Vascular Endothelial Growth Factor-A and Platelet-Derived Growth Factor-B Combination Gene Therapy Prolongs Angiogenic Effects via Recruitment of Interstitial Mononuclear Cells and Paracrine Effects Rather Than Improved Pericyte Coverage of Angiogenic Vessels

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Abstract—Vessel stabilization and the inhibition of side effects such as tissue edema are essential in angiogenic gene therapy. Thus, combination gene transfers stimulating both endothelial cell and pericyte proliferation have become of interest. However, there is currently little data to support combination gene transfer in large animal models. In this study, we evaluated the potential advantages of such a strategy by combining the transfer of adenoviral (Ad) vascular endothelial growth factor (VEGF)-A and platelet-derived growth factor (PDGF)-B into rabbit hindlimb skeletal muscle. AdLaCZ alone or in combination with AdVEGF-A were used as controls. Contrast-enhanced ultrasound, modified Miles assay, and immunohistology were used to quantify perfusion, vascular permeability, and capillary size, respectively. Confocal microscopy was used in the assessment of pericyte-coverage. The transfer of AdPDGF-B alone and in combination with AdVEGF-A induced prominent proliferation of α-smooth muscle actin−, CD31−, RAM11−, HAM56−, and VEGF+ positive cells. Although, pericyte recruitment to angiogenic vessels was not improved, combination gene transfer induced a longer-lasting increase in perfusion in both intact and ischemic muscles than AdVEGF-A gene transfer alone. In conclusion, intramuscular delivery of AdVEGF-A and AdPDGF-B, combined, resulted in a prolonged angiogenic response. However, the effects were most likely mediated via paracrine mechanisms rather than an increase in vascular pericyte coverage. (Circ Res. 2008;103:1092-1099.)

Key Words: angiogenesis ♦ vascular endothelial growth factor ♦ platelet derived growth factor ♦ gene therapy

The stabilization of angiogenic vessels through pericyte recruitment is regarded to be essential for the maintenance of blood flow after angiogenic gene therapy of ischemic diseases. Thus, a gene transfer (GT) that combines vascular endothelial growth factors (VEGFs) and platelet-derived growth factors (PDGFs) and stimulates both endothelial cells and pericytes could be more effective than application of single therapies.

VEGF-A is a strong endothelial mitogen that can induce efficient vascular growth and perfusion. PDGF-B mediates pericyte proliferation and migration and is, thus, associated with vessel stabilization. Interestingly, our previous studies have shown that pericyte proliferation can also be induced after transduction with adenoviral (Ad) VEGF alone, likely through indirect mechanisms, including increased capillary pressure, shear stress, and upregulation of other growth factors. Thus, actual benefits of the combination GT on pericyte proliferation and vascular recruitment associated to therapeutic angiogenesis are unclear.

Currently, very little data are available on combination GT in large animal models. Intramuscular injection of adenoviruses is currently the most efficient method for gene delivery in large animals and holds promise for clinical trials. We compared the effects of intramuscular GT of AdVEGF-A or AdPDGF-B alone or in combination on pericyte activation and the stability of angiogenic vessels in normoxic and ischemic rabbit hindlimbs. We found that the combination GT prolonged angiogenic effects, although pericyte coverage of the neovessels was not enhanced. Rather, AdPDGF-B GT alone or in combination with AdVEGF-A induced recruit-
ment of mononuclear, interstitial cells expressing endogenous VEGF.

Materials and Methods

Ischemia Operation and Gene Transfers

New Zealand White rabbits (mean weight, 2.5 to 3 kg; total n = 141) received intramuscular injections of adenoviruses (10⁹ viral particles [vp]) encoding human VEGF-A⁰⁰ or human PDGF-B. A total dose of 2×10¹³ vp was used for the AdVEGF-A+AdPDGF-B combination GT, β-Galactosidase marker gene (LacZ) alone or in combination with AdVEGF-A was used as a control. Human clinical grade, first generation, serotype 5, replication-deficient adenoviruses produced under GMP conditions and analyzed to be free from contaminants were used. Intramuscular GTs were performed into the semimembranosus muscle of the thigh using a 1-mL syringe and a 25-gauge needle (10¹¹ vp/mL divided into 10 separate 0.1-mL injections) during medetomidine (Domitor, 0.3 mg/kg, Orion) and ketamine (Ketalar, 20 mg/kg, Pfizer) anesthesia. Ligation of the profound femoral artery was performed in a subgroup of animals (n = 58) before GTs as previously described. Animals were euthanized at 6, 14, or 28 days after GT. To reduce the number of animals, the AdVEGF+AdLacZ control was not used in ischemic animals because the results did not statistically differ from transfer of AdVEGF alone to normoxic muscle. All animal experiments were approved by the Experimental Animal Committee at the University of Kuopio.

Contrast-Enhanced Ultrasound Imaging of Perfusion

Perfusion in transduced and contralateral intact rabbit semimembranosus muscles was quantitatively measured with Acuson Sequoia 512 and 15L8 transducer (Siemens), using the power Doppler mode and the administration of a contrast agent 6, 14, or 28 days after GT. Two consecutive longitudinal plane video clips of 10 seconds (power Doppler at 8.5 MHz; dynamic range, 10 dB; power, −18 dB; mechanical index, 0.6; gain, 40; depth, 20 mm) were captured immediately on a bolus injection containing 0.3 mL of second generation contrast agent (sulfur hexafluoride in a phospholipid shell, ~2×10⁶ bubbles per milliliter; mean diameter, 2.5 μm; Sonovue, Bracco) into the ear vein. The perfusion ratio was calculated using Datapro 2.13 (Noesis), using the maximum signal intensities of the transduced and contralateral intact limbs.² Ultra-sound measurements and the analysis of data were performed in a blinded manner.

The Modified Miles Assay for the Evaluation of Tissue Edema

The modified Miles assay was used for the evaluation of tissue edema at death. Evans Blue dye (30 mg/kg, Sigma) was injected intravenously 30 minutes before euthanasia. After euthanasia, the animals were perfusion-fixed with 1 L of 1% paraformaldehyde in 0.05 mol/L citrate buffer (pH 3.5) via the left ventricle. Extravasated Evans blue dye bound to plasma proteins (mostly albumin) was extracted from transduced and contralateral intact semimembranosus muscle samples by incubation in formamide at 60°C for 48 hours. The amount of extravasated Evans blue dye was determined on the basis of absorbance at 610 nm. The results are represented as absorbance ratios between the transduced and contralateral intact muscle samples. The absorbances were normalized to the weight of the muscle sample.

Immunohistology

The Avidin-biotin-HRP system (Vector Laboratories) with 3.prime]-5.prime]-diaminobenzidine (Zymed) color substrate or fluorescein isothiocyanate (FITC) (Zymed) fluorescent dye was used for immunocytochemistry on 7-μm-thick paraffin-embedded sections fixed in 4% paraformaldehyde/15% sucrose for 4 hours. Intraarterially injected rhodamine-labeled Rictinus communis lectin (1 mg in 2 mL of saline administered into the profound femoral artery, Vector) and FITC conjugates were used for immunocytochemistry on 50-μm-thick frozen sections. The endothelium was immunostained using a mouse monoclonal antibody (mAb) against CD31 (DAKO, dilution 1:50). Pericytes and SMCs were stained with an α-smooth muscle actin (α-SMA) mAb (Sigma, 1:250), macrophages were stained with a mAb against rabbit macrophages (RAM11, DAKO, 1:200) and mAb against human macrophages and monocyes (HAM56, DAKO, 1:500) with a trypsin pretreatment. Fibroblasts were identified with desmin and vimentin (Sigma, 1:100 and 1:50, respectively). Protein expressions were studied using a hVEGF antibody (Santa Cruz Biototechnology, 1:500) and a PDGF antibody (R&D, 1:500) with a citrate buffer boiling treatment. Receptor stainings were performed using VEGFR-1 (Santa Cruz Biototechnology, 1:250), VEGFR-2 (R&D, 1:250), and PDGFR-β (Santa Cruz Biototechnology, 1:200) antibodies. General histology and cell morphology were studied using hematoxylin/eosin stainings. Double immunostainings comprising Avidin-biotin-HRP system with FITC (x) and anti–mouse-Alexa 546 (x) were used for the detection of VEGF and HAM56, respectively.

Blood Vessel Measurements

The mean capillary area (micrometers squared) was measured at ×200 magnification from CD31 immunostained sections of semimembranosus muscles obtained from areas covered entirely by skeletal muscle tissue. All measurements were performed in a blinded manner from 10 fields representing maximal angiogenic effects of each muscle section using analySIS software (Soft Imaging System). To avoid ambiguous data caused from trauma effects of the needle injection, the analysis was made outside the needle track area. Means of the measurements are reported. Total area of arteries and veins (percentage of the total muscle area) was quantified from α-SMA–stained sections of semimembranosus muscles at ×40 magnification covering the entire muscle.

Measurements of α-SMA–Positive Cells

The percentage of α-SMA–positive pericytes, SMCs, and myofibroblasts (percentage of the skeletal muscle area) were measured by immunofluorescence (FITC) of α-SMA–stained sections of semimembranosus muscles at ×200 magnification. All measurements were performed using analySIS software (Soft Imaging System) in a blinded manner from 5 fields that represented maximal α-SMA immunofluorescence of each muscle section. Measurements were taken from areas that did not contain large arteries or veins because their SMC layer could affect the results.

Quantification of VEGF-A and PDGF-B Protein Expressions

Muscle samples taken at the time of death were frozen in liquid nitrogen and stored at −70°C. T-Per buffer (Thermo Scientific) with 1× Halt protease inhibitor (Thermo Scientific) was used for protein extraction from homogenized muscle samples. The amount of protein in each sample was quantified with hVEGF-A and hPDGF-B ELISA (R&D Systems) and further normalized to the amount of total protein in each protein extract. The amount of total protein in each sample was quantified with BCA protein assay kit (Thermo Scientific).

Statistical Analyses

The results are expressed as mean ± SEM. Statistical significance was evaluated using the Kruskal–Wallis test, followed by the
Mann–Whitney U test where appropriate. \(P<0.05\) was considered statistically significant.

Preparation of Supplemental Video Files

Three-dimensional reconstructions of the confocal images were prepared with Imaris-software (Bitplane). See the video files in the online data supplement at http://circres.ahajournals.org.

Results

AdPDGF-B Induces Recruitment of Interstitial Cells Six Days After Gene Transfer

The efficacy of the GTs was confirmed using protein expression analysis of the muscle samples (see Figure I in the online data supplement for results). The effect of AdPDGF-B overexpression was first studied in normoxic muscles (Figure 1). AdLacZ-transduced control muscles displayed normal skeletal muscle morphology with small capillaries (Figure 1a, red arrowheads) and the occasional \(\alpha\)-SMA–positive pericytes surrounding the capillaries (Figure 1a, black arrowheads). The main response to AdPDGF-B transduction was the instead of angiogenesis, proliferation of cells in the muscle interstitium (Figure 1b, black arrows). Some enlarged capillaries (Figure 1b, red arrowheads), with increased pericyte coverage (Figure 1b, black arrowheads), were also detected. AdVEGF-A induced abundant capillary enlargement (Figure 1c, red arrowheads) and recruited pericytes around the angiogenic capillaries (Figure 1c, black arrowheads). The AdVEGF-A + AdLacZ controls manifested similar histology to that of AdVEGF-A alone; only a minor recruitment of inflammatory cells, attributable to increased viral dosage, was observed (Figure 1d). In contrast, both the enlargement of capillaries and strong proliferation of interstitial cells (Figure 1e, black arrows) were visible after AdVEGF-A + AdPDGF-B GT. However, recruitment of pericytes to angiogenic vessels was impaired compared to AdVEGF-A or AdVEGF-A + AdLacZ (Figure 1c through 1e). Many \(\alpha\)-SMA–positive cells (Figure 1e, asterisk) and some CD31-positive cells (Figure 1e, red arrows) could be seen in the interstitium of AdVEGF-A + AdPDGF-B–transduced muscles. For AdVEGF-A + dPDGF-B combination GT, 2 doses were tested: \(2\times10^{11}\) and \(10^{11}\) vp. However, both doses yielded similar results (see also supplemental Figure II for the comparison of results obtained from each dose).

The results for normoxic and ischemic animals were very similar when quantified (see also supplemental Figure III for histology from the ischemic muscles). AdPDGF-B alone could not induce significant changes in capillary size (Figure 2a) but moderately increased perfusion in normoxic conditions (Figure 2b). In both normoxic and ischemic muscles, AdVEGF-A, AdVEGF-A + AdLacZ, or AdVEGF-A + AdPDGF-B significantly increased both capillary size and perfusion when compared to AdLacZ alone (Figure 2a and 2b). AdVEGF-A + AdPDGF-B induced more moderate changes when compared to AdVEGF-A alone or combined with AdLacZ, which is probably explained by the moderately lower VEGF expression levels after the combination GT (see supplemental Figure I). AdPDGF-B could not reduce AdVEGF-A–induced edema formation, but,
Angiogenesis Induced by AdPDGF-B or AdVEGF-A + AdPDGF-B Persists Longer Than That Stimulated by AdVEGF-A Alone

Normal skeletal muscle perfusion was detected with contrast-enhanced ultrasound in AdLacZ-transduced normoxic muscles 6 days after GT (Figure 3a). There was a large increase in perfusion after AdVEGF-A, AdVEGF-A + AdLacZ, and AdVEGF-A + AdPDGF-B GTs (Figure 3c through 3e) but only a small increase after AdPDGF-B GT (Figure 3b). Tissue edema was observed between semimembranosus and gracilis muscles in all AdVEGF-A, AdVEGF-A + AdLacZ, and AdVEGF-A + AdPDGF-B (Figure 3c through 3e, asterisks). Fourteen days after GT, perfusion increases induced by AdVEGF-A and AdVEGF-A + AdLacZ were decreased to baseline (Figure 3h and 3i). However, perfusion increases induced by AdPDGF-B and AdVEGF-A + AdPDGF-B were still visible (Figure 3g and 3j; see also supplemental Video 1). Tissue edema was also still detectable in AdVEGF-A + AdPDGF-B 14 days after GT (Figure 3h, asterisk).

In addition to the ultrasound findings, the analysis of CD31-stained muscle sections 14 days after GT (Figure 3k through 3o and 3q) revealed that histological changes induced by AdPDGF-B alone or in combination had also persisted. For AdVEGF-A or AdVEGF-A + AdLacZ most vessels had regressed, although there were some regressing vascular structures still visible (Figure 3m and 3n, arrowheads). However, in AdPDGF-B and AdVEGF-A + AdPDGF-B–transduced muscles, large arteries and veins, and, to some extent capillaries, were still enlarged (Figure 3l and 3o, arrows). Also, cell density was still increased in the muscle interstitium and often localized to persistent angiogenic vessels (Figure 3l and 3o, asterisks).

Quantification of the ultrasound data displayed dramatic changes in skeletal muscle perfusion induced by the GTs (Figure 3p). Whereas perfusion in AdVEGF-A and AdVEGF-A + AdLacZ–transduced muscles quickly decreased after 6 days, a statistically significant increase in perfusion was still observed 14 days after AdVEGF-A + AdPDGF-B GT in both intact and ischemic muscles (Figure 3p). AdPDGF-B–transduced muscles showed very little attenuation of the effect, and ischemic muscles had increased perfusion even 28 days after GT. Ultrasound findings on day 14 were supported by quantification of the histological findings on the same time point, yielding significant increases in vascularity in AdPDGF-B and AdVEGF-A + AdPDGF-B–transduced muscles (Figure 3q).

AdPDGF-B Induces Proliferation of α-SMA–Positive Pericytes and Fibroblasts and Induces Recruitment of CD31+, HAM56+, and VEGF-Positive Cells

As expected, AdPDGF-B induced a significant proliferation of α-SMA–positive cells in rabbit skeletal muscle 6 days after GT (Figure 4a). However, not all cells in the muscle interstitium were positive for α-SMA. Thus, a series of immunostainings were performed to investigate which cells accumulated in AdPDGF-B–transduced muscles. Cell proliferation was confirmed using Ki67 staining (Figure 4b). Hematoxylin/eosin staining allowed the identification of fibroblasts (Figure 4c, arrow) and some granulocytes (Figure 4c, arrowhead). Desmin and vimentin stainings were also performed to confirm the presence of fibroblasts (data not shown). The occasional presence of macrophages was detected with RAM11 staining (Figure 4d, arrowhead). HAM56 staining showed more positivity because it also stained monocytes, in addition to macrophages (Figure 4e). CD34 only stained the vascular endothelium of large arteries in the samples (Figure 4f).
Figure 3. Perfusion increase induced by AdPDGF-B or AdVEGF-A+AdPDGF-B is not decreased to baseline in 2 weeks. Ultrasound images and histology of normoxic muscles in different study groups 1 or 2 weeks after GT. 

a through e, Contrast-enhanced power Doppler ultrasound images showing perfusion in normoxic rabbit semimembranosus muscle (green brackets) 6 days after GT. Perfusion was highly increased in AdVEGF-A (c), AdVEGF-A+LacZ (d), and AdVEGF-A+AdPDGF-B (e); a minor increase was also seen in AdPDGF-B (b). Tissue edema was observed between semimembranosus and gracilis muscles in AdVEGF-A, AdVEGF-A+AdLacZ, and AdVEGF-A+AdPDGF-B (c through e, light blue asterisks). 

f through j, Ultrasound images 14 days after GT to normoxic muscle. Perfusion was decreased to baseline levels in AdVEGF-A (h) and AdVEGF-A+AdLacZ (i). In AdPDGF-B (g) and AdVEGF-A+AdPDGF-B (j), increased perfusion was still visible. Tissue edema was still detectable in AdVEGF-A+AdPDGF-B (j, asterisk).

k through o, CD31-immunostained histological sections of the semimembranosus muscles 14 days after GT. Scale bars, 200 µm in full images and 50 µm in insets. k, No changes resulting from the GT were seen in AdLacZ controls. m and n, Muscle morphology returned to normal also in AdVEGF-A and AdVEGF-A+AdLacZ. Some regressing vascular structures were visible (m and n, arrowheads). In contrast, in AdPDGF-B-transduced (l) and AdVEGF-A+AdPDGF-B-transduced (o) muscles, large arteries and veins to some extent capillaries were still enlarged (o, arrows). Cell density was still increased in the muscle interstitium in AdPDGF-B– and AdVEGF-A+AdPDGF-B–transduced samples (l and o, asterisks).

p, Quantification of the contrast enhanced ultrasound data displayed a statistically significant increase in perfusion 14 days after AdVEGF-A+AdPDGF-B in both intact and ischemic muscles. AdPDGF-B–transduced muscles showed very little attenuation of the effect and the ischemic muscles still had slightly increased perfusion 28 days after GT. q, Quantification of histological changes in vasculature on day 14 showed a significantly higher amount of capillaries and veins in AdPDGF-B–transduced muscles compared to controls. In AdVEGF-A+AdPDGF-B–transduced muscles, the amount of all capillaries, arteries, and veins was increased compared to controls. **P<0.01, *P<0.05 toward AdLacZ at the same time point unless otherwise indicated. See also supplemental Video 1 for day 14 ultrasound videos.
Figure 4. α-SMA−, CD31−, HAM56−, VEGFR-1−, and VEGFR-2−, PDGFR-β− and VEGF-positive cells were found in the muscle interstitium 6 days after AdPDGF-B GT to normoxic muscle. a, α-SMA−positive pericytes could be found both around vascular structures (arrowheads) and in the interstitium (arrow) in AdPDGF-B−transduced muscles. However, a large part of the proliferating cells in the muscle interstitium were not positive for α-SMA. b, Proliferation of the interstitial cells was confirmed using Ki67 staining. Ki67 positivity was found among cells in the interstitium (arrowheads) and also in vascular wall (arrow). c, Hematoxylin/eosin staining displayed a typical fibroblast structure in some cells (arrow), and a few cells could be identified as granulocytes based on the shape of their nuclei (arrowhead). d, RAM11−positive macrophages were an example of inflammatory cells found after AdPDGF-B GT (arrowhead). e, Several monocytes were stained by HAM56 in addition to macrophages. f, CD34 only stained the endothelium of large arteries in the samples. g, CD31−positive cells are normally found as part of vascular structures (arrowheads) but in AdPDGF-B−transduced muscles many could also be detected in the interstitium (arrows). h, Endogenous VEGF protein expression was detected among many of the cells in the interstitium (arrowheads) in addition to vascular cells (arrows). i, Strong PDGFB−protein expression was detected in a few, probably transduced, cells (arrowheads). Lower expression levels were found in vascular structures (red arrows) and in the extracellular matrix (black arrow). VEGF j and HAM56 k (double staining confirmed, after merging of images l), that some monocytes were positive for VEGF. m, VEGFR-1 expression was detected in endothelium (arrowheads) and in the interstitial cells (arrows). VEGFR-2 n and PDGFR-β o were also detected in the proliferating cells (arrows). Scale bars, 100 μm in all images.

Figure 5. AdPDGF-B increases the amount of α-SMA−positive cells within the target muscle, but the recruitment of pericytes to vascular structures is impaired. a, Quantification of the amount of α-SMA−positive cells in normoxic groups on day 6 showed that AdPDGF-B increased the amount of α-SMA−positive cells both when given alone or in combination with AdVEGF-A. However, AdVEGF-A alone could increase the amount of α-SMA−positive cells significantly more than AdPDGF-B alone. b, In confocal images of AdVEGF-A−transduced muscles, α-SMA−positive pericytes (α-SMA, green) were associated with vascular structures (lectin, red). c, In contrast, α-SMA−positive cells could often be detected in the muscle interstitium of AdVEGF-A+AdPDGF-B−transduced muscles without any association with vessels (asterisks). Also, pericytes that could be seen on vessels had long projections directed away from the vessels (arrowheads) after AdVEGF-A+AdPDGF-B GT. Scale bars in b and c, 50 μm. See 3D animations of the confocal images in supplemental Videos 2 and 3 for a more accurate picture of the vascular−pericyte interactions.

The Recruitment of α-SMA−Positive Pericytes to Vessels Is Impaired in AdVEGF-A+AdPDGF-B−Transduced Muscles

Quantification of α-SMA−positive cells from histological samples revealed that AdPDGF-B induced a small but significant proliferation of α-SMA−positive cells in normoxic muscles compared to the AdLacZ control 6 days after GT (Figure 5a). In contrast, AdVEGF-A, AdVEGF-A+AdLacZ and AdVEGF-A+AdPDGF-B showed highly increased numbers of α-SMA−positive cells in the muscle. No significant
difference was observed between AdVEGF-A, AdVEGF-A+/AdLacZ and AdVEGF-A+/AdPDGF-B (Figure 5a). However, confocal images of the transduced muscles with rhodamine lectin (endothelium in red) infusion and α-SMA immunostaining (pericytes in green) demonstrated that the recruitment of pericytes to angiogenic vessels differed between the 2 groups. In AdVEGF-A–transduced muscles, α-SMA–positive pericytes were closely associated with enlarged capillaries (Figure 5b; see also supplemental Video 2). After AdVEGF-A+/AdPDGF-B GT, large numbers of α-SMA–positive cells were not associated with vessels but were scattered in the muscle interstitium (Figure 5c, asterisks; see also Figure 1e). These cells also possessed long extensions projecting away from the vessels (Figure 5c, arrowheads; see also supplemental Video 3).

Discussion

Adenoviral delivery of VEGFs is a potentially useful way to increase perfusion in ischemic muscles. The main problems of AdVEGF-A GT include increased plasma protein extravasation, leading to tissue edema and the instability of the newly formed vessels. PDGFs induce pericyte recruitment and migration and are thus proposed to stabilize vessels and decrease edema. In this study, we tested the effect of intramuscular AdPDGF-B in combination with AdVEGF-A on vessel stability and edema formation in intact and ischemic rabbit skeletal muscles.

Rather than inducing angiogenesis, AdPDGF-B GT induced the proliferation of α-SMA–positive pericytes and fibroblasts and the accumulation of interstitial cells, including inflammatory cells such as monocytes, in both normoxic and ischemic muscles, 6 days after GT. Occasional enlargement of capillaries was detected near the interstitial cells in AdPDGF-B–transduced muscles, possibly indicating a role for the interstitial cells in the angiogenic process as previously described. Capillary enlargement after AdPDGF-B GT was more visible in ischemic animals. However, because capillary size was also increased in the ischemic AdLacZ controls, the increase was most likely mediated by hypoxia regulated endogenous growth factors. In contrast, AdVEGF-A induced efficient angiogenesis and recruitment of pericytes around growing vessels at 6 days. When quantified, AdVEGF-A alone was even found to increase the amount of α-SMA–positive cells significantly more than AdPDGF-B alone. However, as expected, extensive tissue edema accompanied rapid changes in vascular growth. When the 2 growth factors were combined, both angiogenesis and massive proliferation of interstitial cells were observed. The mean capillary area and perfusion were smaller in AdVEGF-A+/AdPDGF-B–transduced muscles compared to AdVEGF-A alone or AdVEGF-A+/AdLacZ GT in both normoxic and ischemic conditions. However, edema was rather increased than decreased in the AdVEGF-A+/AdPDGF-B combination group, especially in ischemic muscles. Thus, at 6 days, the combination GT had no significant improvement of angiogenesis and failed to reduce acute edema.

Combination GT was expected to decrease angiogenesis associated edema via stabilization of angiogenic vessels. However, further analysis of the vascular structures in AdVEGF-A and AdVEGF-A+/AdPDGF-B–transduced muscles showed that the recruitment of pericytes to vascular structures was impaired in the combination group compared to AdVEGF-A alone. Although the number of α-SMA–positive cells did not differ between the groups, pericytes in the AdVEGF-A+/AdPDGF-B GT might be the site of transgene expression in the target tissue. In tumor studies, it has been reported that PDGF secreted by tumor cells leads to abnormal attachment of pericytes to vessels. Also, endothelial PDGF-B retention has been shown to be crucial for proper pericyte investment on the vessels during vascular growth. We showed that following intramuscular GT, transgene production took place in several cell types, including monocytes, fibroblasts, and vascular cells. Our histological analysis also showed deposition of PDGF-B protein in the extracellular matrix. It is possible that overexpression of the transgene outside the vascular wall results in the lack of PDGF gradients from endothelial cells and leads to improper pericyte guidance. AdVEGF-A expression alone can induce relatively efficient pericyte recruitment, even if the transgene is not expressed in the vessel wall as a result of blood flow– and shear stress–mediated mechanisms. In fact, the addition of exogenous PDGF-B appears to cause pericyte detachment from the vessels, as shown in the confocal images of AdVEGF-A+/AdPDGF-B–transduced muscles. Thus, the intramuscular combination GT seems to activate pericyte migration away from the vessel wall rather than toward it. These results address the importance of proper PDGF-B gradients in target tissues to induce efficient pericyte recruitment on vessels.

Fourteen days after GT, the effects of AdVEGF-A returned to baseline levels as the expression of transgene attenuated. However, in AdVEGF-A+/AdPDGF-B–transduced muscles, perfusion and interstitial cell density were still increased. Also, tissue edema was still observed at 14 days after AdVEGF-A+/AdPDGF-B GT. Thus, although the combination GT was unable to decrease edema, it was able to induce longer lasting increases in perfusion, compared to AdVEGF-A GT alone, in both normoxic and ischemic muscles. Interestingly, AdPDGF-B was found to induce significant recruitment of inflammatory cells and CD31–positive nonendothelial cells in the transduced muscles 6 and 14 days after GT. Additionally, the angiogenic changes at both time points were often found near sites of cell accumulation. Importantly, many of the interstitial cells in AdPDGF-B–transduced muscle, such as monocytes and macrophages, expressed endogenous VEGF. Additionally, CD31–positive nonendothelial cells have been previously described to have angiogenic potential. Thus, strengthening of angiogenesis via paracrine secretion of growth factors seems a plausible mechanism for the improved net effect of the combination GT and AdPDGF-B. The role of inflammatory cells in arteriogenesis and bone marrow–derived cells mediating PDGF-CC–induced revascularization have been suggested previously and in line with our findings. However, the risk of fibrosis caused by the accumulation of inflammatory cells and the immediate effect of AdPDGF-B on...
fibroblasts\textsuperscript{18} needs to be considered against the therapeutic potential.

In summary, this study proposes that AdPDGF-B in combination with AdVEGF-A prolongs angiogenic effects via paracrine growth factors secreted from recruited cells. Additionally, this study displays the importance of proper transgene expression in target tissues to induce proper pericyte investment on vessels.

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Disclosures
None.

References
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Supplemental files and descriptions

**Online Figure I.** PDGF-B and VEGF-A ELISAs (R&D) were done to confirm proper transgene expression in normoxic muscles. 

a) The amount of PDGF-B in skeletal muscle 6 days after an adenoviral gene transfer was about 400 pg/mg protein. AdVEGF-A+AdPDGF-B gene transfer resulted in a roughly similar expression. 

b) The amount of VEGF-A extracted from the muscle samples 6 days after an adenoviral gene transfer was also about 400 pg/mg protein. The expression levels did not statistically differ in AdVEGF-A, AdVEGF+AdLacZ or AdVEGF-A+AdPDGF-B transduced muscles. AdLacZ and AdPDGF-B showed low levels of endogenous VEGF-A comparable to the levels found in intact skeletal muscle.

**Online Figure II.** Both full (2x10\(^{11}\)vp) and half doses (2x5x10\(^{10}\)vp) of AdVEGF-A and AdPDGF-B were tested in a pilot study. Results in a) perfusion, b) capillary size, c) arterial coverage and d) venous coverage were found consistent with both doses. Since full doses showed more prominent changes compared to AdVEGF-A, it was selected for further studies.

**Online Figure III.** Histological changes found in ischemic skeletal muscles were highly similar to those in the intact muscles. 

a) Ischemic AdLacZ muscles showed slightly enlarged capillaries (arrowheads) and occasional inflammatory cells at day 6. 

b) Ischemic AdPDGF-B muscles showed high numbers of interstitial cells (arrow) and capillaries (arrowheads) comparable to those in AdLacZ. 

c) Ischemic AdVEGF-A transduced muscles had highly enlarged capillaries. 

d) In AdVEGF-A+AdPDGF-B transduced ischemic muscles capillaries were enlarged (arrowheads) and cells could be often detected in the muscle interstitium (arrows). 

e) Occasional α-sma positive cells (arrowheads) could be detected on capillaries in AdLacZ transduced ischemic muscles at six days. 

f) In AdPDGF-B transduced ischemic muscles the amount of α-sma positive cells was
increased but often these cells were found in the interstitium (arrows), not attached to vessels (arrowhead). g) In AdVEGF-A transduced ischemic muscle enlarged capillaries had an almost complete layer of α-sma positive pericytes around them (arrowheads). h) AdVEGF-A+AdPDGF-B transduced ischemic muscles had a clearly weaker α-sma coating on vessels (arrowheads) than AdVEGF-A, and α-sma positive cells could be found in the muscle interstitium not attached to vessels (arrows). i) 14 days after GT, AdLacZ transduced ischemic muscles had some changes such as necrotic muscle fibers and inflammatory cells (arrow) as compared to normal muscle. j) In AdPDGF-B transduced ischemic muscles the amount of interstitial cells was still increased (arrowheads). A few enlarged capillaries (arrow) were found near interstitial cells in addition to occasional necrotic muscle fibers. k) In AdVEGF-A transduced ischemic muscles most angiogenic changes had returned to baseline conditions at 14 days. Some of the larger arteries and veins were still enlarged (arrows). l) AdVEGF-A+AdPDGF-B transduced ischemic muscles had increased interstitial cell density (arrowheads) and some enlarged capillaries (arrows) visible still at 14 days. Scale bars 100 µm in a-h, 200 µm in 1-l and 50 µm in insets.

**Supplementary video 1.** Contrast enhanced Power Doppler ultrasound videos of normoxic semimembranosus muscles transduced with AdLacZ, AdVEGF-A and AdVEGF-A+AdPDGF-B, 14 days after GT. Gene transfer and time point are indicated at the beginning of each clip. The arrival of the contrast agent is visible a few seconds after each clip starts. In AdLacZ transduced muscle occasional vessels are visible. In AdVEGF-A transduced muscle the number of visible vessels is quite similar to that in AdLacZ. However, in AdVEGF-A+AdPDGF-B transduced muscle more vessels are visible meaning that perfusion is clearly higher than in AdLacZ or AdVEGF-A.
**Supplementary video 2.** 3D-reconstruction of confocal images from AdVEGF-A treated normoxic rabbit skeletal muscle six days after GT. Intra-arterially injected Rhodamin Lectin displaying vascular structures (red). α-sma immunostaining visualizing pericytes and myofibroblasts (green). Branching neovessels are occasionally covered by pericytes that are well attached to the vessel wall.

**Supplementary video 3.** 3D-reconstruction of confocal images from AdVEGF-A+AdPDGF-B treated normoxic rabbit skeletal muscle six days after GT. Intra-arterially injected Rhodamin Lectin displaying vascular structures (red). α-sma immunostaining visualizing pericytes and myofibroblasts (green). In some parts of the vasculature, the amount of α-sma positive cells is increased but the connections of these cells to the vascular structures seem disturbed. Many of the α-sma positive cells have projections directed away from the vessels.
Online Figure II

(a) [Graph showing data with different doses and markers with asterisks.]

(b) [Graph showing data with different doses and markers with asterisks.]

(c) [Graph showing data with different doses and markers with asterisks.]

(d) [Graph showing data with different doses and markers with asterisks.]