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EXPERIMENTAL THERAPEUTIC ANGIOGENESIS AFTER MYOCARDIAL INFARCTION

EFFICACY OF DIFFERENT ANGIOGENIC FACTORS AND DELIVERY METHODS

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ABSTRACT

Angiogenesis, the stimulation of vessel growth, is a promising therapy in ischemic heart disease. A wealth of evidence has shown that several growth factors can induce neovascularization in ischemic models. However, the optimal application of growth factors and delivery method requires further exploration. VEGF, the most investigated therapeutic agent was used to study angiogenic efficacy and safety. In addition, as the angiogenic process is complex with multiple growth factors involved, different combinations of growth factors and with different delivery modalities were also studied.

A rat myocardial infarction model was used to investigate angiogenic effects up to 4 weeks after delivery that was performed one week after the myocardial infarction. VEGF-A_{165}, bFGF and PDGF-BB were administered into the myocardial infarction border zone as gene in plasmid or in adenoviral vector or as protein in a slow release alginate formulation. The amount of delivered growth factor was detected as protein, mRNA or by radiolabelling. Myocardial capillary and arteriolar densities were quantitatively detected by immunohistochemistry. Apoptosis analysis was done using TUNEL staining. Left ventricular function was determined by echocardiography.

While AdhVEGF-A_{165} expressed VEGF-A in the myocardium to a considerably higher extent than PhVEGF-A_{165}, both vectors increased arteriolar and capillary densities and left ventricular function to the same extent. AdhVEGF-A_{165} induced apoptosis and induced higher ectopic expression of VEGF-A than PhVEGF-A_{165}. The combined growth factor delivery of PhPDGF-BB+PhbFGF or PhPDGF-BB+PhVEGF did not show any angiogenic advantage over single growth factor delivery. However, when dual growth factor of PDGF-BB and VEGF-A_{165} were given as protein in alginate gel, more arterioles but not capillaries were observed with the dual protein transfer. Dual protein delivery increased left ventricular function better than single factor delivery.

The findings indicate that at least in the myocardial infarction model used, AdhVEGF-A_{165} has no obvious angiogenic advantage over PhVEGF-A_{165} but more side effects. Combined growth factor delivery for angiogenic therapy might benefit the ischemic tissue more than single growth factor administration. However, the angiogenic effect is dependent not only on growth factors but also on delivery modality and alginate gel might be a promising utility.

Key words: angiogenesis, arteriogenesis, apoptosis, myocardial infarction, VEGF-A_{165}, bFGF, PDGF-BB, adenovirus, plasmid, alginate

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numerals:

I. X. Hao, A. Månsson-Broberg, T. Gustafsson, K. H. Grinnemo, P. Blomberg, A. J. Siddiqui, E. Wärdell, C. Sylvén. Angiogenic effects of dual gene transfer of bFGF and PDGF-BB after myocardial infarction. Biochem Biophys Res Commun. 2004; 315: 1058-63.

II. X. Hao, A. Månsson-Broberg, P. Blomberg, G. Dellgren, A.J. Siddiqui, K.H. Grinnemo, E. Wärdell, C. Sylvén. Angiogenic and cardiac functional effects of dual gene transfer of VEGF-A_{165} and PDGF-BB after myocardial infarction. Biochem Biophys Res Commun. 2004; 322: 292-6.

III. X. Hao, A. Mansson-Broberg , K.H. Grinnemo, G. Dellgren, A.J. Siddiqui, L.Å Brodin, and C. Sylvén. Therapeutic angiogenesis. Comparison of efficacy following plasmid or adenoviral VEGF-A_{165} gene transfer in a rat myocardial infarction model. Submitted.

IV. X. Hao, E.A. Silva, A. Månsson-Broberg, G. Dellgren, A. J. Siddiqui, K.H Grinnemo, E. Wärdell, L.Å. Brodin, D.J. Mooney, C. Sylvén. Therapeutic angiogenic efficacy of combination VEGF-A_{165} and PDGF-BB protein with alginate slow release given intramyocardially after myocardial infarction. Manuscript.

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LIST OF ABBREVIATIONS

Ad	Adenovirus
CABG	Coronary Artery Bypass Grafting
CAR	Coxsackie-Adenovirus Receptor
CMV	CytoMegaloVirus immediate early promoter/enhancer
FGF	Fibroblast Growth Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMP	Good Manufacturing Practice
H	Human
HIF	Hypoxia Inducible transcription Factor
HMV	High Molecular Weight
HSPG	Heparan Sulfate Proteoglycan
ITR	Inverted Terminal Repeats
LAD	Left Anterior Descending coronary artery
LVDd	Left Ventricular Diastolic Diameter
LVDs	Left Ventricular Systolic Diameter
LMW	Low Molecular Weight
MI	Myocardial Infarction
MCP-1	Monocyte Chemoattractant Protein-1
P	Plasmid
PCI	Percutaneous Coronary Intervention
PDGF	Platelet-Derived Growth Factor
PIGF	Placental Growth Factor
SMC	Smooth Muscle Cell
VEGF	Vascular Endothelial Growth Factor
PLSD	Protected Least Significant Difference test

1 Introduction

1.1 General background

1.1.1 The angiogenesis process, health and disease

Angiogenesis is a general term for the process of growth of new vessels. There are three modes contributing to the process: vasculogenesis, angiogenesis (true angiogenesis), and arteriogenesis[1-3](Fig 1).

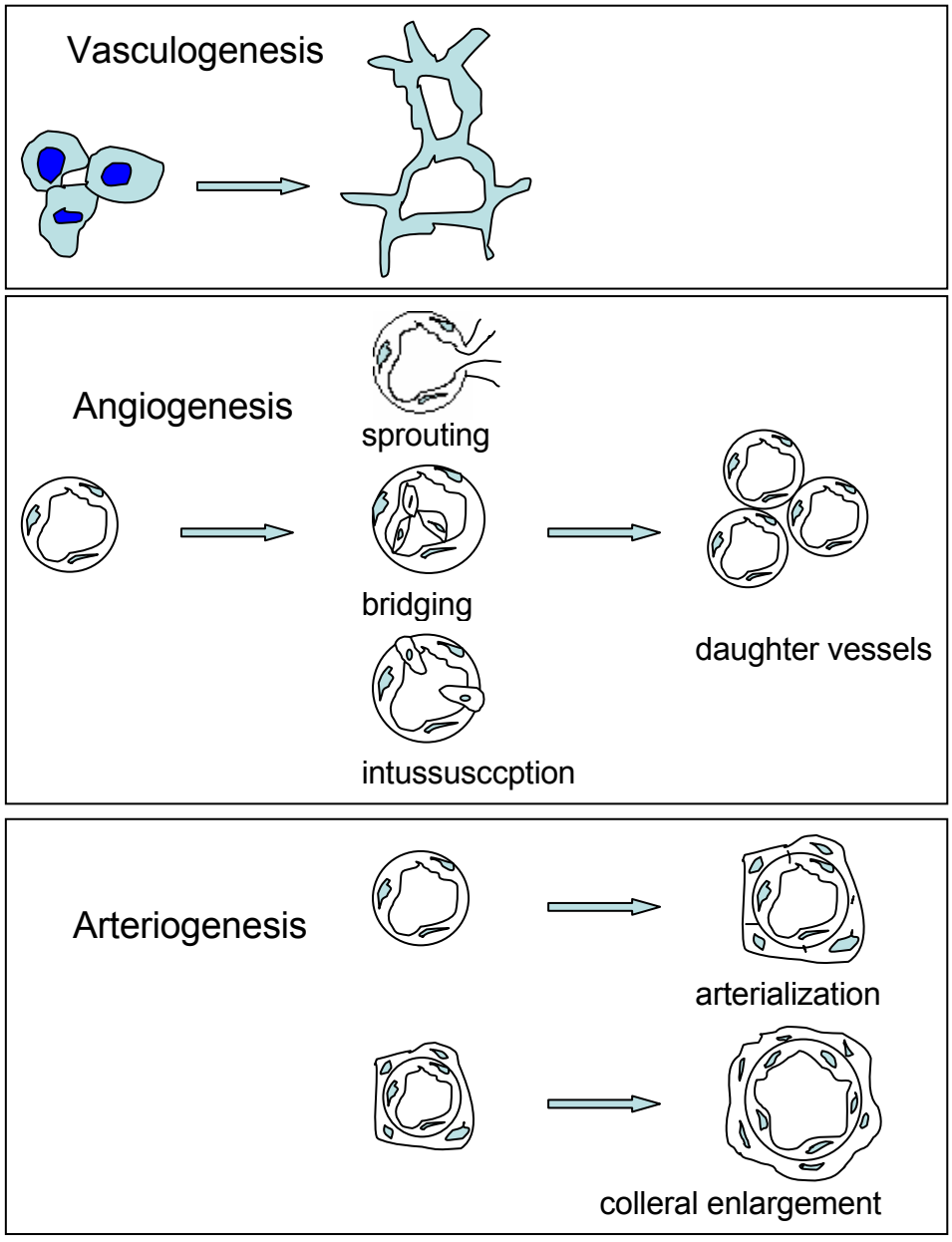


Fig. 1. Three modes of angiogenesis.

Vasculogenesis is vascular de novo formation by circulating vascular precursor cells. It is a crucial process for embryonic development. Recently, this process has also been considered to contribute to the vessel growth in adults[4-6]. Angiogenesis is defined as a process to grow of new capillaries from existing capillaries. Arteriogenesis is mainly a process of vessel maturation that remodels pre-existing capillaries or arteries/arterioles[7,8].

If vessel growth is dysregulated, excessive angiogenesis and insufficient vessel growth or abnormal vessel regression can lead to a varieties of diseases[2,9-11]. For example, cancer, autoimmune disorders, arthritis, and obesity are diseases for excessive angiogenesis.

1.1.2 Angiogenic switch

Angiogenesis is a process regulated by an angiogenic switch[11-13](Fig 2). VEGF, FGF, PDGF are examples of factors that contribute to the stimulation of angiogenesis. Anti-angiogenic molecules such as angiostatin and endostatin, on the contrary, lead to inhibition of angiogenesis.

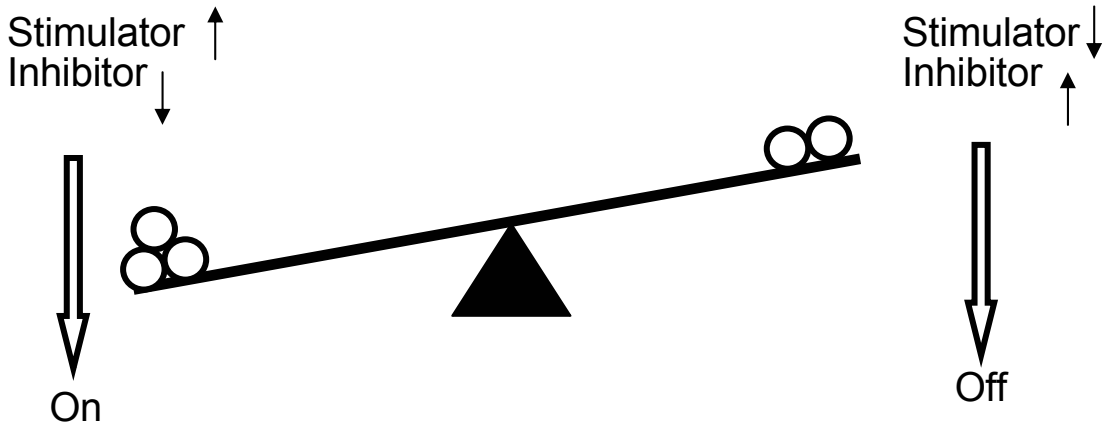


Fig. 2. Angiogenic switch is adjusted by the balance of angiogenic stimulators and inhibitors.

1.1.3 Angiogenic therapy for coronary artery disease

Traditional treatments for coronary artery disease are medical therapies and invasive treatment for revascularization such as CABG or percutaneuous coronary intervention PCI. However, there are a large number of patients who cannot be effectively revascularized using available mechanical techniques. For these patients, therapeutic angiogenesis may be a new option by delivery the angiogenic stimulus[14].

1.2 Therapeutic angiogenic agents

1.2.1 Growth factors

1.2.1.1 VEGFs

The VEGF family comprises 6 members: VEGF-A, -B, -C, -D, -E, and PIGF[15-17](Fig 3). These members bind to the three VEGF receptor tyrosine kinase: VEGFR-1, -2, and -3[18,19]. VEGFR-1 binds VEGF-A, -B, and PIGF. VEGFR-2 binds VEGF-A, VEGF-C, VEGF-D and VEGF-E. VEGFR-3 binds to VEGF-C and -D. The non-kinase receptors, neuropilins, binds to PIGF-2 and $VEGF_{164/5}$ but not to $VEGF_{120/1.}$ Among the VEGF family, VEGF-A has been widely studied for its angiogenic effect. The human VEGF-A gene has four isoforms: VEGF-A_{165}, VEGF-A_{121}, VEGF-A_{189}, and VEGF-A_{206} [20,21]. VEGF-A_{165} is the predominant isoform and binds to cell heparin in the matrix that may prolong its effects[20].

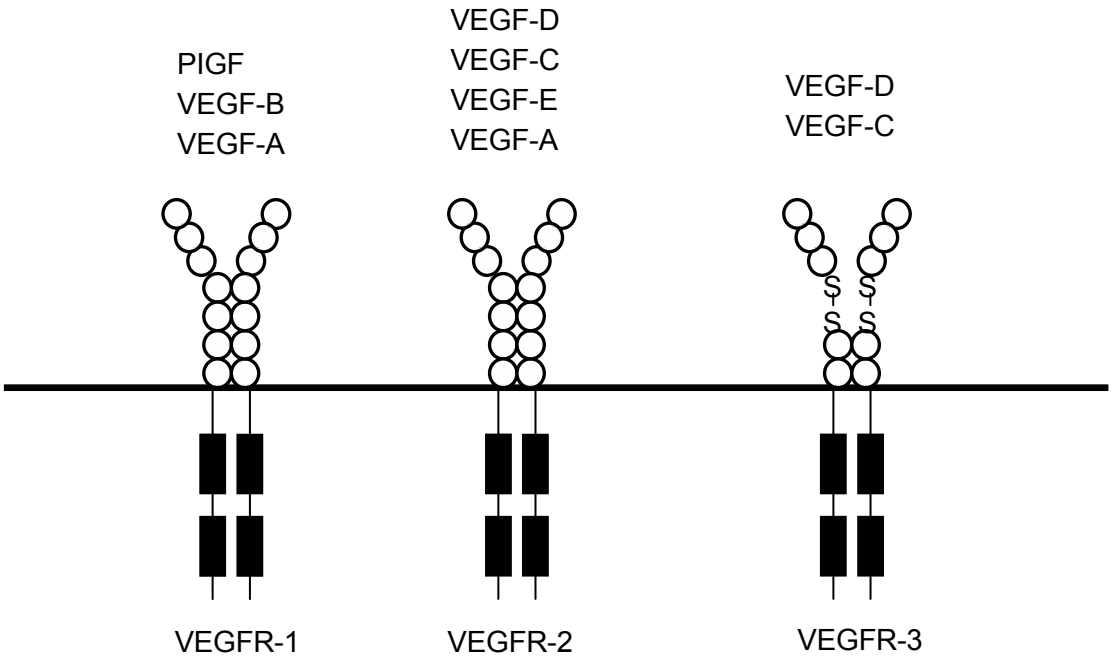


Fig. 3. VEGFs and VEGF receptors.

Although VEGF has been well documented to have its primary effect on endothelial cells[22,23], several studies showed that VEGF also induce arteriogenesis[24-26]. VEGF is also a survival factor for endothelial cells and prevents apoptosis process[15,27,28]. Vascular leakage can also be induced by VEGF; in fact, it was first named Vascular Permeability Factor[29].

1.2.1.2 FGFs

The FGF family includes 23 different members (from FGF 1-23)[1,30]. FGFs bind to FGF receptors that include 4 tyrosine kinases designated as FGFR1, FGFR2, FGFR3, and FGFR4. FGFs act in concert with heparin or heparan sulfate proteoglycan (HSPG) to activate FGFRs[31,32]. The FGFs stimulate the proliferation of cells of meso-, ecto-, and endo-dermal origin, including endothelial cells, smooth muscle cells, and myoblasts[33]. FGF-2 (bFGF) was the first characterized growth factors demonstrated to have an angiogenic effect[33,34].

1.2.1.3 PDGFs

The PDGF family members consist of different polypeptide chains derived from 4 different genes: PDGF-A, B, C, and D[35-37]. The 4 PDGF chains assemble into disulphide-bonded dimers via the process of homo- or heterodimerization. Five different dimeric isoforms have been found so far: PDGF-AA, -AB, -BB, -CC, and -DD. PDGF isoforms exert their cellular effects by binding to α and β receptors. Different PDGF isoforms induce different dimerization of receptors (Fig 4). Three receptor complexes (αα, αβ, and ββ) are formed. All of them can mediate potent mitogenic signals, and αβ is more efficient than the other two. The receptor complexes also have effects on chemotaxis. ββ stimulates chemotaxis, whereas αα inhibits chemotaxis.

PDGF is important for embryogenesis[38]. It has a significant role in the formation of connective tissue during wound healing in the adult. PDGF induces proliferation and migration of endothelial and vascular smooth muscle cell, monocytes, granulocytes, and fibroblasts. PDGF-BB showed a crucial effect on pericyte and smooth muscle cells recruitment[39] but weaker trophic effects on endothelial cells compared with FGFs and VEGF-A[38].

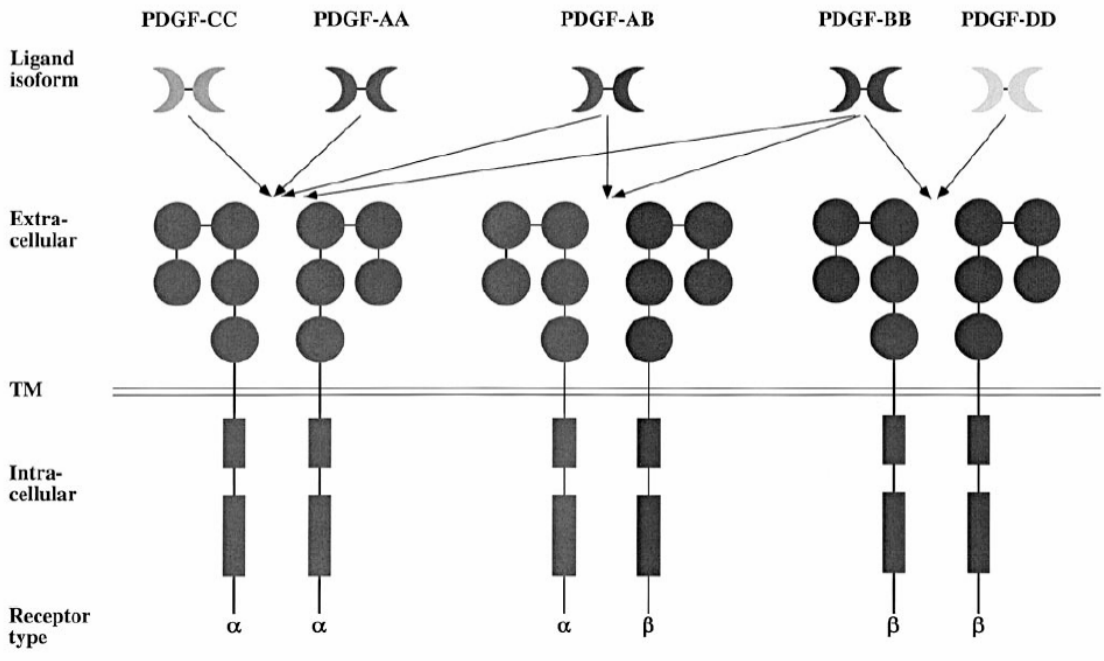


Fig. 4. PDGFs and PDGF receptors. (Adapted from Heldin, 2002, ref 18)

1.2.2 Other factors

Factors such as monocyte chemoattractant protein-1 (MCP-1)[40-42] and granulocyte-macrophage colony-stimulating factor (GM-CSF)[5,43] and hypoxia inducible transcription factor (HIF)-1_{α} also contribute to angiogenesis[44-46].

1.3 Delivery modalities for angiogenic agents

Growth factors can be delivered as protein[47,48], gene[49], or polymeric biomaterial combined with protein[50,51] or gene[52-54].

1.3.1 Protein

Protein has been used in clinical trials for the delivery of growth factors to induce myocardial angiogenesis[47,48]. Protein can be delivered in precise doses, but its half-time is short. As it is usually delivered systemically, potential high plasma concentration

with systematic adverse effect is unavoidable in order to obtain the local biological effect[55-57].

1.3.2 Gene

Gene transfer delivers the vectors into the cell to express the growth factor required. Plasmid and adenovirus are the vectors that have been commonly used for therapeutic angiogenesis[58,59]. Growth factor expression with gene therapy is transient but exposes the tissue for a longer time than direct protein administration.

Plasmids are small circular extrachromosomal DNA multiplied in prokaryotic cells (*Escherichia coli*)(Fig 5)[60]. Plasmids are safe and easily obtained in large quantities compared to viral vectors. However, they have lower transfection efficiency than adenovirus due to their low entry into the nucleus. The exact mechanism for entry is unclear, but it has been suggested to occur via endocytosis. Only a small amount of plasmid enter into the nucleus where it remains extrachromosomal[61,62].

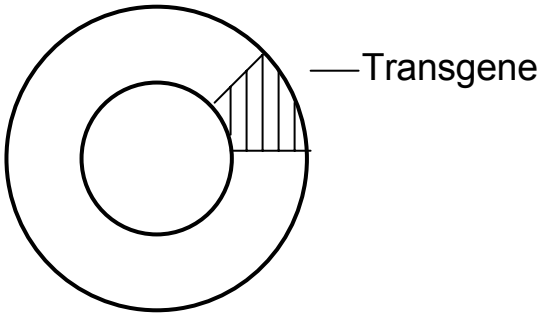


Fig. 5. Plasmid Structure.

Adenovirus can be taken up into the cells via the Coxsackie-adenovirus receptor (CAR) and through $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins[63], which makes it have a higher expression efficiency than plasmid[27,39,64,65]. Adenovirus remains extrachromosomal after entering the nucleolus. It does not integrate into the host genome[66] and has a transient expression. One disadvantage is the host immune reactions aroused by adenovirus[67,68]. In addition, adenovirus production and control are more costly than plasmid production.

Adenovirus has been modified when it is used as a vector. The adenoviral gene is partly deleted in order to prevent the replication of the virus, to increase the transgene carrier ability, and to decrease immune reactions from the host[66,69,70]. First generation of adenovirus vectors have been generated by E1 and/or E3 gene region deletions[66,71]. The transgene can be introduced into the deleted E1 region. Based on first generation, second generation of adenovirus has been obtained by deleting the E2 and/or E4 genes to reduce the immune reaction and prolong the gene expression time. The third generation of the adenovirus deprives nearly all of the genome that is called gutless adenovirus (Fig 6).

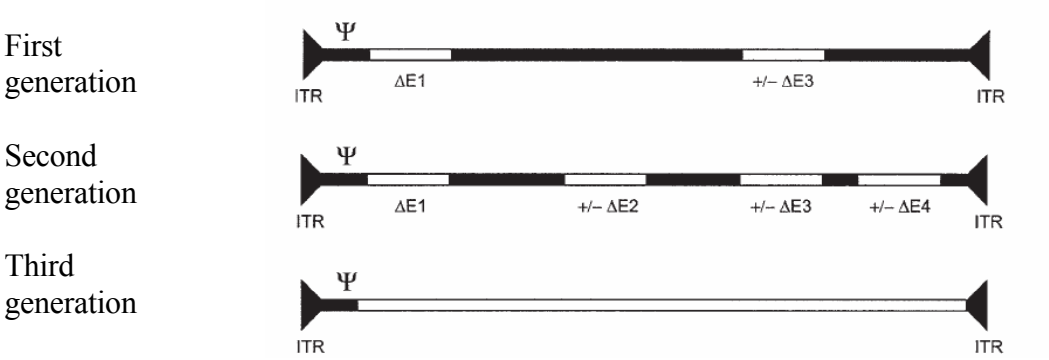


Fig. 6. Adenoviral genome structures of the first, second, and third generation. Regions that have been deleted are indicated by open boxes (Adapted from Kovesdi, 1997, reference 34).

1.3.3 Polymeric biomaterial

Another alternative for grow factor delivery is to deliver proteins or genes with polymeric biomaterials[72-74]. Growth factor concentration and delivery kinetics can be controlled in a tailored way. Different factors with different release kinetics can be administered by a single delivery.

1.4 Challenges

Angiogenic therapy has been carried out for about a decade. However, the optimal agent and delivery modality are still under investigation. Clinical studies have been emphasized on VEGF. The most commonly used delivery modalities for VEGF have been plasmid and adenoviral vectors. However, the benefit and safety issues of the two delivery methods have not been directly compared. In addition, as the angiogenic process is complex, multiple growth factors can be instrumental. Whether combined growth factor delivery has an advantageous angiogenic effect over single factor delivery has not been fully explored.

2. Aims of study

1. To investigate the angiogenic effect of the combination of PhbFGF and PhPDGF-BB compared to each factor alone in a rat myocardial infarct model.

2. To investigate the angiogenic effect of the combination of PhVEGF-A_{165} and PhPDGF-BB compared to each factor alone in a rat myocardial infarct model.

3. To compare the angiogenic effects of PhVEGF-A_{165} and AdhVEGF-A_{165} and adverse effects as regards apoptosis and ectopic expression of the transgene in a rat myocardial infarction model.

4. To investigate the angiogenic effect of intramyocardial dual delivery of VEGF-A_{165} and PDGF-BB proteins with alginate gel compared to single factor delivery after myocardial infarction.

3. Materials and methods

3.1 Animals

Sprague–Dawley (SD) rats weighing around 300 g (paper I and II) or Fisher rats weighing around 200-250g were used (paper III and IV) (B&K Universal, Sollentuna, Sweden).

3.2 Materials

3.2.1 Plasmids

PhVEGF-A_{165}: PhVEGF-A_{165} is a eukaryotic expression vector encoding for the 165 amino acid isoform of human VEGF-A, driven by a cytomegalovirus immediate early promoter/enhancer (CMV).

PhPDGF-BB: The human PDGF-BB gene was amplified by PCR. The amplified fragment was digested with *Bsi*WI and *Bam*HI and inserted into PhVEGF-A_{165} where the VEGF gene had been cut out.

PhFGF-2: The amplified human FGF-2 gene was inserted into digested PhVEGF-A_{165}. This procedure is similar to the procedure for PhPDGF-BB.

Plasmid LacZ: The amplified LacZ gene was inserted into PhVEGF-A_{165}. This procedure was similar to the procedure for PhPDGF-BB.

Placebo plasmid: VEGF gene fragment was cut out from the PhVEGF-A_{165} plasmid. Then the ends were annealed and the resulting plasmid was used as a placebo plasmid.

3.2.2 Adenovirus

AdhVEGF-A_{165}: Human GMP-grade first-generation adenovirus encoding human VEGF-A_{165} driven by a CMV promoter.

AdLacZ: Human GMP-grade first-generation adenovirus encoding *LacZ* marker gene driven by a CMV promoter.

3.2.3 Proteins

The proteins are recombinant human VEGF-A_{165} and PDGF-BB (R&D, USA).

3.2.4 Alginate

Ultra pure alginate (ProNova Biomedical, Norway) at high molecular weight (HMW) and low molecular weight (LMW) were used. LMW alginate was obtained by gamma (γ)-irradiating HMW alginate. Then both HMW and LMW alginate underwent an oxidization process to oxidize 1% alginate sugar residues. To form the binary alginate hydrogel, alginate solution was mixed with 2% LMW and HMW at the ratio of 75:25. Then the solution was cross-linked with aqueous slurries of a calcium sulfate solution to form the alginate gel. Protein was added into the LMW alginate solution before the binary alginate solution was mixed.

3.3 Methods

3.3.1 Myocardial infarction model

Rats were anaesthetized with midazolam (5mg/kg) and medetomidine hydrochloride (0.1mg/kg), and they were intubated and ventilated. The rat chest was surgically opened through the fourth intercostal space to expose the heart. Left anterior descending coronary artery (LAD) was identified and ligated. Pallor and regional wall motion abnormality of the left ventricle confirmed occlusion. One week after the LAD ligation, the chest was reopened and intramycardial growth factor transferring was performed by injection. Rats were randomly selected for the treatments.

3.3.2 Growth factor transfer in vivo

Transfer was carried out in the heart with or without myocardial infarction. The transfer was performed intramyocardially with single or multi-spots injection. In

myocardial infarction heart, periinfarct region along the border of the myocardial infarction was chosen as injection site.

Different growth factors were transferred with the different modalities: PhFGF-2, PhPDGF-BB, PhFGF-2+PhPDGF-BB (paper I); PhVEGF-A_{165}, PhPDGF-BB and PhVEGF-A_{165}+ PhPDGF-BB (paper II); PhVAdVEGF-A_{165}, AdLacz, PhVEGF-A_{165}, and PlacZ (paper III); protein hVEGF-A_{165}, hPDGF-BB or hVEGF-A_{165}+hPDGF-BB in alginate hydrogel (paper IV). Transfer volume was 100µl. Delivery dose was 20 µg plasmid in paper I and II and 40 µg in paper III. Adenovirus dose was $5\mathbf{X}10^9$ pfu. 3µg hVEGF-A_{165} and hPDGF-BB were delivered in paper IV.

3.3.3 hVEGF-A, PDGF-BB protein expression and hbFGF gene expression

VEGF or PDGF gene expression was determined in hearts by ELISA. Twenty-four hours after plasmid and 6 days after adenovirus gene transfer, hearts were harvested and minced with a homogenisator knife in homogenization buffer. Thereafter, the homogenized substance was centrifuged for 10 minutes at 14000 X rpm at 4ºC. The supernatant was collected and frozen at -70ºC. Later all frozen samples were analyzed by ELISA for human VEGF-A or human PDGF-BB by immunoassay according to the manufacturer's instructions (Quantkine, R&D system).

Because no antibodies specifically binding human bFGF were available, bFGF expression was determined by real-time PCR. Normal hearts injected with bFGF plasmid were snap-frozen in liquid nitrogen and total RNA was extracted using the Trizol reagent (Life technologies, USA) according to the manufacturer's recommendations. The RNA was treated with Dnase (Invitrogen, USA) to remove possible genomic and plasmid DNA contamination. Then RNA was reverse transcribed to prepare cDNA by Superscript Rnase H reverse transcriptase (Superscript, Invitrogen USA/Gibco-BRL) with random hexamer primers according to the manufacturer's

specifications. Real-time PCR was used for measurement of specific mRNA transcript from the bFGF plasmid (ABI-PRISMA 7700 Sequence Detector, Perkin–Elmer Applied Biosystems, Foster City, CA, USA). Because of endogenous control for potential variation in RNA loading and quantification, 18S were used. The fluorescent threshold was set in the exponential phase of product formation.

3.3.4 PDGF and VEGF release kinetics

^{125}I-VEGF-A_{165} and ^{125}I-PDGF-BB were purchased from PerkinElmer Life Sciences (USA). Binary alginate gel with the respective radiolabelled growth factor was prepared as the procedure described above. The resulting mixture was then cast between two glass plates separated with 1-mm spacers and allowed to gel for 30 minutes. The gels were divided into four samples and subsequently incubated in 3-ml of PBS buffer solution (Invitrogen, PBS solution with 0.1g/l of $MgCl_2 \cdot 6H_2O$ and 0.132g/l of $CaCl_2 \cdot 2H_2O$) at 37 °C. At each experimental time point, the radiolabelled growth factors in the extracted buffer solution were measured using a gamma counter - 1470 WIZARD (PerkinElmer, USA).

3.3.5 Capillary and arteriolar densities

Hearts were collected and frozen on OTC compound. Frozen sections were prepared. For the analysis of capillary density, the sections were incubated with Griffonia Badeiraea Simplicifolia Isolectin B4 (GSL-I-B4, Vector Laboratories) followed by a second incubation with ABC Complex. Finally, capillaries were visualized by DAB with supplementation of 0.03% hydrogen peroxide. Capillaries were counted at a magnification of 40X taken by a LCD camera (Olympus, Japan) connected with a microscope. Pictures of eight fields around the injection site were taken and capillary count was analyzed blinded with an image analysis program (Micro Image, Olympus). For the analysis of arteriolar density, the lectin stained sections were incubated with

primary antibody against α-actin (Sigma). Rabbit anti-mouse secondary antibody (FITC, Dako) was used to visualize the blood vessels. Blood vessels stained around the injection site were counted using a fluorescence microscope (20X). All analyses were performed in a blinded manner.

3.3.6 Apoptosis assay

TUNEL assay was performed on frozen sections with an ApopTag Fluorescein in Situ Apoptosis Detection Kit (CHEMICON International, CA, USA) according to the manufacturer's specifications. Heart slides were treated with digoxigenin-dNTP and termial deoxynucleotidyl transferase followed by incubation with anti-digoxigenin conjugated with fluorescein. Counterstaining with DAPI was performed to visualize nuclei. Five fields of apoptotic positive cells were counted under 400X magnification in a blinded fashion. Two areas along the border of myocardial infarction were analysed: the injection site and the contra lateral site of the myocardial infarction where no injection had been made.

After TUNEL staining, slides were incubated with anti-human desmin to detect if the apoptotic cells were cardiomyocytes. Then fluorescent-labelled (TRITC) secondary antibody was added for visualization of cardiomyocytes.

3.3.7 Cardiac function

Cardiac function and dimensions were assessed by echocardiography using a Vingmed Vivid 5 (Vingmed A/S, Norway) ultrasound system equipped with a 10 MHz transducer. Echocardiography was performed on the rats 3 days after ligation and at 1 and 4 weeks after plasmid injection. The heart was imaged in the 2-D mode in the long axis view at the level of the largest left ventricular diameter. The left ventricular end-diastolic dimension (LVDd) and the left ventricular end-systolic dimension (LVDs) were measured (paper I and II). Colour Tissue Velocity Image was performed at the

apical chamber of view at the frame rates close to 230 fps (paper III and IV). The probe position was adjusted under the guidance of Pulsed Tissue Doppler to the maximum velocity of the mitral valve. The recording was obtained by positioning a sample volume in each basal septum wall segment. Time velocity integrals were measured from 2-3 beats to minimize the variability. The final value of regional systolic myocardial function was calculated as a mean value from 2-3 peak time velocity integrals.

3.4 Statistical analysis

Data are presented as mean ± SEM. Comparison between the groups was made by means of Student t-test, 1-way ANOVA followed by Fisher's PLSD test. Paired t-test was performed to compare the difference between baseline and after treatment. Values were considered to be statistically significant at a value of $p \leq 0.05$.

4. Results

4.1 Paper I

Aims

To investigate the angiogenic effect of the combination of PhbFGF and PhPDGF-BB myocardial gene transfer compared to each factor alone in a rat myocardial infarct model.

Results

Protein expression of hPDGF-BB in rat hearts 24 hours after injection of PDGF-BB and bFGF + PDGF-BB was 98 ± 6.6 and 93 ± 9.2 pg/ml, respectively. mRNA expression of bFGF in rat hearts 24 h after gene transfer in bFGF and bFGF + PDGF-BB transferred hearts was 0.086 ± 0.0048 and 0.066 ± 0.0016 arbitrary units, respectively ($P<0.5$). PhbFGF, PhPDGF-BB and their combination all had a more pronounced angiogenic effect on the arteriolar than the capillary level (Fig. 7). PhbFGF stimulated both capillary and arteriolar growth while PhPDGF-BB preferentially stimulated arterioles. The combination increased the amount of both capillaries and arterioles and in addition gave rise to stable capillaries compared to single factor transfer ($p<0.05$) but did not further enhance angiogenesis.

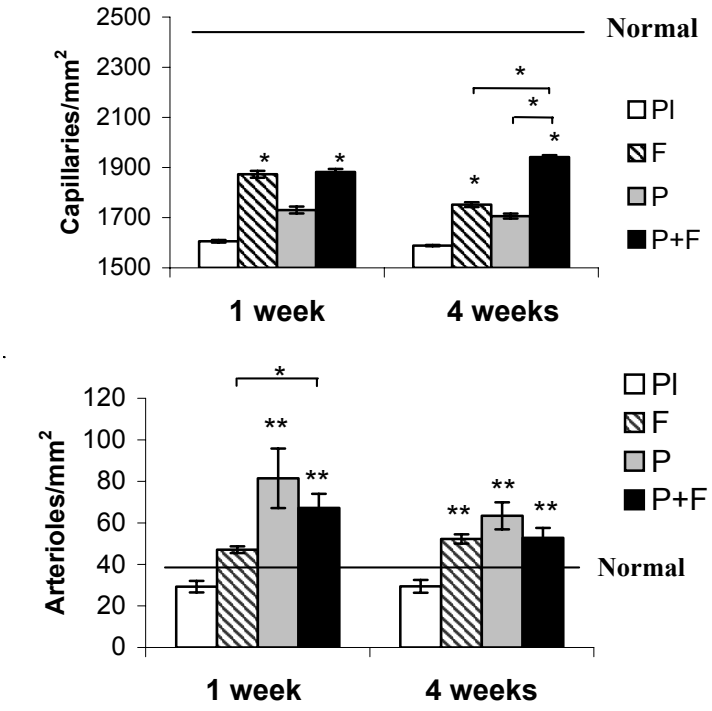


Fig. 7. Capillary (above) and arteriolar (below) densities in the border region of myocardial infarction 1 and 4 weeks after plasmid injection. Values are mean ± SEM. ANOVA followed by Fisher's PLSD test. *P<0.05, **P<0.001. C: control; F: bFGF; P: PDGF-BB; P+F: bFGF PDGF-BB.

Cardiac function showed that gene transfer in the four groups did not influence the remodelling that caused increments of both LVDs and LVDd (Fig. 8). Cardiac function did not improve with the treatments.

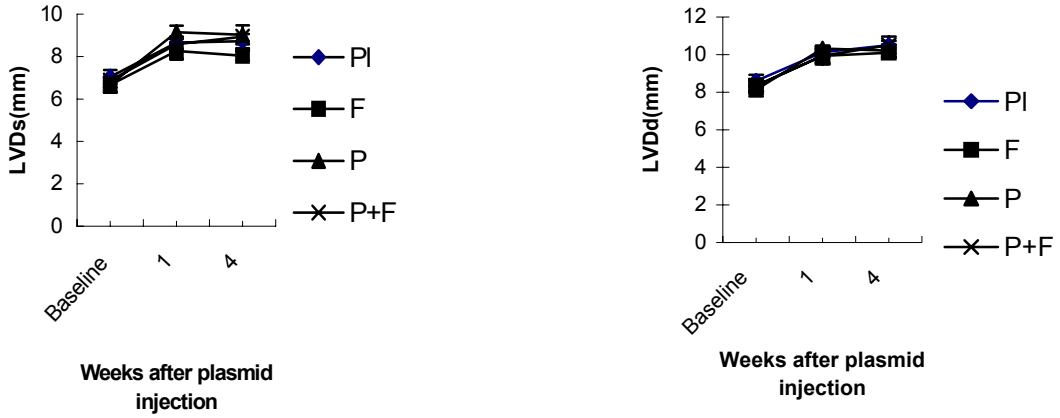


Fig. 8. Left ventricular systolic diameter, LVDs (left) and diastolic diameter, LVDd (right) measured by echocardiography. No significant difference was found between groups at baseline, 1 and 4 week after plasmid injection. Values are mean ± SEM

Conclusions

Simultaneous combined delivery of PhFGF-2 and PhPDGF-BB has no angiogenic advantage over single plasmid delivery.

4.2 Paper II

Aims

To explore the angiogenic effect of the combination of PhVEGF-A_{165} and PhPDGF-BB myocardial gene transfer compared to each factor alone in a rat myocardial infarct model.

Results

VEGF-A and PDGF-BB were both expressed 24 hours after gene transfer of PhVEGF-A_{165} and PhPDGF-BB (211 ± 45 and 116 ± 21 pg/ml, respectively). PhVEGF-A_{165} +PhPDGF-BB expressed VEGF-A and PDGF-BB with no difference to the single

plasmid treatments (141 ± 15 pg/ml for VEGF-A, $p = 0.18$; 92 ± 20 pg/ml for PDGF-BB, $p = 0.44$).

PhVEGF-A_{165} increased capillary density more than PhPDGF-BB, and PhPDGF-BB preferentially stimulated arteriolar growth (Fig. 9). The combination increased both capillaries and arterioles but did not enhance angiogenesis any more than single plasmid treatment did.

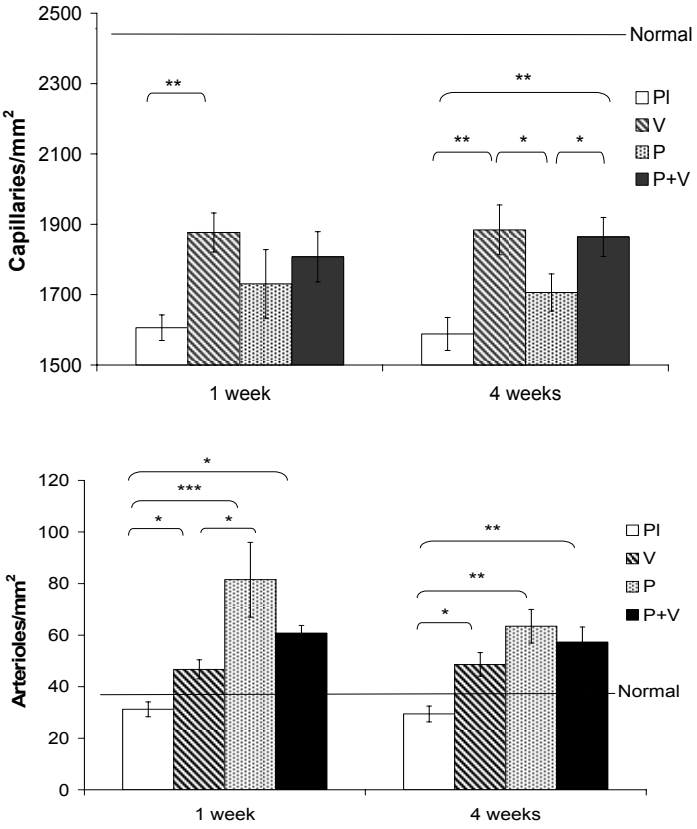


Fig. 9. Capillary (above) and arteriolar (below) densities in the border region of myocardial infarction 1 and 4 weeks after plasmid injection. Values are mean ± SEM. ANOVA followed by Fisher's PLSD test. *P<0.05, **P<0.001. C: control; F: bFGF; P: PDGF-BB; P+F: bFGF PDGF-BB.

VEGF-A_{165} counteracted left ventricular dilatation after 1 week in terms of LVDd and LVDs ($p < 0.01$, Fig. 10). The combination showed a similar effect to VEGF-A_{165}, but the combination only had significant effect on LVDd (p<0.05). Cardiac function

remodelling of the left ventricle after 4 weeks was not counteracted by any of the treatments.

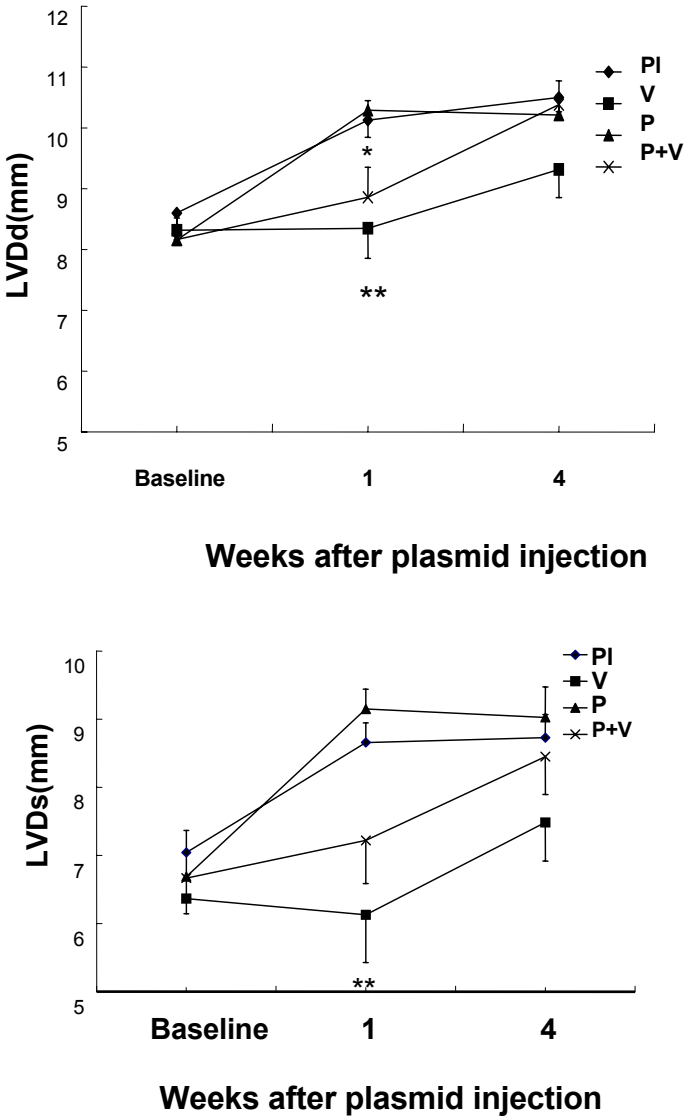


Fig. 10. Left ventricular systolic diameter, LVDs (above) and diastolic diameter, LVDd (below) measured by echocardiography. No significant difference was found between groups at baseline, 1 and 4 week after plasmid injection. Values are mean ± SEM.

Conclusions

Simultaneous combined delivery of PhFGF-2 and PhPDGF-BB stimulates angiogenesis both at the capillary and arteriolar levels and transiently counteracted cardiac remodelling after myocardial infarction. However, it is not advantageous compared to single plasmid delivery.

4.3 Paper III

Aims

To compare the angiogenic effect of PhVEGF-A_{165} and AdhVEGF-A_{165} and adverse effects as regards apoptosis and ectopic expression of the transgene expression in a rat myocardial infarction model.

Results

AdVEGF-A_{165} induced substantially higher myocardial VEGF-A expression than PhVEGF-A_{165} (17280±5467 versus 890±452 pg/ml in normal myocardium, P<0.001; 938±290 versus 60±13 pg/ml in periinfact myocardium, p<0.01, Fig. 11). However, AdVEGF-A_{165} and PhVEGF-A_{165} induced angiogenesis to a similar extent (Fig. 12). Arteriolar density was increased 40-80% with the two vectors after 1 and 4 weeks of gene transfer (AdVEGF-A_{165} or PhVEGF-A_{165} versus PLacZ, p< 0.05). Capillary density was also increased 15-20% after one week (p<0.05, AdVEGF-A_{165} or PhVEGF-A_{165} versus PlacZ). After 4 weeks of treatment, only AdVEGF-A_{165} showed a tendency to increased capillary density. AdLacZ induced transient increment of capillary and arteriolar densities after 1 but not 4 weeks.

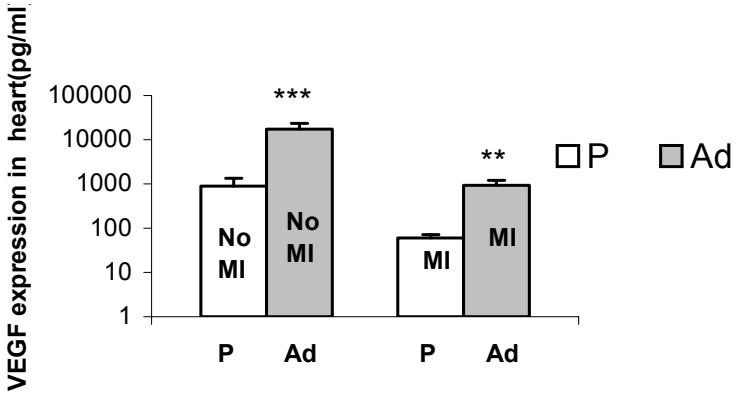


Fig. 11. VEGF-A expression in heart after gene transfer of phVEGF-A_{165} (24 hours), and AdhVEGF-A_{165} (6 days) without and with myocardial infarction. Values are mean ± SEM. P: PhVEGF-A_{165}, Ad: AdVEGF-A_{165}. **p<0.01, ***p<0.001, AdVEGF-A_{165} versus PhVEGF-A_{165}.

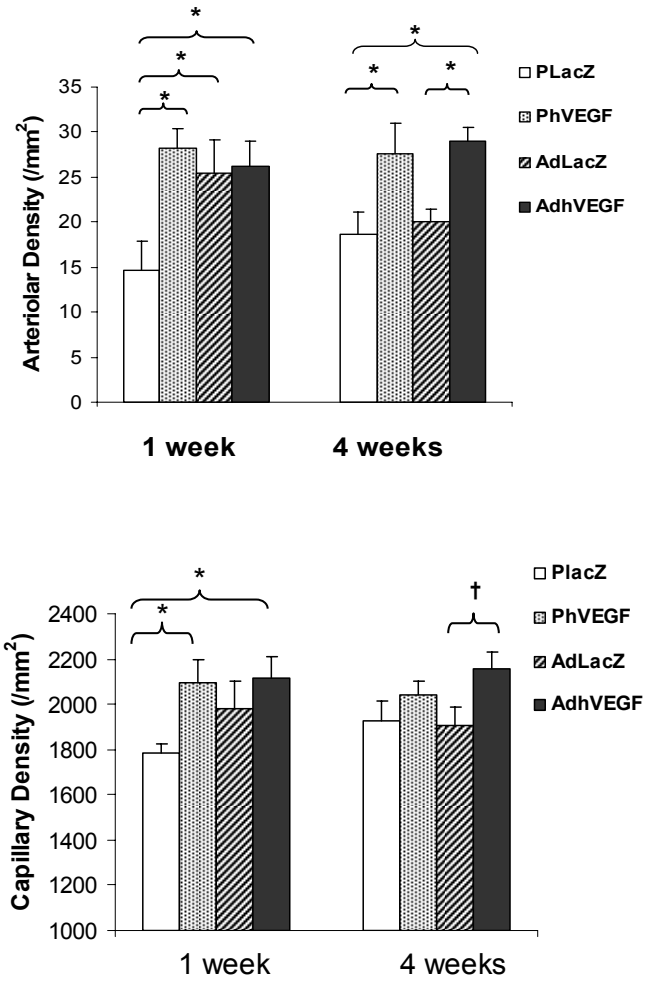


Fig. 12. Arteriolar (above) and capillary densities (below) 1 and 4 weeks after gene transfer of PhVEGF-A_{165}, PLacZ, AdhVEGF-A_{165}, and AdLacz. Values are mean ± SEM. *p<0.05, ANOVA followed by Fisher's PLSD test; †p<0.05, Student's t-test, where no significance was found with ANOVA.

Cardiac function deteriorated around 30 % with time with PLacZ treatment compared to baseline (p<0.05, Fig. 13), whereas it did not change with AdLacZ and improved with AdhVEGF-A_{165} (p<0.05) or Ph VEGF-A_{165} (p<0.05) to 47-57 %. After 4 weeks of treatment, ventricular velocity-time integral increased both after PhVEGF-A_{165} and AdhVEGF-A_{165} delivery compared to PlacZ (P<0.05).

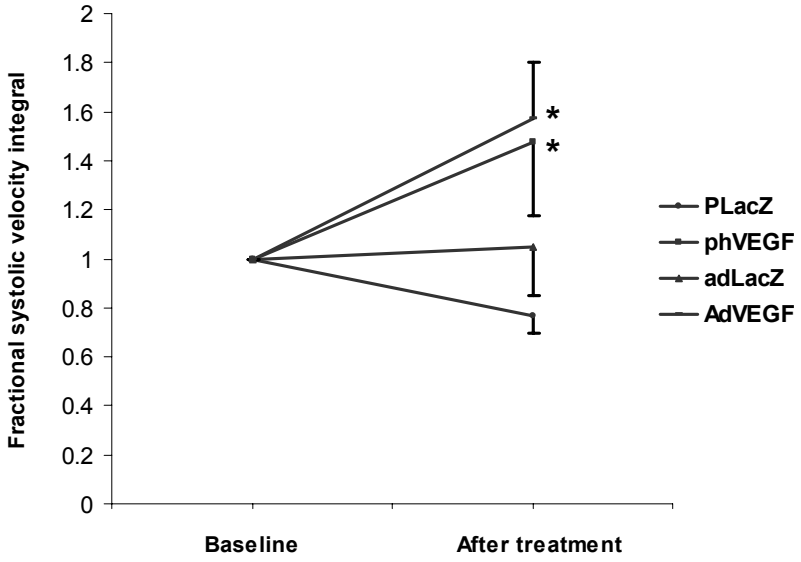
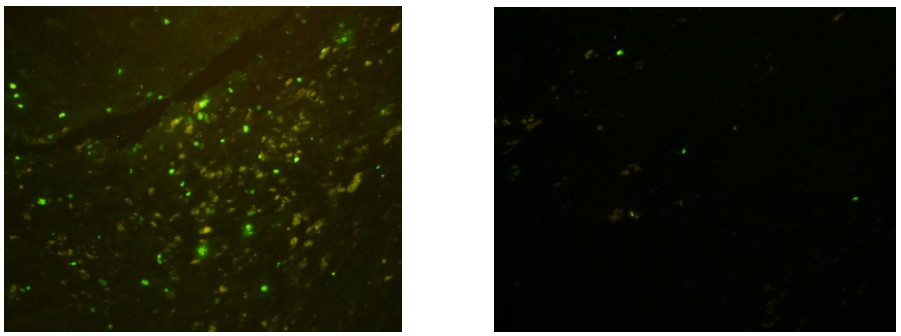


Fig.13. Change of fractional systolic velocity-time integral 4 weeks after gene transfer of PhVEGF-A_{165}, PlacZ, AdhVEGF-A_{165}, and AdLacz. *P<0.05, versus PLacZ, ANOVA followed by Fisher's PLSD text.

Plasmid and saline injection did not significantly increase the apoptotic cell number compared to the non-injected site, but both AdLacZ and AdhVEGF-A_{165} induced a higher number of apoptotic cells (31±6 and 24±8 cells/fields, respectively; P<0.001). No significant difference was observed between AdLacZ and AdhVEGF-A_{165} (Fig. 14). Double staining showed that some of the apoptotic cells were cardiomyocytes (Fig. 15).

A. B.



C.

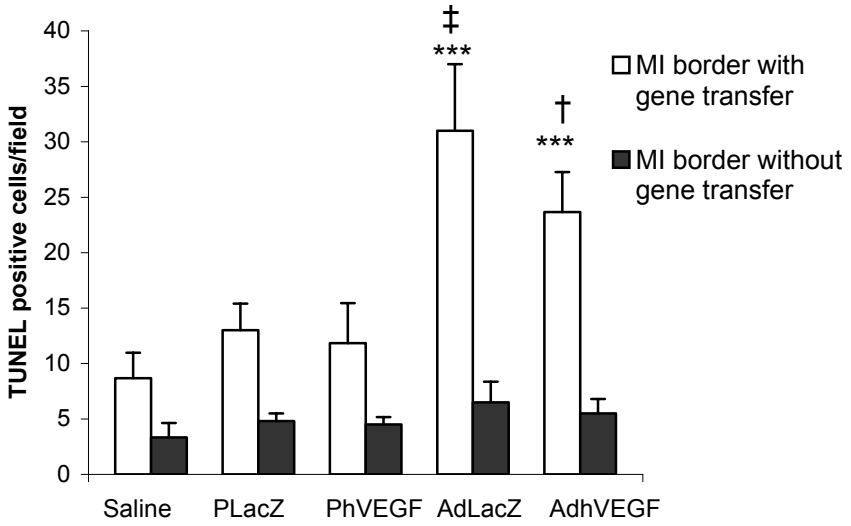


Fig.14. Apoptotic cells in the border zone of the myocardial infarction one week after gene transfer in injected and contra lateral non-injected area. TUNEL positive cells after treatment by AdLacZ in myocardial infarction border zone in (A) injected and (B) non-injected area, 400X. (C) Counted positive cells in non-injected and injected area after treatment with saline, PLacZ, PhVEGF-$A_{165,}$ AdLacZ and AdhVEGF-A_{165}. Values are mean ± SEM. *$p<0.001$, versus non-injected area, †$p<0.05$, ‡ $p<0.01$ versus saline.

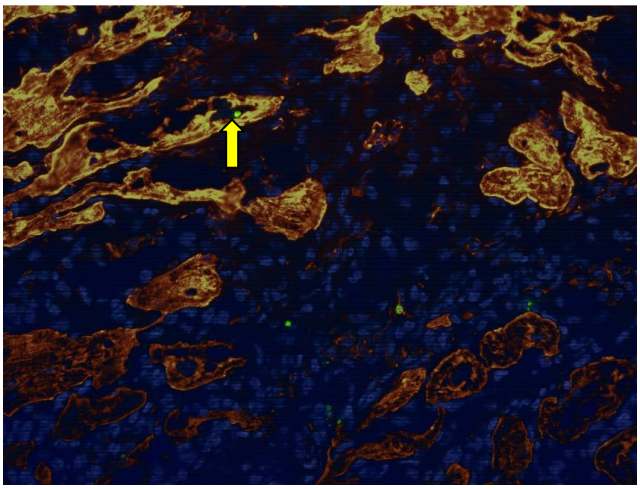


Fig. 15. Double staining for TUNEL and anti-desmin. Double stained cell is marked with arrow.

Ectopic expression was present for both PhVEGF-A_{165} and AdhVEGF-A_{165} treatment but substantially higher after AdhVEGF-A_{165} gene transfer (Fig. 16).

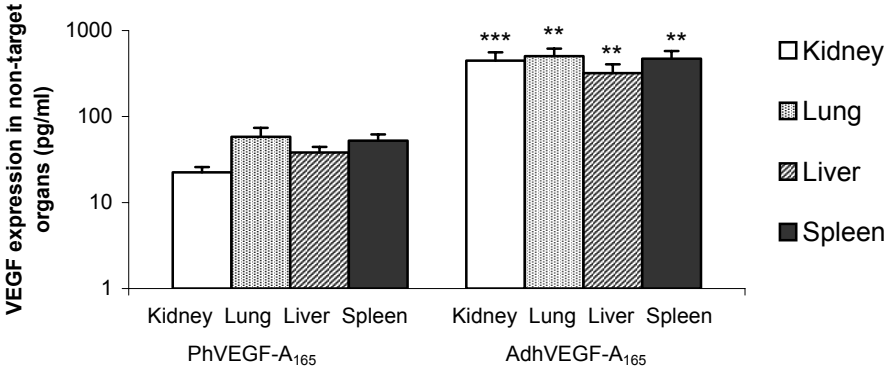


Fig. 16. Ectopic expression of VEGF-A in non-target organs after intramyocardial gene transfer of phVEGF-A_{165} and AdhVEGF-A_{165}. Values are mean ± SEM. **p<0.01, ***p<0.001, AdhVEGF-A_{165} versus PhVEGF-A_{165}.

Conclusions

Intramyocardial AdhVEGF-A_{165} gene transfer induced substantially higher hVEGF-A protein expression than PhVEGF-A_{165} in the myocardium. PhVEGF-A_{165} and AdhVEGF-A_{165} stimulated vessel growth and improved left ventricular function to a similar extent. AdhVEGF-A_{165} induced more apoptotic cells and also higher ectopic expression of VEGF than PhVEGF-A_{165} gene transfer. Thus at least in this myocardial infarction model AdhVEGF-A_{165} has no obvious angiogenic advantage over PhVEGF-A_{165}.

4.4 Paper IV

Aims

To investigate the angiogenic effect of dual delivery of VEGF-A_{165} and PDGF-BB proteins with alginate gel compared to single factor delivery after myocardial infarction.

Results

PDGF-BB had slower release kinetics than VEGF-A_{165} in alginate gel (Fig. 17).

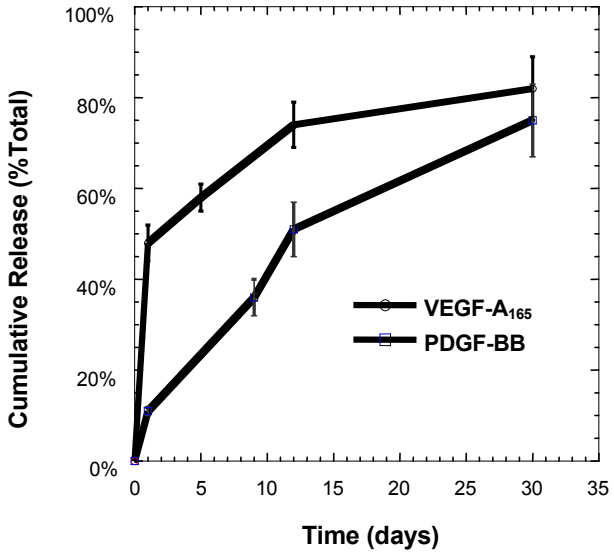


Fig. 17. In vitro release kinetics of VEGF-A_{165} and PDGF-BB from alginate gel. Values are given as mean ± SEM.

Both VEGF-A_{165} and PDGF-BB alone increased arteriolar density ($p<0.001$, PDGF-BB versus alginate blank; $p<0.01$, VEGF-A_{165} versus alginate blank; Fig. 18). However, only VEGF-A_{165} increased capillary density compared to blank alginate ($p<0.05$). Dual growth factor administration led to even higher elevated density of arterioles ($p\leq0.05$, dual versus PDGF-BB; $p<0.05$, dual versus VEGF-A), whereas no further increment was found in capillary density.

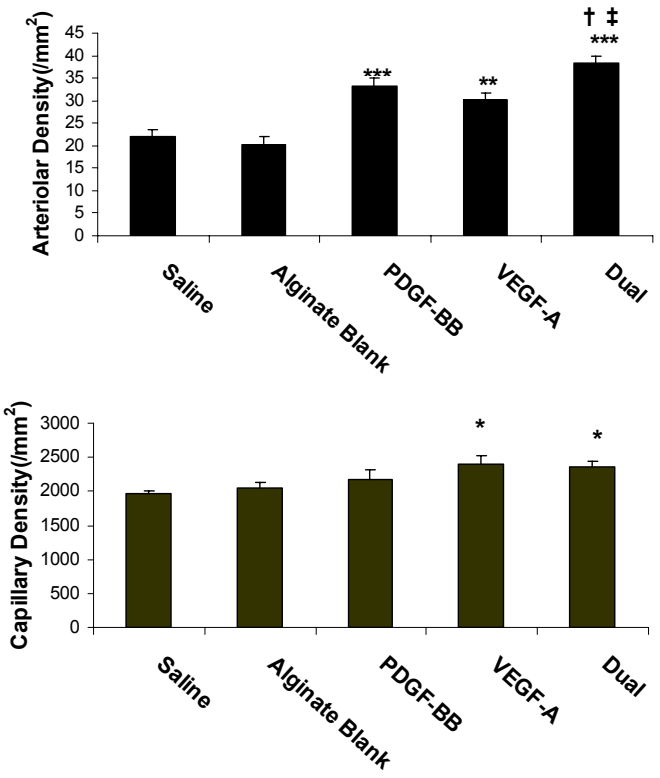


Fig.18. Arteriolar (above) and capillary densities (below) 4 weeks after delivery of PDGF-BB, VEGF-A_{165} or the dual growth factors in alginate hydrogel. * $p<0.05$, versus alginate blank; ** $p<0.01$, versus alginate blank; ***$p<0.001$, versus alginate blank. † $p\leq0.05$, versus PDGF-BB; ‡ $p<0.05$, versus VEGF-A_{165}.. ANOVA, followed by PLSD test. Values are given as mean ± SEM.

Systolic velocity-time integral deteriorated with time around 20% after alginate blank administration ($p<0.001$, Fig. 19). With both VEGF-A_{165} and PDGF-BB treatments the

systolic velocity-time integral was unchanged compared to baseline but increased to a higher level than alginate blank delivery 4 weeks after treatment ($p<0.05$). Dual protein delivery increased 30% of systolic velocity-time integral compared with baseline ($p<0.01$) and displayed a superior effect than either of the single factor delivery ($p<0.05$).

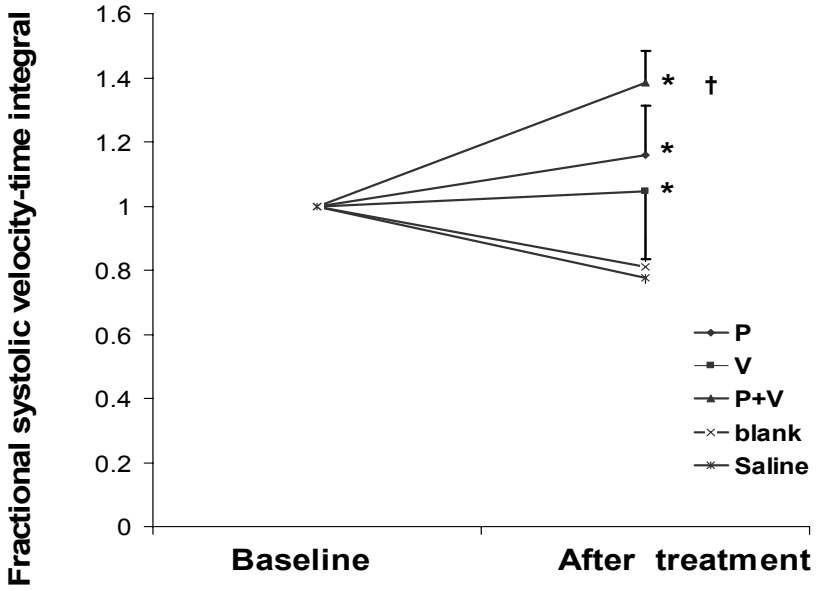


Fig. 19. Fractional systolic velocity-time integral 3 days before and 4 weeks after delivery of PDGF-BB, VEGF-A_{165} or the dual growth factors in alginate hydrogel 4 weeks after treatments. * $p<0.05$ versus alginate blank. † $p<0.05$ versus PDGF-BB or VEGF-A_{165}.

Conclusions

The alginate hydrogel delivery system is applicable for delivery in a myocardial infarction model. Dual growth factor delivery of VEGF-A_{165} and PDGF-BB induce higher angiogenic effect on arteriolar level and improved cardiac function than single factors. This may indicate clinical utility.

5. General Discussion

Angiogenic efficiency and safety are two main issues for therapeutic angiogenesis. The goal of the therapy is to build up sufficient vessels to compensate for the decreased blood flow in ischemic tissue. It is critical to identify the most efficient therapeutic agents and to develop an optimal delivery modality to induce a favourable angiogenic effect. Delivery modalities with the least side effects should also be considered.

VEGF is the most widely investigated growth factor both clinically and experimentally. Adenovirus and plasmid are the most commonly used modalities for its delivery. We investigated both efficiency and side effects of the two vectors. Although both previous and our own study showed that AdVEGF-A_{165} induced substantially higher myocardial VEGF-A expression than PhVEGF-A_{165}[65,75], a similar angiogenic effect was found with PhVEGF-A_{165} and AdVEGF-A_{165}. It appears that at least with this experimental design the overexpressed VEGF-A_{165} does only have a turn-on or ceiling effect.

Increment of systolic velocity-time integral after both AdhVEGF-A_{165} and phVEGF-A_{165} treatment suggested increased perfusion and function of the myocardium. This indicates that the increased vessel densities induced by AdhVEGF-A_{165} and phVEGF-A_{165} were functional vessels. However, the increment extent was similar for the two treatments.

Adenovirus displayed more side effects than plasmid. TUNEL stained positive cells showed little or no apoptotic effect with plasmid treatment, whereas apoptosis was induced by adenovirus. Adenovirus has been documented to modulate apoptosis[76,77]. In our experiment, we used the modified first generation adenovirus with deletion of E1 and E3. Still apoptosis was observed. VEGF is known to counteract cell apoptosis [78], but in our study VEGF did not counteract the apoptosis effect aroused by adenovirus although a tendency to decrease was identified with VEGF overexpression.

We determined apoptosis 7 days after gene transfer. At least some of the apoptotic cells were cardiomyocytes. An impairment of left ventricular function due to the iatrogenic myocardial apoptosis in the ischemic periinfarct region cannot be excluded although it could not be demonstrated in the present study.

Ectopic VEGF-A gene expression in different organs was found both with adenovirus and plasmid gene transfer. However, AdhVEGF-A_{165} induced much higher VEGF expression than plasmid.

Thus intramyocardial adenoviral VEGF-A_{165} gene transfer induced a similar angiogenic effects as plasmid, but adenovirus delivery led to more apoptosis and ectopic VEGF-A expression. This indicates AdhVEGF-A_{165} has no obvious advantage over PhVEGF-A_{165} at least this myocardial infarction model.

To optimize therapeutic angiogenesis, multiple growth factors might be needed. Previous studies showed that combination of PDGF and bFGF or PDGF-BB and VEGF induce higher vessel density than single factors[79,80]. In studies I and II, neither of the combined plasmid therapy showed any advantageous angiogenic effect compared with the single plasmid delivery. The different result from the previous studies might depend on two main factors: growth factor and delivery modality.

Treatment with bFGF + PDGF-BB proteins was reported to have a synergistic effect on vascular growth in peripheral models, including induction of more stable vessels[80]. In our study, capillary density was maintained with the combination of PhbFGF and PhPDGF-BB, but no synergistic effect was observed on the arteriolar level. The observed different results with the combination of bFGF and PDGF-BB might depend on the delivery ratio of PDGF-BB and bFGF. Expression of PDGF receptors α and β differed with different ratio of delivered bFGF and PDGF-BB [80,81,82]. PDGF-BB was reported to act as an angiogenic stimulant via the β receptor or as an

inhibitor of angiogenesis via the α receptor [38,81,83]. Different PDGF α and β receptor levels might contribute to the angiogenic switch and determine the angiogenic efficiency. In fact, the PDGF-β receptor has only been found in capillaries in contrast to arterioles, which express both the PDGF-α and the PDGF-β receptors[38]. This may explain in our study the combination treatment showed advantage over single ones on capillary densities but not on arteriolar densities.

VEGF-A_{165} mainly stimulates endothelial cell growth that induces angiogenesis, although VEGF overexpression also induces arteriolar growth[24-26]. Although the mechanisms for arteriogenic effect of VEGF-A_{165} has not been completely elucidated, studies showed that VEGFR2 are expressed on vascular smooth muscle cells (SMC)[84] and VEGF can stimulate vascular SMC migration[85,86]. PDGF-BB is considered to stimulate smooth muscle growth[87,88]. For optimization of the arteriogenic process, PDGF-BB should be delivered in the late phase of VEGF-A_{165} delivery thus boosting VEGF- A_{165} formation of arterioles by recruitment of smooth muscle cells on newly formed capillaries. However, Dorafshar et al. reported that in vitro VEGF inhibits PDGF-BB effects on proliferation of vascular smooth muscle cells in a dose-dependent manner[89]. VEGF-A_{165} was suggested to act as an antagonist to PDGF at the receptor level because the 3-D structure of VEGF is similar that of PDGF-BB with a related amino acid sequence. In our first study with simultaneous plasmid transfer of VEGF-A_{165} and PDGF-BB, no increased angiogenesis was induced compared to single factors. When VEGF-A_{165} and PDGF-BB were delivered as proteins with an alginate hydrogel preparation where PDGF-BB had a slower release than VEGF-A_{165}, more arterioles were formed than after single factor delivery.

In the alginate experiment, we used an injectable alginate delivery system for delivery of growth factors. As natural alginate is stable, we modified alginate into a degradable

alginate by an oxidation process. PDGF-BB and VEGF-A_{165} are released with alginate slow degradation for a long duration. Molecular weight of the alginate is also adjusted to control the release kinetics[90]. As low molecular weight alginate has greater degrading speed than high molecular weight alginate, a two-phase release was found with a binary alginate gel. Fast release of both VEGF- A_{165} and PDGF-BB is due to the degradation of low molecular weight alginate and second slower release is related to the slower high molecular weight alginate degradation. PDGF-BB has a slower release than VEGF-A_{165}, which is optimal for arteriogenesis. This is related to their different affinity to alginate with different charge density.

Dual growth factor delivery induced higher arteriolar density than VEGF-A_{165} alone. This confirms that PDGF-BB can enhance the VEGF-A_{165} angiogenic effect at the arteriolar level. PDGF-BB may further stimulate and enhance the VEGF-A_{165} arteriogenic process by recruitment of SMCs and pericytes to the newly formed capillaries induced by VEGF-A_{165} and proliferation of recruited SMC by VEGF-A_{165}.

The studies showed that in the present therapeutic angiogenesis phase, AdhVEGF-A_{165} does not have superior angiogenic efficacy to PhVEGF-A_{165} but more side effect at least in the myocardial infarction model. Combined growth factor delivery that used for angiogenic therapy might benefit the ischemic tissue more than single growth factor administration. However, the angiogenic effect is closely related with delivery modality and alginate gel might be a promising utility.

6. Conclusions

1. Simultaneous gene transfer of PhPDGF-BB and PhFGF-2 stimulated angiogenesis both at the capillary and arteriolar levels, but it did not have any advantageous effect over single gene transfer on angiogenesis or cardiac function.

2. Simultaneous gene transfer of PhPDGF-BB and PhVEGF-A_{165} stimulated angiogenesis both at the capillary and arteriolar levels and transiently counteracted cardiac remodelling after myocardial infarction. However, it did not have any advantageous effect over single gene transfer on angiogenesis or cardiac function.

3. AdhVEGF-A_{165} does not have any superior obvious angiogenic efficacy to PhVEGF A_{165} but more side effects in a myocardial infarction model.

4. Dual growth factor delivery of VEGF-A_{165} and PDGF-BB in alginate hydrogel is applicable in a myocardial infarction model and induces higher arteriolar densities and improved myocardial function than single factors. This may indicate clinical utility.

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