Effect of adventitial VEGF<sub>165</sub> gene transfer on vascular thickening after coronary artery balloon injury

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Abstract

Objective: Experimental studies have provided evidence that neovascularization is an important feature of plaque growth, and angiogenic gene therapy may, therefore, increase plaque growth. This study examined the effect of local (peri)adventitial vascular endothelial growth factor165 (VEGF) gene transfer on vascular thickening after coronary balloon injury. Methods: Two coronary arteries of 15 pigs were subjected to balloon injury followed by either (peri)adventitial VEGF165 or β-galactosidase (LacZ) plasmid/liposome-mediated gene transfer via needle injection catheter. At days 3, 14 and 28, histologic sections of coronary arteries were analyzed. Results: Transferred VEGF165 gene and increased adventitial neovascularization were detected in coronary arteries after balloon injury and VEGF injection. The mean intima + media (I + M) area increased after coronary balloon injury and VEGF (1.13 ± 0.17 and 2.54 ± 0.52 mm<sup>2</sup>) or LacZ (1.37 ± 0.19 and 2.96 ± 0.41 mm<sup>2</sup>) gene transfer, with no significant difference between both groups at 3 and 28 days, respectively. No significant difference in I + M neovascularization was observed at day 28 between the treatment groups (microvessel area density 0.24 ± 0.08% with VEGF and 0.26 ± 0.14% with LacZ, respectively). I + M endothelial cell proliferation index ranged from 7% to 22% (VEGF) and 18% to 24% (LacZ). Conclusions: Catheter-mediated (peri)adventitial VEGF165 gene transfer induces adventitial neovascularization but not an increase of vascular thickening/I + M growth and vascularization in a porcine model of coronary artery injury.

Keywords: Angiogenesis; Atherosclerosis; Gene therapy; Growth factors

1. Introduction

Vascular endothelial growth factor (VEGF) is a proangiogenic cytokine essential for vasculogenesis and postnatal angiogenesis, including cardiovascular diseases [1,2]. VEGF has been used in clinical studies to stimulate collateral vessel formation for the treatment of ischemic heart disease, denoted therapeutic angiogenesis [3]. In spite of the potential beneficial therapeutic effect of VEGF, experimental studies have raised concerns about the safety of VEGF-mediated angiogenesis. These studies have shown evidence that plaque/lesion neovascularization is necessary for plaque growth [4,5]. A recent study by Celletti et al. [6] addressed the question whether angiogenesis is not only necessary but sufficient for plaque growth. They found that a single intramuscular injection of recombinant human VEGF protein in a hypercholesterolemic rabbit model results in an increase in aortic plaque size. However, as we have only begun to understand new facets in the complexity of VEGF biology, it is unknown if these results can be transferred to the setting of local VEGF gene therapy targeting the cardio-
vascular system. Moreover, the clinical relevance of the reported data has to be elucidated as experimental and clinical studies suggest that local therapy may be superior over systemic therapy and gene therapy over administration of recombinant proteins [7,8]. There is still no evidence for lesion acceleration after human coronary/myocardial or peripheral angiogenic gene therapy [9–11]. We, therefore, examined the effect of local (peri)adventitial VEGF165 gene transfer, comparable to the therapeutic modality used in primarily successful human angiogenic gene therapy studies, after mechanical coronary lesion induction on intima + media (I + M) microvessel (MV) angiogenesis and vascular thickening/I + M growth [12].

2. Methods

2.1. Animal model

All studies were carried out with approval of the Animal Care Committee of the State Office Berlin and in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). Fifteen domestic, juvenile, crossbred swine were fed a standard pig chow without cholesterol supplementation. One hour prior to surgery, each animal was sedated with ketamine (25 mg/kg) intramuscularly, and ketamine (25 mg/kg) and xylazine (83 mg/kg) were given intravenously for general anesthesia. Atropine (1 mg) was administered intramuscularly to reduce orotracheal secretions. Throughout the procedure, the electrocardiogram and arterial blood pressure were monitored. Arterial access was obtained via percutaneous puncture of the femoral artery and an 8-french arterial sheath was inserted.

Amiodarone (5 mg/kg) was given prophylactically to prevent ventricular arrhythmias. A heparin bolus (5000 units) was given intravenously. Two of the three major coronary arteries of each animal were subjected in a randomized fashion to balloon injury followed by periadventitial/perivascular injection of either therapeutic (VEGF165) or control (β-galactosidase = LacZ; plasmid and liposomes are described subsequently) genes in the injured coronary artery segment using a needle injection catheter (Bavaria Medizin Technologie, Oberpfaffenhofen, Germany). The remaining coronary artery served as a noninjured control artery. This study design was chosen to validate the effect of VEGF gene transfer in comparison to an appropriate control using exactly the same procedure (LacZ group) and in comparison to an uninjured artery to show that the effect of VEGF gene transfer was limited to the target artery without systemic effects in the coronary circulation (three major coronary arteries of the same animal for comparison).

Coronary angioplasty was performed under fluoroscopic guidance in the proximal segment of one of the three main coronary arteries (e.g., left anterior descending, left circumflex and right coronary arteries) using standard guidewire and balloon technique with a standard balloon angioplasty catheter (diameter 4 mm; Medtronic, Düsseldorf, Germany) to ensure a balloon to artery ratio of 1.3–1.5:1. Coronary angiography was performed after administering 200 μg of intracoronary nitroglycerin before and after percutaneous transluminal coronary angioplasty (PTCA) using anionic contrast media to confirm normal blood flow and vessel patency. The arterial sheath was then removed and manual compression over the puncture site applied until the arterial wound was closed. Anesthesia was terminated and the pigs were observed in the recovery room prior to returning to their stalls. To label proliferating cells, each pig received a 50 mg/kg intravenous dose of 5-bromo-2′-deoxyuridine (BrDU; Roche, Mannheim, Germany) 1 h prior to euthanasia under general anesthesia (five pigs per time point at days 3, 14 and 28 after PTCA and gene transfer) with a euthanasia solution T 61 (Intervet, Unterschleißheim, Germany).

2.2. Histopathologic processing

Porcine hearts were harvested immediately after sacrifice and perfused with Ringers’ lactate at 100 mm Hg for 10 min via pressure tubing seated in the ascending aorta. This was followed by perfusion fixation with 10% neutral buffered formalin at 100 mm Hg for 60 min. Hearts were immersion-fixed in 10% neutral buffered formalin overnight, and processed the following day as previously described [13]. Briefly, 30 mm of artery from the proximal angioplasty sites for each coronary artery was dissected from the heart en bloc and embedded in paraffin for sectioning at 5-μm intervals. For each representative arterial segment, a minimum of eight serial sections were stained with hematoxylin and Elastica van Gieson for morphometric analysis as described below. A section of small bowel was resected from each pig and used as positive control tissue for BrDU immunolabeling.

2.3. Plasmid/liposomes

For in vivo gene transfer, the following plasmid and liposome complexes were made: 50 μg pCMV-VEGF165 plasmid [containing the human VEGF cDNA (96% homologous with pig VEGF) nucleotides 57–629 in a pCIS expression vector, Genentech, South San Francisco, CA, USA] were slowly complexed with 50 μg of Lipofectin (Gibco-BRL, Grand Island, NY, USA) and diluted to a total volume of 600 μl with Ringer solution [14]. For transfection of diluted control arteries, a similar plasmid/liposome complex containing *Escherichia coli* LacZ cDNA CMV-expression plasmid (nucleotides 1–3100) was used [15]. The mixtures were kept at room temperature for at least 30 min and used for gene transfer within 2 h. Plasmids were isolated using Maxi columns (Qiagen, Hilden, Germany), purified using phenol/chloroform extractions and ethanol precipitation and analyzed for the absence of any microbiological or endotoxin contamination (Limulus assay; Sigma, St. Louis, MO, USA).
2.4. RT-PCR analysis

Twelve sections of paraffin-embedded tissue were deparaffinized with xylene and then washed with 100% ethanol. Total RNA was extracted using Rneasy Mini Kit columns (Qiagen) including DNase treatment. Furthermore, possible contamination with genomic DNA was excluded by control experiments omitting the reverse transcriptase. The samples were reverse-transcribed to the first-strand cDNA using Superscript II RT and random primers (Invitrogen, Karlsruhe, Germany). A 40-cycle PCR (cycle parameters: 30 s at 95 °C, 30 s at 62 °C, 30 s at 72 °C) was performed with Ampli Taq DNA Polymerase (Perkin Elmer Applied Biosystems, Foster City, CA, USA) and 100 μM primer pairs. Primers were designed to distinguish between endogenous porcine VEGF and transected human VEGF165 by selecting the antisense primer from the polyadenylation signal of the expression vector and the sense primer from the coding region. Primer sequences were used as follows: sense primer 5’-agaacgg-tcctcttggaatt-3’ and antisense primer 5’-tgacattataaggct-caataa-3’. To prove that the selected primers work reliably in the reverse-transcribed probes, in vitro experiments were performed by transfecting HaCat ceratinocytes with the same VEGF165 plasmid used for in vivo gene transfer, then isolating total RNA and performing RT and PCR as described above.

2.5. Morphometric and microvessel analysis

Two independent investigators who were blinded to the nature of tissue sections (treatment) examined all slides. Morphometric analysis was performed on all arterial segments (minimum of eight sections per segment) and the cross section with the most severe luminal narrowing was identified for study analysis (including calculation of the injury score) using digitized images. The images were magnified in a Leica DMRD microscope (Leica, Bensheim, Germany) and captured by a Sony 3CCDTM ( Charged Couple Device ) true color (red-green-blue/RGB) video camera (Sony, Tokyo, Japan) mounted on the microscope by a Leica 0.5-in. C-mount adapter with 0.35 magnification level (Leica) and connected to a frame grapper. The grabbed images (704 × 548 pixel RGB format with a 24-bit resolution and 16 million colors) were digitized by a Matrox Comet 24-bit color graphic card. Images were analyzed employing the software LUCIA G (version 3.52ab, Nikon, Düsseldorf, Germany) in an Intel Pentium MMXTM 233-MHz-based personal computer and Microsoft. Because of interruptions of the internal elastic lamina post-balloon angioplasty, it was impossible to consider the intima and media as entities, and therefore, a combined intima plus media (I + M) area was measured on Elastica van Gieson stained slides. I + M (= tissue area between the lumen area and the external elastic lamina) was defined as vascular thickening. To analyze I + M angiogenesis, the total number of MVs in the I + M area, as well as the percentage of I + M area occupied by MVs (MV area density) and MV size, was studied. Tissue slides of noninjured arteries without gene transfer, arteries after PTCA and VEGF or LacZ (control) gene transfer from all time points were immunolabeled with an anti-vWF antibody. Fifteen uninjured arteries and 30 balloon-injured arteries (15 with VEGF and 15 with LacZ gene transfer) were analyzed using the following protocol. All optical fields within the I + M area were evaluated by image analysis at 200 × magnification. In addition to counting the total number of MVs of all optical fields, the perimeter of MV within the I + M were traced and the MV area measured. The I + M microvascular area density was calculated for each artery as summed MV area of the I + M divided by the total I + M area (quotient expressed as a percentage by multiplying by 100). To determine the average MV size for a group of arteries, we divided the MV perimeter by the MV number to obtain the MV size index of each artery (expressed in μm). A large index indicates that the average microvessel size is large.

2.6. Endothelial cell proliferation

To quantify the levels of endothelial cell proliferation that accompany I + M MV angiogenesis, we quantitatively analyzed at 400 × magnification the total number of endothelial cells (ECs) and the total number of BrDU immunoreactive ECs on adjacent cross sections of the same artery. The proliferation index (percentage of BrDU immunoreactive ECs) was then calculated for the I + M of each artery.

2.7. Immunohistochemistry

Immunohistochemical labeling was carried out on adjacent tissue sections using previously described methods [16]. Five-micrometer tissue sections were deparaffinized using Roti-Histol (Carl Roth, Karlsruhe, Germany) and then rehydrated in a decreasing ethanol series.

2.7.1. Staining of ECs

After pretreatment with 0.25% pepsin–HCl solution, slides were incubated with a 1:250 rabbit anti-vWF polyclonal antibody (DAKO Diagnostika, Hamburg, Germany) for 45 min at 37 °C. A biotinylated swine anti-rabbit antibody was applied for 45 min followed by an streptavidin/biotinylated alkaline phosphatase complex (both from DAKO Diagnostika) for 30 min. Alkaline phosphatase activity was visualized with Naphthol AS-MX Phosphate ( Fast Red, DAKO Diagnostika ).

2.7.2. Staining of replicating cells

After a 5-min incubation with 3% hydrogen peroxide and a 15-min pretreatment with HCl followed by a 7-min digest with 0.1% trypsin, slides were incubated with a 1:25 mouse anti-BrDU monoclonal antibody (Amersham Biosciences Europe, Freiburg, Germany) for 2 h at 37 °C. A ready-to-use biotinylated horse anti-rabbit/mouse antibody was applied for 30 min followed by a 30-min incubation with ABC.
Reagent (RTU Vectastain universal Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA). Horseradish peroxidase activity was visualized with 3-amino-9-ethyl carbazole (AEC Substrate Kit, DAKO Diagnostika). Porcine small bowel served as positive control tissue. Incubations with PBS containing 1% BSA and isotype-matched immunoglobulins were used as negative controls for all immunostainings. Hematoxylin was used as nuclear counterstain.

2.8. Statistical analysis

Quantitative data were expressed as mean ± S.E.M. For comparison of multigroup variables (for each group at each time point), the variance of means was analyzed using a one-way ANOVA. If the F-test results were significant, post hoc comparisons were carried out using a Fisher LSD test to perform multiple pairwise comparisons among and an unpaired t-test was used to compare the treatment groups. Statistical significance was defined by a p value < 0.05.

3. Results

3.1. VEGF transgene expression

Expression of the transfected VEGF cDNA in VEGF- and LacZ-transfected arteries 3 days after balloon injury and needle injection catheter delivery as analyzed using RT-PCR. Lane 1, Control PCR without previous reverse transcription; lane 2, VEGF-transfected coronary artery with an expected 196-bp amplified fragment indicating the expression of the transgene; lane 3, LacZ-transfected artery showing no transgene expression; lane 4, same as lane 2 but 5’ primer omitted; lane 5, same as lane 2 but 3’ primer omitted; lane 6, positive control plasmid for VEGF transgene; lane L, DNA size markers using a 100-bp ladder.

![Fig. 1. VEGF transgene expression in porcine coronary arteries. Expression of the transferred VEGF cDNA in VEGF- and LacZ-transfected arteries 3 days after balloon injury and needle injection catheter delivery as analyzed using RT-PCR. Lane 1, Control PCR without previous reverse transcription; lane 2, VEGF-transfected coronary artery with an expected 196-bp amplified fragment indicating the expression of the transgene; lane 3, LacZ-transfected artery showing no transgene expression; lane 4, same as lane 2 but 5’ primer omitted; lane 5, same as lane 2 but 3’ primer omitted; lane 6, positive control plasmid for VEGF transgene; lane L, DNA size markers using a 100-bp ladder.](image)

Table 1

<table>
<thead>
<tr>
<th>Number of vessels</th>
<th>Injury score</th>
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<tr>
<td>VEGF 15</td>
<td>1.77 ± 0.530</td>
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<tr>
<td>LacZ 15</td>
<td>1.80 ± 0.414</td>
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Injury score expressed as extend of fragmentation of IEL, media and EEL at sites of maximal injury in coronary arteries in VEGF- and LacZ-treated groups: 0.5 points for minimally disrupted IEL + intact media and EEL; 1.0 points for lacerated IEL + intact media and EEL; 1.5 points for lacerated IEL + less than half thickness of media lacerated + intact EEL; 2.0 points for lacerated IEL + more than half thickness of media lacerated + intact EEL; 2.5 points for lacerated IEL + media totally lacerated + EEL minimally disrupted [32]. Data are shown as means ± S.D.

3.2. Injury score

Table 1 shows the mean injury score of the evaluated arterial segments. Noninjured control arteries did not show any fragmentation of the arterial wall. The severity of injury to coronary arteries was similar in both VEGF- and LacZ-treated groups.

![Fig. 2. Histomorphology of VEGF- and LacZ-transfected coronary arteries. Representative photomicrographs 28 days after coronary angioplasty and VEGF (A) or LacZ (B) gene transfer using the needle injection catheter showing the intima plus media lesion development/vascular thickening. Both slides were stained with Elastica van Gieson (bar = 1 mm).](image)
3.3. Vascular thickening/intima plus media area

Fig. 2 shows representative photomicrographs of coronary arteries 28 days after PTCA plus VEGF (Fig. 2A) or PTCA plus LacZ (Fig. 2B) gene transfer. As detailed in Fig. 3, the mean I+M area is significantly increased with time after PTCA in both VEGF- and LacZ-treated groups compared with noninjured arteries without gene transfer. No statistically significant difference was observed between the VEGF- and LacZ-treated groups at day 28 after PTCA (2.54 ± 0.52 mm² with VEGF vs. 2.96 ± 0.41 mm² with LacZ, p = 0.61, respectively).

3.4. Microvessel angiogenesis

3.4.1. Microvessel number

Fig. 4 shows magnifications of the coronary arteries illustrated in Fig. 2 of the vascular thickening/I+M growth with I+M microvessel angiogenesis (EC immunohistochemical staining with vWF-antibody) 28 days after PTCA plus VEGF (Fig. 4A) or PTCA plus LacZ (Fig. 4C) gene transfer with the needle injection catheter. Adjacent cross sections of the coronary arteries shown in Fig. 2 immunolabeled with an antibody to vWF showing ECs (endothelial cells) of intima + media (A) and adventitia (B) microvessels from VEGF-transfected artery and ECs of intima + media (C) and adventitia (D) microvessels from LacZ-transfected artery. Note the significant microvessel angiogenesis in the adventitia of VEGF-transfected compared to LacZ-transfected arteries and the similar angiogenic microvessel response in the intima + media area after (peri)adventitial VEGF and LacZ plasmid/liposome transfer, respectively (bar = 200 μm).
transfer and of the adventitia with adventitial microvessel angiogenesis (EC immunohistochemical staining with vWF-antibody) of VEGF- (Fig. 4B) and LacZ- (Fig. 4D) treated coronary arteries. These figures demonstrate that there was a pronounced adventitial microvessel angiogenesis in the VEGF gene therapy arm compared to the LacZ gene transfer (control) arm 28 days after intervention. In contrast, histologically, no difference was observed with respect to I + M microvessel angiogenesis at day 28 after PTCA in both treatment groups. Image analysis of MVs in the I + M revealed no statistical significant difference in MV number between the VEGF and control gene (LacZ) treated coronary arteries (8.67 ± 3.48 for VEGF vs. 8.80 ± 3.10 for LacZ, \( p = 0.97 \) respectively; Fig. 5A) at the only time point after intervention (28 days) when I + M microvessel angiogenesis occurred regularly. I + M MV angiogenesis was absent until day 14 and only observed in two animals of each treatment group at day 14 after intervention.

3.4.2. Microvessel area density and microvessel size index

As MV number may not reflect the relative I + M vascularity in relation to an increased I + M area over the time course after PTCA, we analyzed the I + M area comprised of MVs (= MV area density). Further, since changes in the MV area density are not only due to alterations in microvessel number but also due to changes in the microvessel size, we therefore calculated a microvessel size index. Similar to MV number, there was no difference in MV area density (0.24 ± 0.08% for VEGF vs. 0.26 ± 0.14% for LacZ, \( p = 0.91 \), respectively) or MV size index (126 ± 16 \( \mu \)m for VEGF vs. 98 ± 25 \( \mu \)m for LacZ, \( p = 0.36 \), respectively) between both treatment groups 28 days after PTCA (Fig. 5B,C).

3.4.3. Endothelial cell proliferation

One important feature of angiogenesis is the EC proliferation. In all coronary arteries where I + M MV angiogen-

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**Fig. 5.** Intima + media microvessel image analysis. I + M angiogenesis was absent until day 14 after balloon injury and VEGF or LacZ gene transfer and only occurred reproducibly 28 days after intervention. There was no significant difference in microvessel number (A) and microvessel area density (B) in the I + M area between VEGF (black bars) and LacZ (open bars) transfected arteries at 28 days after balloon injury. The microvessel size index of the I + M microvessels 28 days after intervention (C), reflecting not only angiogenic but also potential vasodilatory effects was similar in the VEGF- and LacZ-treated groups.

**Fig. 6.** Endothelial cell proliferation of intima + media microvessels. Immunostaining for BrdU of a histological cross section of a coronary artery 28 days after coronary angioplasty and VEGF gene transfer demonstrating proliferating endothelial cells (arrows) of microvessels in the I + M (bar = 40 \( \mu \)m).
esis occurred, we analyzed the total number of ECs (endothelial staining with vWF antibody), the total number of BrdU immunopositive (=proliferating) ECs as well as the resulting EC proliferation index. Proliferation of microvessel ECs in the I+M (Fig. 6) was only observed in one LacZ-treated animal 14 days after intervention (EC proliferation index 9%) and in two VEGF-treated animals, and three LacZ-treated animals 28 days after intervention with an EC proliferation index ranging from 7% to 25% (7–22% for VEGF and 18–24% for LacZ).

4. Discussion

VEGF gene therapy is a promising approach to induce therapeutic angiogenesis for the treatment of ischemic myocardial disease [3]. Recent experimental studies have shown that plaque neovascularization is necessary for plaque growth, and that systemic application of VEGF protein enhances atherosclerotic plaque progression [5,7,17]. Despite the ample evidence that VEGF functions as a vascular protective factor, the conflicting data from these experimental studies have raised concerns about the safety of VEGF angiogenic therapy and its potential detrimental effect via modulation of plaque microvessel angiogenesis in atherosclerosis [18,19]. The harmful effects of VEGF therapy were shown in small animal models looking at peripheral arteries or aortas and using a systemic approach of VEGF protein application. We therefore examined the effect of local (peri)adventitial VEGF gene delivery on vascular thickening/I+M area and I+M vascularity in porcine coronary arteries, resembling more closely the characteristics of the coronary/myocardial VEGF gene therapy applied in clinical studies [3]. For catheter-based local gene delivery, the needle injection catheter was used in this study. In contrast to the majority of balloon catheter delivery systems, the needle injection catheter allows an active delivery with high efficiency, providing a peri(adventitial) gene depot without causing a vessel wall trauma due to its ultrathin needles [20,21].

Using the balloon injury model to induce lesion development, our study showed that local (peri)adventitial delivery of VEGF165 DNA induces a biological effect and results in adventitial neovascularization but does not increase I+M neovascularization nor vascular thickening/I+M growth.

4.1. VEGF and lesion neovascularization and vascular thickening

Reduction in aortic plaque growth in apolipoprotein-E-deficient mice by antiangiogenic therapy with inhibition of intimal neovascularization suggests an important role of plaque or lesion microvascularity in plaque/lesion development [5]. However, the role of VEGF in lesion development, possibly promoting plaque vascularization as a potential prerequisite for plaque expansion, was not investigated. Recent studies by Celletti et al. [6,17] gave insight into the potential role of VEGF in this pathophysiological process, and showed that VEGF administration by itself stimulates plaque vascularity and growth. The present study did not show an increase in I+M lesion microvascularity and vascular thickening/I+M growth after (peri)adventitial VEGF165 in comparison to control (LacZ) gene administration post coronary balloon injury. In context with these different observations, it is important to note that in the above-mentioned studies, VEGF has been given systemically, inducing an increase in bone marrow and blood macrophages/monocytes. Macrophages modulate lesion formation and atherogenesis due to the induction of growth factors (prothrombotic, proliferative and angiogenic) and cytokines [22,23]. Therefore, it is possible that the observed increase in plaque vascularity after systemic VEGF administration results from systemic VEGF-mediated macrophage recruitment and not from direct local VEGF effects on the vascular wall. As local arterial wall macrophage accumulation was only observed 3 weeks after systemic VEGF administration (despite an early macrophage increase in bone marrow and blood), it seems unlikely that VEGF-induced macrophage accumulation in the plaque would also occur, at least to the same degree after local VEGF gene transfer. In addition to species differences and the variable response to injury of coronary and peripheral arteries or aortas (chronic atherosclerotic and acute mechanical vessel wall reaction), this may be an important reason for the difference in plaque/I+M lesion vascularity seen in our data compared to the other studies. In accordance with this, local perivascular VEGF gene transfer using the collar model in rabbits resulted in neovascularization only in the adventitia and not in the neointima, whose area was reduced 14 days after gene transfer [24]. Our study showed similar results with increased neovascularization only in the adventitia but not in the I+M lesion, and no subsequent increase of the vascular thickening/I+M growth 14 and 28 days after (peri)adventitial VEGF gene transfer. The fact that we did not see a reduction in vascular thickening may be explained by the difference in lesion characteristics and severity induced by balloon injury compared to the silastic collar as well as by the differences in species and arteries.

4.2. VEGF and spatial patterns of microvessel angiogenesis

Despite a pronounced angiogenesis of adventitial microvessels, no increase in I+M microvascularity was observed in our study. The adventitial angiogenesis was paralleled by VEGF mRNA expression in the outer compartment of the adventitia detected by in situ hybridization, whereas no VEGF mRNA expression was found in I+M 14 days after balloon injury and VEGF gene transfer (data not shown). The spatial patterns of vessel wall neovascularization and various parameters evaluating the changes in I+M microvascularity (MV number, size and area density and EC
proliferation) have been studied intensively and raise the question concerning a potential different biological role of adventitial and I + M vascularization in lesion development. It has not been investigated in the VEGF gene delivery studies showing plaque acceleration where increased vascularity was located (at luminal surface, in the plaque or adventitia). In previous studies with PTCA of porcine coronary arteries, it has been shown that regression of adventitial MVs correlates with arterial narrowing and an inverse correlation of adventitial MV area density and neointimal hyperplasia despite a positive correlation of MV number and neointimal increase was observed [16,25]. These results suggest a beneficial effect of the adventitial microvascularization and emphasize the importance of analyzing the percentage of vessel wall area covered by MVs rather than solely the MV number to assess the effect of MV neovascularization. Our study did not show any changes neither in I + M MV number nor in MV area density after PTCA and peri(adventitial) VEGF in comparison to control (LucZ) gene transfer, suggesting that local delivery of the VEGF165 gene to the outer compartments of coronary arteries does not cause acceleration of lesion formation.

4.3. VEGF therapy regimen in clinical and experimental studies

The first Phase I clinical trial with local intramyocardial VEGF gene delivery showed a reduction in angina and an increase in cardiac perfusion in a small number of patients [12]. These promising results could not be confirmed in the randomized Phase II VIVA trial but VEGF delivery was intracoronary (not intramural but intraluminal) with the unavoidable flush away effect with blood flow. Besides, VEGF protein, and not cDNA, was used in this study [26]. Recent Phase I and I/II clinical trials used a catheter-mediated intramyocardial VEGF gene transfer and showed again a favorable outcome with reduced angina and improved myocardial perfusion in a higher number of patients [27,28]. Moreover, these trials and a Phase I study using a local catheter-mediated intracoronary (intramural) VEGF gene transfer did not show any evidence that atherosclerosis is accelerated after local gene transfer [10]. Despite these promising results, the discussion about the safety of VEGF gene transfer for therapeutic angiogenesis remains controversial. The critical question is, how experimental data, showing reduced plaque size in peripheral or aortic vessels after antiangiogenic therapy in hypercholesterolemic animal models and increased plaque size after VEGF therapy, relate to the above cited clinical data. In contrast to the successful clinical trials, angiogenic VEGF or antiangiogenic therapy in the experimental models was performed using a systemic delivery of proteins and the effect was not investigated in coronary arteries. Our experiments focused on the local delivery of VEGF gene into peri(adventitial) or perivascular tissue of coronary arteries and as reported in the clinical studies VEGF gene transfer did not show any evidence that lesion formation is accelerated after local gene transfer [11].

Another important issue in the evaluation of the safety of VEGF therapy is, apart from the delivery regimen (systemic versus local), the dosage of VEGF used for therapeutic angiogenesis. Unregulated/constitutive VEGF overexpression in a mouse model of myoblast-mediated VEGF gene delivery into the myocardium was associated with formation of intramyocardial vascular tumors and death [29]. These results emphasize that beneficial and harmful effects of VEGF therapy depend on the level of systemic and local VEGF concentration and underscore the importance of regulating VEGF expression for therapeutic angiogenesis. Taking this into consideration, it is important to know that low gene transfection efficiency and low local VEGF concentrations can achieve meaningful biological effects [30]. Therefore, in our study, a low dose of a single peri(adventitial) injection of 50 μg VEGF165 plasmid was used. In comparison, in experimental and clinical studies without any observed side effects or lesion acceleration, 25–200 μg VEGF were locally delivered and levels of circulating angiogenic growth factors were either not measurable or in the picogram range [24,27,31].

4.4. Limitations

Even though the response to balloon injury of coronary arteries resembles many characteristics of the pathophysiology of coronary artery disease (e.g. smooth muscle cell/myofibroblast proliferation and migration, inflammatory reaction, intramural thrombus formation as seen in the unstable plaque), the principal limitation of our study is that our porcine coronary arteries did not have a calcified, severely atherosclerotic plaque that may influence the biological effect of VEGF despite its local delivery away from the lesion/plaque.

5. Conclusions

Local delivery of low concentrations of VEGF gene into the outer compartment of coronary arteries induce adventitial angiogenesis but does not promote I + M microvessel angiogenesis nor accelerates vascular thickening/I + M growth in a porcine balloon injury model. This observation is consistent with results of clinical studies using the local gene transfer strategy for VEGF-induced therapeutic angiogenesis where no unwanted side effects (hemodynamically significant lesion acceleration) were observed. Our study is not in contradiction to the reports of earlier studies with an increase in plaque size after systemic VEGF protein application but underscores that vessel wall neovascularization is a finely tuned mechanism that depends on local VEGF concentration. Successful and safe VEGF therapy likely depends on local targeted delivery with a narrow therapeutic concentration range that differentiates the beneficial from
the negative VEGF effects. Long-term follow-up periods in clinical and experimental studies of local VEGF gene transfer are warranted to confirm the safety of VEGF therapy.

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