 0.01$; p values were compared with Bio-CABG group). Angiography via the gastroepiploic artery, and a microvascular corrosion cast, showed direct visible collaterals between the gastroepiploic and occluded left circumflex coronary arteries in the Bio-CABG group (**Figure 3**).

These results show that a bypass from the gastroepiploic artery to coronary arteries can be achieved without surgical

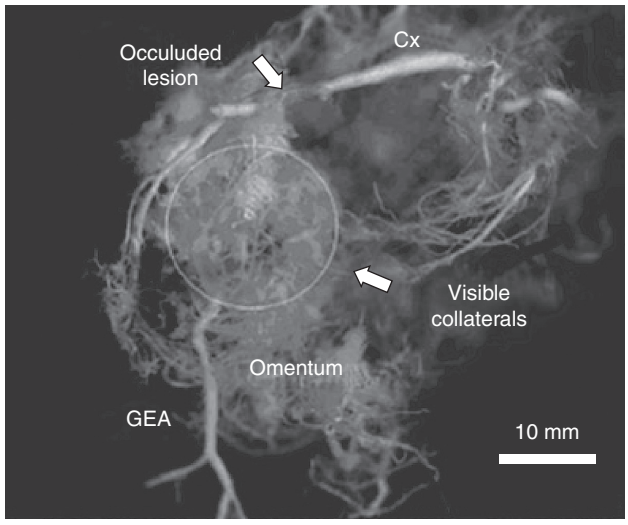

Figure 3. A microvascular corosion cast image of collateral formation in the Bio-CABG group.
Reprinted from [41], Copyright (2006), with permission from Elsevier.
Cx: Circumflex coronary artery; GEA: Gastroepiploic artery.

anastomosis through the sustained release of FGF-2. This new revascularization concept, namely, biologic coronary artery bypass grafting, can be applicable for revascularizing either severely diseased or tiny coronary vessels that are not feasible to revascularize by conventional methods.

7.3 Hybrid therapy with cell transplantation

7.3.1 Prevascularization by FGF-2 increases the efficacy of cardiomyocyte transplantation

The area in and around the infarcted regions is a poor environment for transplanted cells because the blood supply is insufficient. Therefore, the efficacy of prevascularization in ischemic regions before cell transplantation has been studied [42].

Rats with myocardial infarction were randomized into four groups: the control group received a culture medium injection to the left ventricular wall, the CM group received fetal cardiomyocyte transplantation, the FGF group received sustained-release of FGF-2, and the FGF-CM group received sustained-release FGF-2 pretreatment, followed by cardiomyocyte transplantation. In the FGF and FGF-CM groups, neovascularization was found in the scar tissue 1 week later. The left ventricular maximum time-varying elastance was higher in the FGF-CM group than in the CM and FGF groups (0.52 ± 0.23, 0.30 ± 0.08, and 0.27 ± 0.20 mmHg/μl; FGF-CM, CM and FGF groups, respectively; $p < 0.01$). Histologically, more transplanted cells survived in the FGF-CM group than in the CM group. Prevascularization with FGF-2 enhanced the benefits of cardiomyocyte transplantation.

7.3.2 Combination of FGF-2 or HGF and skeletal myoblast transplantation

Further studies were conducted to determine whether simultaneous administration of control-released FGF-2 enhances the efficacy of skeletal myoblast (SM) transplantation in a rat MI model. Four weeks after ligation of the LAD, the rats were randomized into three groups: neonatal SMs (5×10^6) were subepicardially transplanted in the MI area, and the MI and peri-MI area was covered by a gelatin sheet containing 100 μg FGF-2 (FGF/SM group); SM transplantation and a gelatin sheet with saline were applied in the same manner (SM group); culture medium alone was injected with a gelatin sheet with saline (control group). Four weeks later, left ventricular end-diastolic diameter and MI size were the smallest, end-systolic elastance was highest, Tau was lowest and vascular density in the MI and peri-MI area was highest in the FGF/SM group. The graft volume was much larger and the number of large arterioles in the graft is more in the FGF/SM group than that in the SM group.

In addition, the efficacy of controlled-released HGF in the skeletal myoblast transplantation was investigated [43]. Lewis rats with chronic MI were divided into four groups. In the HGF/SM group, neonatal SMs (5×10^6) were transplanted in the MI area with a gelatin sheet incorporating 40 μg of HGF. The SM Group received SM transplantation and the placement of a saline sheet. The HGF groups and the control group received culture medium injection plus HGF and saline sheet application, respectively. At 4 weeks, the HGF/SM group showed the smallest left ventricular diastolic dimension with echocardiography (HGF/SM group, 0.95 ± 0.01 cm; SM group, 0.97 ± 0.01; HGF group, 1.00 ± 0.01, $p < 0.05$; control group, 1.01 ± 0.01, $p < 0.01$; p values were compared with HGF/SM group), end-systolic elastance was highest (HGF/SM, SM, HGF, control group: 1.00 ± 0.10, 0.59 ± 0.07, 0.37 ± 0.05, and 0.34 ± 0.03 mmHg/μl, respectively, $p < 0.01$, versus groups SM and HGF, and control group) and Tau was the lowest after cardiac catheterization (HGF/SM, SM, HGF, control group: 13.8 ± 1.9, 15.6 ± 1.1, 20.7 ± 0.7, and 21.0 ± 0.6 ms; $p < 0.01$, versus HGF, control group). Inside the graft, the vascular density was higher (34.5 ± 3.2, 13.2 ± 0.9, 28.5 ± 2.4, and 14.5 ± 1.3/mm^2; HGF/SM, SM, HGF, control group, respectively; $p < 0.0001$ in ANOVA) and the percentage of fibrotic area was smaller in the HGF/SM group than in SM group.

Therefore, FGF-2 and HGF can enhance the efficacy of skeletal myoblast transplantation to infarcted hearts.

7.4 Animal studies for limb ischemia

7.4.1 Controlled-release FGF-2

The efficacy of sustained-release FGF-2 in a rabbit hindlimb ischemic model have been investigated [44]. An intramuscular injection of 30 μg and 100 μg of FGF-2-incorporated microspheres improved limb perfusion in a dose-dependent fashion. Angiography of the femoral artery in rabbits of the FGF-2 treated group showed marked collaterals that communicated from the proximal end of the resected femoral artery to the distal popliteal artery. Neither FGF-2

nor VEGF concentrations in the serum increased after FGF-2 treatment. These findings demonstrate that controlled-release FGF-2 dose-dependently improves blood perfusion in ischemic limbs by inducing growth of rich collateral vessels without systemic effects.

7.4.2 *Combination therapy of FGF-2 and HGF*

Combination therapy with different growth factors and also combination therapy with growth factors and adjuvant treatment were studied, because clinical trials of therapeutic angiogenesis with single growth factors have so far shown unsatisfactory results.

Further studies investigated whether the simultaneous intramuscular injection of FGF-2 and HGF could enhance blood vessel formation in the murine ischemic hindlimb, compared with FGF-2 alone or HGF alone [45]. Four weeks after treatment, blood perfusion recovery in the ischemic limb by the dual release of 5 μg of FGF-2 and 20 μg of HGF (94.2 ± 10.9%) was higher than that by either single growth factor (FGF-2, 51.2 ± 5.8%; HGF, 52.5 ± 8.0%), and it was equivalent to that observed with 80 μg of FGF-2 alone (95.1 ± 7.6%) or 80 μg of HGF alone (92.8 ± 7.6%). A histological evaluation at 4 weeks showed higher capillary density from the dual release than those from either single release (868 ± 173 vessels/mm^2, 204 ± 68 vessels/mm^2, 185 ± 98 vessels/mm^2; FGF-2+HGF, FGF-2, HGF, respectively; $p < 0.01$). The percentage of mature vessels assessed by α-smooth muscle actin staining was also higher in dual release than those in either single release (43.8 ± 7.8%, 9.5 ± 3.0%, 11.7 ± 3.8%; $p < 0.01$). This study demonstrates that the sustained dual release of a lower dose of FGF-2 and HGF can achieve equivalent blood perfusion recovery and more mature vasculature in the ischemic limb than a higher dose of FGF-2 alone or HGF alone. This trial suggests that the combination of several growth factors may, therefore, be a more effective treatment than a single growth factor.

7.4.3 *Adjuvant therapy and controlled-release FGF-2*

The effectiveness of adjuvant therapy using a serotonin blocker (sarpogrelate), in addition to sustained-release FGF-2, was investigated in a rabbit hindlimb ischemic model [46]. Two weeks after femoral artery removal, the rabbits were assigned to one of four experimental groups and treated for 4 weeks: control group: no treatment; S group: supplemented with a diet containing sarpogrelate; FGF group: single intramuscular injection of a sustained-release form of 50 μg FGF-2 microspheres; FGF/S group: combined treatment with sustained-release 50 μg of FGF-2 and sarpogrelate. Compared with the FGF group, the FGF/S group showed a significant improvement both in the resting regional blood flow, as assessed by colored microspheres (4.0 ± 0.7, 5.8 ± 1.0, 7.2 ± 0.6, 9.7 ± 2.0 ml/min per 100 g tissue; control, S, FGF, FGF/S group, respectively; $p < 0.01$) and by microangiography (0.41 ± 0.06, 0.42 ± 0.04, 0.68 ± 0.07, 0.79 ± 0.06; $p < 0.01$).

These studies demonstrated that adjuvant therapy (i.e., salpogrelate, in addition to sustained-release FGF-2), thus produced more effective neovascularization than single sustained-release FGF-2.

7.5 A clinical trial for limb ischemia

A Phase I – IIa study was conducted in seven patients with critical limb ischemia [47]. They were intramuscularly injected with 200 μg of FGF-2-incorporated gelatin hydrogel microspheres into the gastrocnemius of an ischemic limb. Endpoints were safety and feasibility of treatment after 4 and 24 weeks. One patient was excluded from the study after symptomatic improvements for social reasons. After evaluation of the other six patients, significant improvements were observed in a test of distance walked in 6 min (295 ± 42, 491 ± 85 m; pretreatment, after 24 weeks, respectively; $p = 0.023$) and in transcutaneous oxygen pressure (53.5 ± 5.2, 65.5 ± 4.0 mmHg; $p = 0.03$). The resting pain scale also improved (3.5 ± 0.2, 1.0 ± 0.6; $p = 0.022$). The ankle-brachial pressure index improved at 4 weeks, but not at 24 weeks (pretreatment, 0.62 ± 0.12; 4 weeks, 0.73 ± 0.14, $p = 0.024$, versus pretreatment; 24 weeks, 0.68 ± 0.11). Among five patients who had a non-healing foot ulcer, three patients showed complete healing, one showed reduced healing and one and no change at 24 weeks. Serum concentrations of FGF-2 were undetected or within normal level in all patients.

8. Conclusion

In summary, in randomized controlled trials for ischemic heart disease, the VIVA trial (intracoronary injection of $VEGF_{165}$ protein) showed symptomatic improvement but without an ETT improvement, and the REVASC trial (intramyocardial injection of adenovirus encoding $VEGF_{121}$) showed symptomatic and ETT improvement without perfusion improvement. The KAT trial (intracoronary injection of adenovirus encoding $VEGF_{165}$) showed perfusion improvement, but no statistical analysis was performed to compare these findings with the control group. The Euroinject One Trial (intramyocardial injection of adenovirus encoding $VEGF_{165}$) showed improvement in regional wall motion without perfusion improvement. The FIRST (intracoronary injection of FGF-2 protein), AGENT and AGENT 2 (intracoronary injection of adenovirus encoding FGF-4) did not show any significant improvement, compared with the control group.

In randomized controlled trials for limb ischemia, the TRAFFIC trial (intra-arterial injection of FGF-2 protein) showed an improvement in walking time. The trial performed by Kusumanto *et al.* (intramuscular injection of $VEGF_{165}$ plasmid) showed an improvement in both the hemodynamics and skin ulcers. TALISMAN (intramuscular injection of

FGF-1 plasmid) reduced amputation without any other endpoints. HGF-STAT (intramuscular injection of HGF plasmid) showed an improvement in $TcPO_2$. The RAVE trial (intramuscular injection of adenovirus encoding $VEGF_{121}$) and the trial performed by Makinen *et al.* (intra-arterial injection of adenovirus or plasmid encoding $VEGF_{165}$) did not show any significant improvement.

In conclusion, although animal studies of therapeutic angiogenesis using growth factor have shown great effectiveness, and Phase I clinical trials have also shown some efficacy, many randomized controlled Phase II and III trials have not yet shown sufficient efficacy, especially regarding perfusion. In addition, no trial has yet shown an improvement in mortality.

9. Expert opinion

Although animal studies of therapeutic angiogenesis using growth factors have shown great effectiveness, most clinical studies have shown less than sufficient results. One of the reasons is that most animal studies have been performed using normal young animals, but clinical trials typically enroll older patients with advanced atherosclerosis. The reason for the decreased effectiveness of growth factor therapy is that angiogenic response is decreased in older patients [48], and patients with concomitant diseases such as diabetes, hyperlipidemia [49]. Therefore, more animal studies with diabetic, hyperlipidemic or other atherosclerotic disease models are needed in order to elucidate the mechanisms of decreased effectiveness and develop more effective therapies for patients with advanced atherosclerosis.

Although Phase I clinical trials have shown efficacy, many randomized controlled Phase II or III trials have not shown a sufficient effect. One of the reasons for this discrepancy is the placebo effect. Therefore, in order to assess the efficacy of a treatment, a randomized controlled trial is needed.

According to a subgroup analysis of the FIRST and AGENT trial, more severely ill patients tended to improve more than less severely ill patients. And people who really need therapeutic angiogenesis are severely ill patients. As a result, we should consider that trials only include severely ill patients.

In addition, many questions such as which growth factor is the most effective, what dose is optimal, and which delivery system is the best, still remain to be clarified.

Our controlled release system of growth factors does not use genes, and the quantity and duration of growth factor administration is controllable. Gelatin is a safe material and different forms can be used in this system. This drug delivery system is administered locally without increasing the serum concentration of the growth factor. Therefore, this drug delivery system is a promising system for future therapeutic angiogenesis.

Because many randomized Phase II or III trials using a single growth factor do not show sufficiently positive results, therapeutic angiogenesis with a single growth factor may, therefore, not be sufficient. The combination of several growth factors, or a combination of a growth factor and cell transplantation, might be a promising approach. More animal studies and clinical studies are needed to develop a more efficient therapy.

Declaration of interest

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