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Angiogenic and cardiac functional effects of dual gene transfer of VEGF-A_{165} and PDGF-BB after myocardial infarction

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Abstract

Therapeutic angiogenesis is a potential treatment modality for myocardial ischemia. phVEGF-A_{165}, phPDGF-BB, or a combination of the two were injected into the myocardial infarct border zone in rats 7 days after ligation of the coronary left anterior descending artery. Cardiac function was measured by echocardiography. Hearts were harvested 1 and 4 weeks after plasmid injection. phVEGF-A_{165} increased capillary density more than phPDGF-BB, and phPDGF-BB preferentially stimulated arteriolar growth. The combination increased both capillaries and arterioles but did not enhance angiogenesis any more than single plasmid treatments did. VEGF-A_{165} and the combination of phVEGF-A_{165} and phPDGF-BB counteracted left ventricular dilatation after 1 week but did not counteract further deterioration.

Keywords: Angiogenesis; Arteriogenesis; VEGF-A_{165}; PDGF-BB; Plasmid; Myocardial infarction

Therapeutic angiogenesis with growth factors is a promising therapy for myocardial infarction. However, because angiogenesis is a complex process, treatment with combination of relevant growth factors could be even more effective than single growth factor therapies. Single growth factor transient overexpression of VEGF-A_{165} after plasmid or adenoviral vector gene transfer promotes angiogenesis and has a favourable effect both experimentally and in the clinical phase I/II studies [1–3]. PDGF-BB has been demonstrated to have angiogenic effects experimentally [4,5]. However, VEGF may not be sufficient for the establishment of mature vessels [6]. PDGF-BB may work in concert with VEGF and recruit smooth muscle cells to the nascent vasculature [7,8]. Local dual delivery of recombinant VEGF-A_{165} and PDGF-BB proteins with a polymeric slow release formulation resulted in the formation of a mature vascular network subcutaneously and also in the leg muscle [9].

Appropriate protein slow release formulations, however, have not been developed for myocardial applications. Direct naked plasmid transfer induced gene expression in the myocardium [10,11]. Plasmid administered together with a water-soluble lipopolymer increased gene expression in the myocardium [12]. However, such transfection reagents can be cytotoxic and thus may not be suitable for tentative clinical intramyocardial gene delivery. Therefore, we used naked plasmid in this study to investigate if dual plasmid gene transfer of VEGF-A_{165} and PDGF-BB would more efficiently stimulate angiogenesis and cardiac function than single gene transfer in a myocardial infarction model.

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Methods

Animals

Sprague–Dawley (SD) rats weighing around 300 g were used (B&K Universal, Sollentuna, Sweden). The study was approved by the Stockholm Southern Ethics Review Board for Animal Experiments. Animals were treated in accordance with Institutional Guidelines for care of laboratory animals.

Plasmids

phVEGF-A_{165} is a eukaryotic expression vector encoding for the 165 amino acid isoform of human VEGF-A, driven by a human cytomegalovirus immediate early promoter/enhancer (HCMV IE) [13]. Human PDGF-BB gene (a generous gift from Dr. Y. Cao, Karolinska Institutet, Stockholm, Sweden) 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. Placebo plasmid was made by annealing the ends of the stripped VEGF plasmid without insertion of any new DNA. For the propagation of plasmids, *Escherichia coli* DH5α from Life Technologies (Gaithersburg, MD) was used as host. Plasmids were purified using the Qiagen kit (Hilden, Germany) according to the manufacturer's instructions. Purity was checked by gel electrophoresis and by optical density at 260 and 280 nm.

Gene transfer in vivo

Normal heart. Gene transfer was first performed in the normal heart to evaluate VEGF-A and PDGF-BB protein expression after plasmid transfer. Rats were anaesthetised with a combination of midazolam (5 mg/kg) and medetomidine hydrochloride (0.1 mg/kg) and subsequently endotracheally intubated. Hearts were exposed via thoracotomy and were given placebo plasmid, phVEGF-A_{165}, phPDGF-BB, or the combination of phVEGFA_{165} and phPDGF-BB ($n = 6$ in each group). One animal in the combination group died after operation. Each plasmid was given in an amount of 20 μg in 100 μl saline. The plasmid solution was given intramyocardially with a curved 30-gauge needle into 4 or 5 sites at the anterior wall. After the injection, the chest incision was closed, and the anaesthesia was reversed by combined injection of atipamezol hydrochloride (5 mg/kg) and flumazenil (0.1 mg/kg). Twenty-four hours after the plasmid injection, rats were sacrificed using carbon dioxide, and the heart was harvested and minced with a homogenizing knife in a homogenisation buffer (25 mM Hepes, pH 7.4, 1.5 mM EDTA, 0.5 mM PMSF, 0.5 mM benzamidine, and 10 μg/ml trypsin inhibitor). Next, the homogenate was centrifuged for 10 min at 14000 rpm at 4 °C. The supernatant was collected and frozen at −70 °C.

Myocardial infarction heart model. The heart was exposed by the above procedure. The left anterior descending artery (LAD) was then ligated just below the left appendage with a 7.0 polypropylene suture. Pallor and regional wall motion abnormality of the left ventricular confirmed that the LAD occlusion had occurred. One week after LAD ligation, 48 rats were randomly separated into two cohorts (24 rats/cohort). Each cohort was separated into four groups for plasmid injection as described above ($n = 6$/group). The chest was reopened and 20 μg of each plasmid was injected at 4–5 sites along the border of the infarct from the middle ventricle to the top and apex. The chest wall was then closed. Animals were sacrificed after 1 week (cohort 1) and 4 weeks (cohort 2) of plasmid treatments.

hVEGF-A and hPDGF-BB gene expression in vivo

Frozen samples from hearts were analysed using ELISA for human VEGF-A and PDGF-BB using immunoassay as per the manufacturer's instructions (Quantkine, R&D system).

Cardiac function measurement

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.

Histological morphometry

One week and 1 month after plasmid injection, hearts were harvested and weighed. The hearts were cut into two equal halves and were embedded in OCT compound (Histolab, Sweden). The slices were immediately frozen in liquid nitrogen and then kept in −70 °C. Five micrometer sections were made by cryostat. Slides from the centre of the infarct were stained with hematoxylin and eosin to differentiate the viable myocardium (red) versus scar (blue). The area of scarred myocardium and the total left ventricular tissue area were quantified using computer-based image analysis. Infarct size was computed as scarred area/total tissue area × 100% at 1.25× magnification. Infarct wall thickness was derived by averaging three measurements taken from the respective regions.

Immunohistochemistry

Capillary density was analysed in the sections that were incubated with Griffonia Badeiraea Simplicifolia Isolectin B4 (GSL-I-B4, Vector Laboratories), which was followed by a second incubation with ABC Complex. Finally, capillaries were visualised using DAB with 0.03% hydrogen peroxide. Capillaries were counted at a magnification of 40× using a LCD camera (Olympus, Japan) connected to a microscope. Ten fields of pictures around the injection site were taken and the capillary count was analysed 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 visualise the blood vessels. The blood vessels stained around the injection site were counted under 20× magnification in a fluorescence microscope by an individual who was blinded in regard to the group assignments.

Statistical analysis

Data are presented as means ± SEM. Comparison between the four groups was made using a 1-way ANOVA followed by Fisher's PLSD test. Values were considered significantly different at a value of $p < 0.05$.

Results

hVEGF-A_{165} and hPDGF-BB gene expression

VEGF-A and PDGF-BB were both expressed 24 h 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, $p = 0.44$).

Cardiac function and morphology

At the baseline, there was no difference in LVDd and LVDs between the four plasmid injection groups. VEGF-A_{165} counteracted left ventricular dilatation after 1 week in terms of LVDd and LVDs ($p < 0.05$, Figs. 1A and B, Table 1). The combination showed a similar effect to VEGF-A_{165} but only had significant effect on LVDd. However, cardiac function remodelling of the left ventricle was not counteracted by any of the treatments after 4 weeks. Thus, there was no difference found between all the growth factors treated and placebo groups. Cardiac function after PDGF-BB treatment alone did not differ from the placebo. Single or combinations of growth factor gene transfer did not influence heart weight, anterior wall thickness, or infarct area either 1 or 4 weeks after treatment (Table 2).

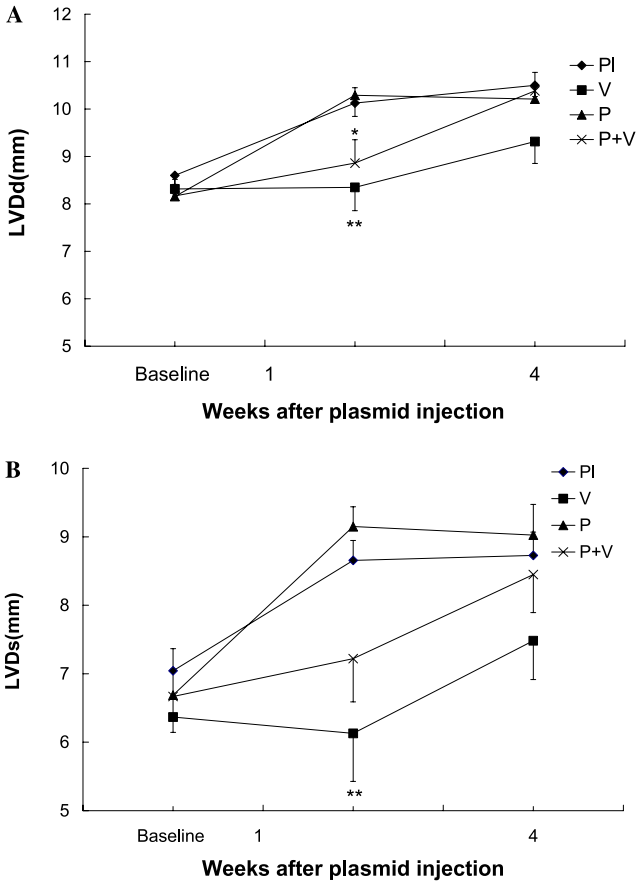

Fig. 1. (A) Left ventricular diastolic diameter (LVDd) and (B) left ventricular systolic diameter (LVDs) measured by echocardiography 1 and 4 weeks after plasmid injection. Values are given as means with SEM. Pl, placebo plasmid; V, phVEGFA-$_{165}$; P, phPDGF-BB; and V + P, phVEGF-A_{165} combined with phPDGF-BB.

Capillary density

Capillary density in the periinfarct area was decreased by about 30% 1 and 4 weeks after placebo plasmid transfer (Fig. 2A). One week after treatment, phPDGF-BB only induced a trend to increased capillary density compared to placebo, while phVEGF-A_{165} increased capillary density by about 20% ($p < 0.01$). However, capillary density did not attain the reference range for normal. The combination did not show any advantageous effect on capillary density over VEGF-A_{165} alone. After 4 weeks of treatment, gene transfer of the two growth factors or their combination induced similar increment of capillary density that, however, was lower than the reference range for normality (Fig. 2A).

Arteriolar density

Myocardial arteriolar density in the periinfarct area was decreased by about 25% 1 and 4 weeks after placebo plasmid transfer (Fig. 2B). After 1 week of treatment, all growth factor gene transfer groups induced a higher arteriolar density compared to the placebo (VEGF-A_{165}: 65%, $p < 0.05$; PDGF-BB: 116%, $p < 0.001$; and VEGF-A_{165} + PDGF-BB: 95%, $p < 0.01$), attaining a level above the reference range for normal ($p < 0.05$). PDGF-BB increased the arteriolar density more than VEGF-A_{165} ($p < 0.05$). The combination did not show

Table 1
Cardiac function at baseline (3 days after LAD ligation and 4 days before plasmid injection) and after 1 and 4 weeks after plasmid gene transfer

	Pl	V	P	P+V
LVDd (mm)				
Baseline	8.60 ± 0.32	8.31 ± 0.15	8.16 ± 0.25	8.17 ± 0.35
1 week	10.13 ± 0.28	8.35 ± 0.50**	10.33 ± 0.16	8.86 ± 0.50*
4 weeks	10.5 ± 0.23	9.32 ± 0.46	10.21 ± 0.33	10.38 ± 0.39
LVDs (mm)				
Baseline	7.04 ± 0.32	6.37 ± 0.22	6.69 ± 0.38	6.67 ± 0.25
1 week	8.65 ± 0.29	6.12 ± 0.70**	9.15 ± 0.29	7.22 ± 0.63
4 weeks	8.73 ± 0.34	7.48 ± 0.57	9.03 ± 0.45	8.45 ± 0.56

LVDd, left ventricular diastolic diameter; LVDs, left ventricular systolic diameter; Pl, placebo plasmid; V, phVEGF-A_{165}; P, phPDGF-BB; and V + P, phVEGF-A_{165} combined with phPDGF-BB. Values are given as means ± SEM.

* $P < 0.05$.

** $P < 0.01$.

Table 2
Cardiac morphology of the gene transfer groups one and four weeks after treatment

	Pl ($n = 6$)	V ($n = 6$)	P ($n = 6$)	V + P ($n = 6$)	Normal ($n = 5$)
Heart weight (g)					
1 week	1.17 ± 0.12	1.26 ± 0.05	1.64 ± 0.27	1.24 ± 0.16	1.70 ± 0.05
4 weeks	1.88 ± 0.49	1.61 ± 0.11	2.44 ± 0.22	1.54 ± 0.04	
AWT (mm)					
1 week	1.20 ± 0.1	1.31 ± 0.14	1.29 ± 0.12	1.46 ± 0.29	3.8 ± 0.20
4 weeks	0.91 ± 0.11	1.40 ± 0.12	1.12 ± 0.15	1.44 ± 0.27	
Infarct size (%)					
1 week	25 ± 2	22 ± 6	24 ± 6	22 ± 5	25 ± 2
4 weeks	24 ± 2	20 ± 2	22 ± 3	21 ± 5	

AWT, anterior wall thickness in the infarction area; Pl, placebo plasmid; V, phVEGF-A_{165}; P, phPDGF-BB; and V + P, phVEGF-A_{165} combined with phPDGF-BB.

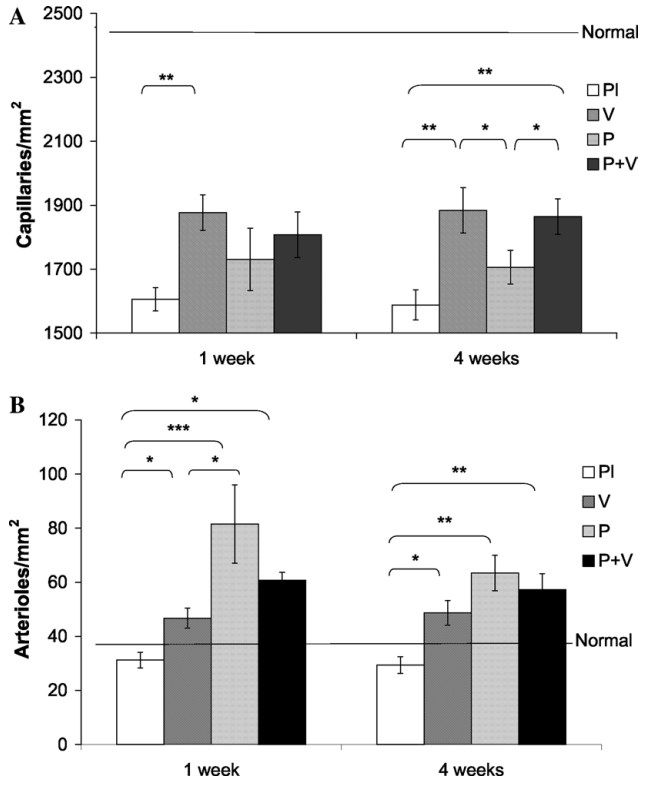

Fig. 2. (A) Capillary and (B) arteriolar densities in the border region of the myocardial infarction 1 and 4 weeks after plasmid gene transfer. Values are given as means ± SEM. ANOVA showed statistical inhomogeneity between the four treatments. Intergroup differences according to Fisher's PLSD test against placebo are shown if not otherwise indicated. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, compared to the placebo. Pl, placebo plasmid; V, phVEGF-A_{165}; P, phPDGF-BB; and V + P, phVEGF-A_{165} combined with phPDGF-BB.

any advantageous effect on arteriolar density over PDGF-BB alone. After 4 weeks of treatment, gene transfer of the two growth factors or their combination induced similar increment of arteriolar density and thus no difference between the treatment with PDGF-BB or VEGF-A_{165} was found (Fig. 2B).

Discussion

This study was carried out using a rat myocardial infarction model. The main findings of the present study are that phVEGF-A_{165} induced angiogenesis at the capillary level more than phPDGF-BB, and phPDGF-BB preferentially stimulated arterioles. Simultaneous gene transfer of phVEGF$_{165}$ and phPDGF-BB increased both capillaries and arterioles but did not increase the vessel densities any further than observed after single factor gene transfer. phVEGF$_{165}$ transiently counteracted cardiac remodelling. A similar effect on cardiac function was found with the combination of phVEGF$_{165}$ and phPDGF-BB.

Endothelial cells are the primary targets for VEGF [14] and smooth muscle cells are the typical target cells for PDGF [4], observations that agree with the different angiogenic effects of VEGF-A_{165} and PDGF-BB found in this study. In addition to increased capillary density, VEGF showed angiogenic effect on arteriolar growth as well. This confirms that VEGF also stimulates pericyte growth [8].

Richardson et al. [9] reported that combination of VEGF-A_{165} and PDGF-BB proteins in a polymeric scaffold system induced more and functionally better blood vessels in a subcutaneous model and in a leg muscle model. The different result in our study might be due to differences in delivery systems and experimental organ models. The plasmid we used is expressed in the rat heart for 3–7 days [15]. Proteins can be delivered with a slow release formulation for a longer duration but are unavailable for myocardial application. Richardson et al. used sequenced protein delivery where PDGF-BB had a slower delivery with a second phase. Dorafshar et al. [16] reported that in vitro VEGF inhibits PDGF-BB effects on proliferation of vascular smooth muscle cells in a dose-dependent manner. VEGF was suggested to act as an antagonist to PDGF at the receptor level because the 3-D structure of VEGF is similar

that of to PDGF-BB with a related amino acid sequence. Such a mechanism might also explain why in the present study simultaneous delivery of phVEGF-A_{165} and phPDGF-BB did not induce increased angiogenesis compared to single factors, whereas in the Richardson et al. [9] study angiogenesis might be more efficient due to the delayed delivery of PDGF-BB.

Both capillary and arteriolar densities were decreased in the border zone of the myocardial infarction as noted in the previous study we performed [17]. At the capillary level, although an increment was induced by phVEGF-A_{165} and the combination, it did not attain the reference limit for normality. Contrariwise, all growth factors increased arteriolar density with an increment above the reference limit for normality.

It has been shown that reestablishment of blood flow to the infarcted region has beneficial effects in attenuating ventricular enlargement [18]. After 1 week of injection of phVEGF-A_{165}, decreased LVDd and LVDs were found that might be due to the increased blood flow. However, phVEGF-A_{165} could not counteract further remodelling of the heart. Hemodynamic factors with systolic bulging of the infarction scar with aneurysm formation may be important determinants for the process. Similar to phVEGF-A_{165}, the combination treatment of phVEGF-A_{165} and phPDGF-BB attenuated ventricular enlargement at 1 week but not after 4 weeks.

In conclusion, simultaneous gene transfer of PDGF-BB and VEGF-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 transfers on angiogenesis or cardiac function.

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