Urokinase Gene Transfer Augments Angiogenesis in Ischemic Skeletal and Myocardial Muscle

Dmitry O Traktuev[1,2], Zoya I Tsokolaeva[2], Alexander A Shevelev[2], Konstantin A Talitskiy[2], Victoria V Stepanova[2], Brian H Johnstone[1], Tahmina M Rahmat-Zade[2], Alexander N Kapustin[2], Vsevolod A Tkachuk[2], Keith L March[1] and Yelena V Parfyonova[2]

[1]Indiana Center for Vascular Biology and Medicine, Indiana University, Indianapolis, Indiana, USA; [2]Institute of Experimental Cardiology, Russian Cardiology Research Center, Moscow, Russia

Urokinase plasminogen activator (uPA) is required for both endogenous and vascular endothelial growth factor (VEGF)–augmented angiogenesis in normal tissues, leading us to hypothesize that uPA augmentation by gene transfer might promote angiogenesis in ischemic tissues. Overexpression of uPA was studied in rat myocardial infarction (MI) and mouse hind limb ischemia models and compared with VEGF overexpression effects. Animals were divided into control and three experimental groups ($n = 6$), receiving intramuscular injections of plasmids as follows: (i) control (empty vector or expressing β-galactosidase); (ii) uPA; (iii) $VEGF_{165}$; (iv) a 1:1 mixture of uPA and $VEGF_{165}$. The capillary densities in both ischemic models were greater ($P < 0.05$) in tissues treated with uPA, VEGF, or a combination of both than in controls. Infarct size was reduced in hearts from uPA and VEGF experimental groups compared with controls ($P < 0.05$). Local overexpression of uPA induced a marked increase in the number of macrophages and myofibroblasts present within infarcts. Hind limb blood flow was greater in all experimental groups by day 10 ($P < 0.05$). Overall, the effects of uPA and VEGF were uniformly comparable. Additional analysis revealed association of local edema with VEGF but not with uPA treatment. This study established that uPA gene therapy effectively induces functionally significant angiogenesis in models of acute MI and hind limb ischemia.

Received 14 April 2007; accepted 17 May 2007; published online 24 July 2007. doi:10.1038/sj.mt.6300262

INTRODUCTION

Angiogenic gene or protein therapy has been considered as an approach with significant potential for treating patients with ischemic heart and limb diseases. However, this promise has not yet been realized in patients, even with potent angiogenic factors such as vascular endothelial growth factor (VEGF). One limitation to the implementation of gene therapy with VEGF, a prototypic angiogenic factor, has been clinical evidence of dose-dependent vascular permeability, with attendant local edema formation[1,2] as well as hypotension.[3]

It has been demonstrated that mice deficient in urokinase, also known as urokinase plasminogen activator (uPA), possess impaired endogenous ability to restore blood flow compared with wild-type mice with experimentally created hind limb ischemia.[4] Moreover, in uPA knockout mice, VEGF failed to stimulate enhanced angiogenesis in infarcted hearts,[5] suggesting a critical dependence of VEGF angiogenic activity on uPA.

Urokinase is a proteolytic enzyme named for its important role in plasminogen activation and consequent degradation of thrombi, for which it has been widely employed clinically in the context of thrombotic occlusion.[6] Another primary effect of uPA is the direct and indirect (through plasmin) activation of matrix metalloproteinases (MMPs), which then act to degrade the extracellular matrix (ECM) and basement membrane, allowing cells to move through these barriers.[7] In addition, these activated proteases liberate and activate various growth and differentiation factors secreted by cells in inactive forms or sequestered on the cell surface or within the ECM, thus contributing to the evolution of a migratory or invasive cell phenotype.[8–10] Finally, uPA modulates signaling and cell adhesion through uPA receptor and various integrins.[11–13] Direct demonstration of the importance of uPA in angiogenesis has arisen from a number of *in vitro* studies[14,15] as well as by inactivation of the gene *in vivo* as noted above;[4,5] these results have shown that uPA has potent direct and indirect pro-angiogenic activities.

Prior work in our laboratory has shown that local application of uPA protein in the context of vascular injury modulates conduit vessel remodeling.[16,17] However, no prior studies have addressed the potential of local forced expression of uPA to promote angiogenesis *in vivo*. To evaluate the possibility that over expression of uPA, using a clinically practicable approach, could enhance angiogenesis and arteriogenesis and result in enhanced blood flow as well as tissue protection in the ischemic tissue, we studied the effects of plasmid-based gene transfer of uPA in two complementary experimental models of ischemia. uPA expression was induced by local plasmid injection into ischemic tissue of rat myocardium and mouse hind

The first two authors contributed equally to this work.

Correspondence: *Yelena V. Parfyonova, Laboratory of Angiogenesis, Cardiology Research Center of Russia, 3rd Cherepkovskaya 15a, Moscow 121552, Russia. E-mail: yeparfyon@cardio.ru*

limb muscle. Effects of uPA over expression were directly compared with that of either VEGF alone or VEGF in combination with uPA.

RESULTS

Evaluation of myocardial tissue transfection efficiency

Preliminary control experiments were performed to establish the efficiency of intramuscular plasmid transfection, using a plasmid-based β-galactosidase reporter construct. Histochemical visualization of β-galactosidase activity in myocardium revealed that 0.768 ± 0.011% of myocytes in the infarct border zone expressed β-galactosidase at day 7 after plasmid injection (**Figure 1e**).

Expression of human uPA and human VEGF in cardiac muscle

To confirm the functionality of the plasmids carrying therapeutic genes, temporal RNA and protein expressed from plasmid-encoded VEGF or uPA were examined. Using human-specific primers as well as human-specific antibodies, we selectively evaluated levels of human transgene expression in rodent backgrounds. Maximal expression of both transgenes occurred at day 3 after gene transfer (the earliest time point examined). The expression of transgenes was detected in all experimental animals at day 14; however, levels substantially decreased and were undetectable by day 28 (**Figure 1a** and **b**).

The dynamics of protein expression (levels) directly paralleled that of RNAs, being detectable during the first 2 weeks after plasmid injection and becoming undetectable by the fourth week (**Figure 1c**).

Immunohistochemical tissue evaluation revealed that human VEGF and uPA proteins were present in cardiomyocyte cytoplasm and in interstitial cells (**Figure 1d**).

The effects of transgene expression on infarcted myocardium

Vascular density in ischemic tissues. The level of angio- and arteriogenesis in infarcted heart was evaluated in each of three distinguishable zones of myocardium: (i) infarcted zone, (ii) border zone, and (iii) remote non-infarcted myocardium.

At day 14 after treatment, the densities of von Willebrand factor–positive capillaries in the border zone around the forming scar in the uPA, VEGF, and uPA/VEGF groups were greater than in the control groups ($P < 0.05$) and were similar to each other (**Table 1**). A higher density of α-smooth muscle actin–positive (α-SMA$^+$) vessels with distinguishable lumens was measured in uPA, VEGF, and uPA/VEGF groups than in both control groups ($P < 0.05$). Again there was no difference between the three treatment groups (**Table 1**).

The density of capillaries (von Willebrand factor–positive vessels) in the total scar area was higher in the VEGF and uPA/VEGF groups than in the control and saline groups ($P < 0.05$). The density of capillaries in the uPA group was significantly higher than in the saline control group and trended higher than in the plasmid control group ($P = 0.08$). The density of α-SMA$^+$ vessels in the infarcted scar area was higher in the VEGF and uPA treatment groups ($P < 0.05$) than in both control groups (**Table 1**). In the

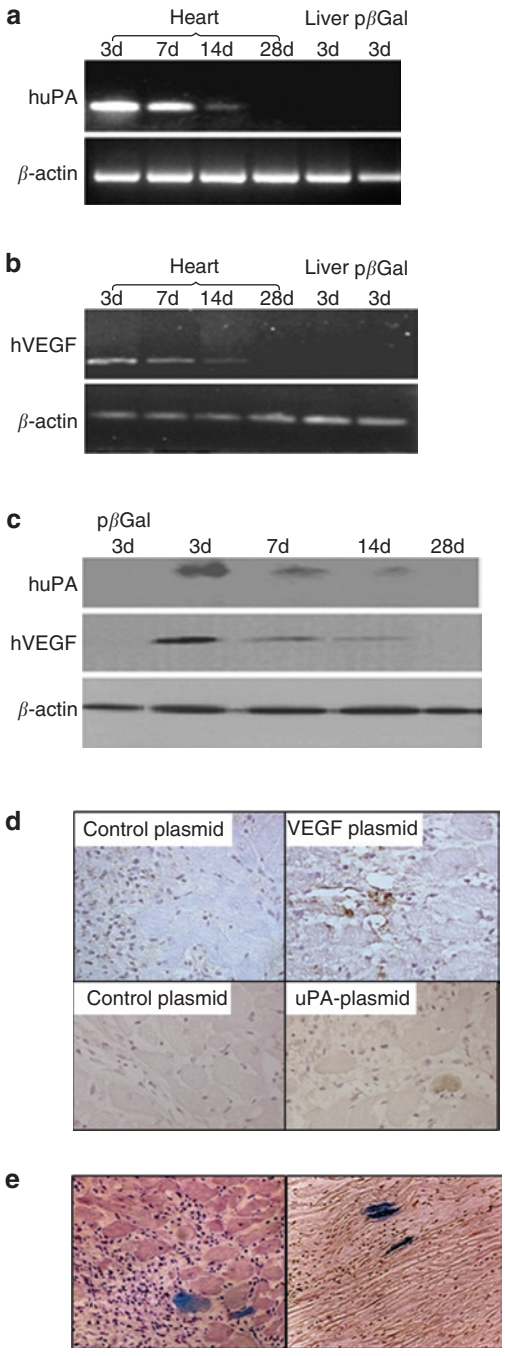

Figure 1 Analysis of β-galactosidase expression and time course of vascular endothelial growth factor (VEGF) and urokinase plasminogen activator (uPA) transgene overexpression in the rat myocardium tissue. (**a**, **b**) Reverse transcription polymerase chain reaction analysis of human uPA and $VEGF_{165}$ messenger RNA expression in infarcted myocardium in groups treated with corresponding plasmid ("liver": a sample of liver harvested from the rats that received the corresponding plasmid; pβGal: a sample of heart tissue harvested from the rats that received β-galactosidase plasmid injection). (**c**) Western blot analysis of human uPA and human $VEGF_{165}$ protein expression in infarcted rat hearts after corresponding plasmid injections. (**d**) Immunohistochemical analysis of VEGF and uPA proteins in myocardial sections. (**e**) β-galactosidase expression in the infarct border zone 7 days after infarction and β-galactosidase plasmid injection: cardiomyocytes expressing β-galactosidase (dark blue) are situated near the necrosis area infiltrated by inflammatory cells (left panel) and around the injection site (right panel).

Table 1 Evaluation density of vWF (endothelial cells) and α-SMA$^+$ vessels in the rat myocardium 14 days after surgery/treatment[a]

	Saline ($n = 6$)	Control plasmid ($n = 8$)	uPA ($n = 6$)	VEGF ($n = 7$)	uPA/VEGF ($n = 6$)
Capillaries					
Border zone	201.2 ± 16.7	224.7 ± 8.9	254.5 ± 8.5[b,c]	288.2 ± 15.9[b,c]	272.6 ± 18.7[b,c]
Scar region	179.6 ± 12.2	195.6 ± 8.1	218.5 ± 7.9[c]	255.8 ± 23.2[b,c]	232.6 ± 12.8[b,c]
Remote region	137.9 ± 10.1	159.8 ± 8.3	140.3 ± 9.5	149.9 ± 10.1	154.7 ± 11.7
Arterioles					
Border zone	19.3 ± 4.5	26.8 ± 2.3	43.5 ± 3.0[b,c]	38.0 ± 2.6[b,c]	35.7 ± 2.9[b,c]
Scar region	25.1 ± 1.4	31.4 ± 3.6	52.1 ± 3.7[b,c]	62.2 ± 2.9[b,c]	42.4 ± 3.8[c]
Remote region	14.0 ± 3.6	14.5 ± 1.9	16.9 ± 1.7	14.2 ± 2.1	14.2 ± 1.7

Abbreviations: α-SMA$^+$, α-smooth muscle actin positive; uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.
[a]Density presented as the number of vessels per high-powered field at ×200 magnification. [b]$P < 0.05$ for experimental groups versus control plasmid group (Student's *t*-test). [c]$P < 0.05$ for experimental groups versus saline group (Student's *t*-test).

Table 2 Body weight and cardiac dimensions at day 14 after MI and plasmid injections

	Control plasmid ($n = 11$)	uPA ($n = 13$)	VEGF ($n = 10$)	uPA/VEGF ($n = 10$)
Body weight (g)	334.4 ± 14.2	332.7 ± 15	327.7 ± 14.9	336.0 ± 8.6
Left ventricular cavity circumference (mm)	22.9 ± 1.1	22.04 ± 1.4	20.5 ± 1.8	22.7 ± 1.3
Post–MI scar area (% of total left ventricular area)	28.8 ± 2.1	23.2 ± 1.6[a]	21.18 ± 1.2[a]	24.4 ± 2.9
Left ventricular wall thickness (mm)	3.12 ± 0.11	3.36 ± 0.12	3.12 ± 0.09	2.9 ± 0.27
Interventricular septum thickness (mm)	3.08 ± 0.06	3.0 ± 0.19	3.02 ± 0.17	3.18 ± 0.25
Scar thickness (mm)	1.01 ± 0.2	1.06 ± 0.19	1.08 ± 0.05	1.04 ± 0.14

Abbreviations: MI, myocardial infarction; uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.
[a]$P < 0.05$ for experimental groups versus control plasmid group (Student's *t*-test).

uPA/VEGF group, the density of α-SMA$^+$ vessels was higher than in the saline group alone.

No differences were found in von Willebrand factor–positive and α-SMA$^+$ vessel densities within remote non-infarcted regions of the heart (Table 1).

Infarct dimensions and left ventricle remodeling. Analysis of the cardiac tissue protective effects of uPA, VEGF, and uPA/VEGF was performed by staining thin sections of the myocardium with Mallory trichrome reagent at day 14 after surgery/treatment. This method allows visualization of areas of fibrosis resulting from tissue necrosis. The ratio of infarcted area to total left ventricular area was smaller ($P < 0.05$) in uPA and VEGF plasmid treated groups than in the control (Table 2 and Figure 2). Treatment with both plasmids led to a slightly reduced infarct area that was not statistically different from control. There was no significant difference in the size of infarcted areas among the uPA, VEGF, and uPA/VEGF groups. Extended analysis of remodeling phenomena revealed that left ventricular chamber circumference, left ventricular wall thickness, interventricular septum thickness, and scar thickness did not differ among the groups (Table 2).

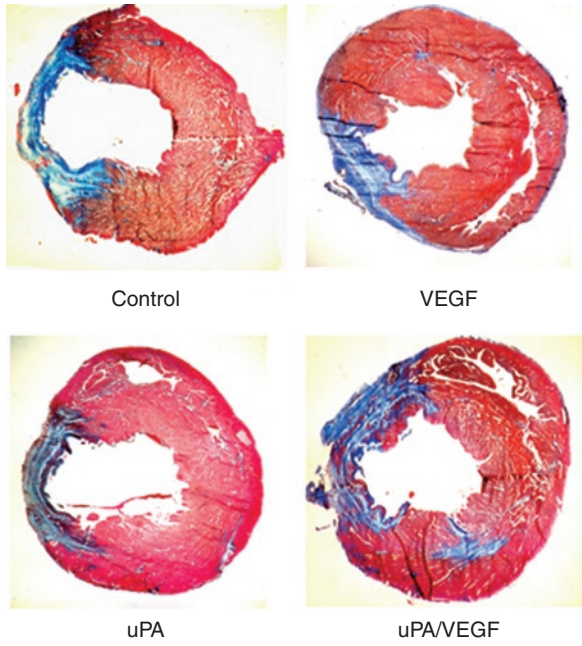

Figure 2 Effect of plasmid treatments on the size of rat heart infarcts. Representative photographs of Mallory trichrome–stained rat heart sections from all experimental groups at day 14 after injury/treatment. uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.

Myofibroblast accumulation in peri-infarct zones. To investigate whether uPA overexpression enhanced myofibroblast accumulation, the presence of non-vessel-associated α-SMA$^+$ cells was assessed in the peri-infarct zone at day 14 after surgery/treatment. Enhanced staining of α-SMA$^+$ myofibrolasts was observed specifically in the forming scar area in the hearts of all groups (Table 3). Significantly higher densities of myofibroblasts were observed in the myocardial sections of uPA-treated hearts compared with the control and VEGF groups ($P < 0.05$).

Table 3 Quantitative evaluation of myofibroblast accumulation within three distinct regions of myocardium at day 14 after infarction and plasmid injections[a]

Zone	Control plasmid (*n* = 6)	uPA (*n* =6)	VEGF (*n* = 6)	uPA/VEGF (*n* = 6)
Border zone	59.2 ± 2.3	144.2 ± 18.7[b,c]	94.8 ± 1.6[b]	109.0 ± 5.6[b]
Remote non-infarcted subendocardium	1.3 ± 0.3	1.1 ± 0.1	1.5 ± 0.04	1.0 ± 0.07
Remote non-infarcted interventricular septum	2.1 ± 0.6	1.5 ± 0.07	1.3 ± 0.16	1.4 ± 0.14

Abbreviations: uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.
[a]Cell numbers (mean ± SEM) per high-power field at ×400 magnification. [b]$P < 0.05$ for experimental groups versus control plasmid group (Student's *t*-test). [c]$P < 0.05$ for uPA versus VEGF plasmid group.

Table 4 Quantitative evaluation of monocyte/macrophage accumulation within three distinct regions of myocardium at day 3 after infarction and plasmid injections[a]

Zone	Control plasmid (*n* = 4)	uPA (*n* = 6)	VEGF (*n* = 5)	uPA/VEGF (*n* = 4)
Infarct zone	84 ± 10.2	186.7 ± 21.8[b]	134.4 ± 15.8	165.9 ± 30.1[b]
Remote non-infarcted subendocardium	1.12 ± 0.35	2.3 ± 2	0.5 ± 0.07	0.5 ± 0.07[b]
Remote septal region	0.5 ± 0.08	0.4 ± 0.07	0.4 ± 0.01	0.4 ± 0.06

Abbreviations: uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.
[a]Cell numbers (mean ± SEM) per high power field at ×400 magnification. [b]$P < 0.05$ for experimental groups versus control plasmid group (Mann–Whitney rank test).

Mononuclear cell accumulation in infarcted hearts. To evaluate the extent of monocyte/macrophage infiltration into recovering myocardium after treatment, the number of $CD68^+$ cells (a surface marker specific for monocytes/macrophages) was determined in thin sections of infarcted regions and compared with the results for remote, non-infarcted regions. As expected, infiltrating $CD68^+$ cells had accumulated in peri-infarct and infarct zones by day 3 after infarction/treatment in all experimental groups (Table 4) and were predominantly associated with vessels (**Supplementary Figure S1**). The number of $CD68^+$ cells in the infarct area was higher in the uPA and uPA/VEGF groups than in the controls (Table 4). The level of $CD68^+$ cell accumulation in remote myocardium was not different among groups and was similar to that in the infarcted zone of hearts treated with VEGF.

The effect of transgene in a mouse ischemic hind limb model

The test plasmids were further evaluated in the complementary ischemic hind limb model. The time course of limb reperfusion after iliofemoral interruption was measured by laser Doppler imaging and expressed as a ratio of blood flow in the ischemic limb

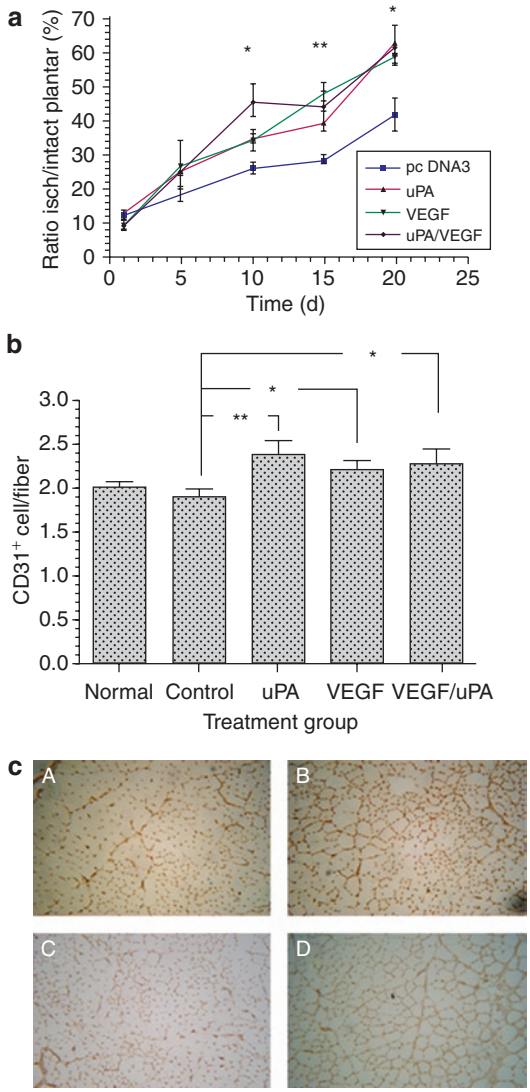

Figure 3 Effect of local urokinase plasminogen activator (uPA) and vascular endothelial growth factor (VEGF) overexpression on angiogenesis in ischemic (isch) mouse limb. (**a**) Blood flow restoration measured on the plantar aspect of the ischemic hind limb of mice receiving control (pcDNA3), uPA, VEGF, and combined uPA + VEGF plasmids. Perfusion data are expressed as the percentage ratio of perfusion in the ischemic limb to perfusion in the non-ischemic limb. (**b**) Analysis of $CD31^+$ cell densities (capillaries) in mouse ischemic hind limb muscles (m. tibialis anterior) at day 20 after plasmid treatment (*$P < 0.05$, **$P < 0.01$). (**c**) Representative photographs of $CD31^+$ cells in ischemic muscles that received control (pcDNA3), uPA, VEGF, and combined uPA + VEGF plasmids, respectively (*$P < 0.05$, **$P < 0.01$).

to blood flow in the contralateral non-ischemic limb (Figure 3a). Perfusion was improved in each treatment group compared with the empty vector control group at days 10, 15, and 20 after induction of ischemia ($P < 0.05$ for all), but was not different among treatments. Capillary density (defined as $CD31^+$ capillaries per visible myofiber) in tibialis anterior (TA) muscles at day 20 (Figure 3b and c) increased in parallel with tissue perfusion. The ischemic/non-ischemic leg capillary density ratio was enhanced by 1.26-fold, 1.16-fold, and 1.2-fold in the groups treated with murine uPA, human VEGF, and human VEGF/murine uPA plasmids, respectively, compared with control ($P < 0.01$, $P < 0.05$, and

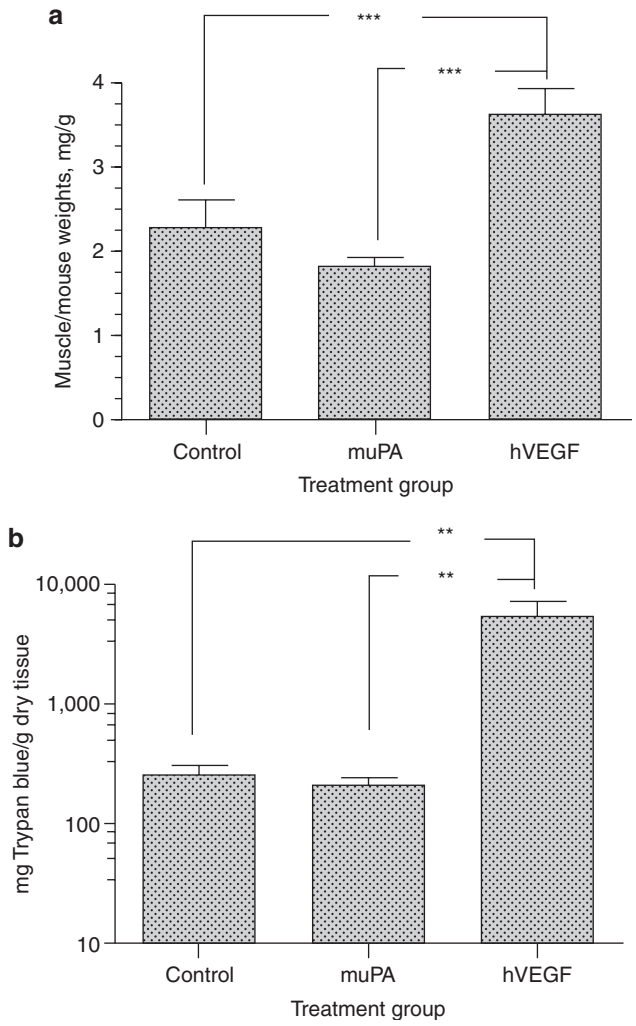

Figure 4 Analysis of edema development in the ischemic muscles (m. tibialis anterior) in response to local overexpression of vascular endothelial growth factor (VEGF) or urokinase plasminogen activator (uPA). Each mouse received local injections of a control or VEGF- or uPA-encoding plasmid, followed by electroporation at the zone of injection. On the fourth day mice received intravenous injections of Evans blue, followed by muscle harvest and dye extraction after 30 minutes. (**a**) Muscle weight immediately after isolation. (**b**) Evans blue dye accumulation in the treated muscle ($n = 3$ for each group) (**$P < 0.01$, ***$P < 0.001$). muPA, murine plasminogen activator.

$P < 0.05$, respectively). Capillary densities in the TA muscles were not different among the three treatment groups.

Analysis of edema development after uPA or VEGF plasmid injections

It has been demonstrated that forced VEGF overexpression was associated with increased vascular permeability, leading to edema.[18] This phenomenon has not been previously evaluated in the context of uPA overexpression. To amplify potential edematous tissue formation, an electroporation was performed after DNA injection to enhance the uptake of plasmid.[19] Extravasation was monitored by systemic injection of Evans blue dye. Interestingly, uPA transfection did not increase Evans blue accumulation in TA muscles (207 ± 25.3 mg/g tissue) in comparison with control plasmid (253 ± 48.4 mg/g tissue), whereas approximately 25-fold more dye leakage from vessels was noted in the VEGF-treated group (5,299 ± 1,734 mg/g tissue) compared with both the control and uPA groups ($P < 0.01$ for each) (**Figure 4a** and **b**). Thus, it appears that, unlike VEGF, uPA stimulates angiogenesis without a commensurate increase in edema formation.

DISCUSSION

The potential clinical utility of gene therapy using individual angiogenic growth factors to treat peripheral limb or myocardial ischemia has been reported by several groups.[20–22] These controlled clinical trials have tested single-gene therapies given as single doses. Thus far, these trials have shown little sustained benefit to patients with ischemic diseases.[23–25] Our study demonstrates a robust effect of uPA overexpression on vessel growth and tissue perfusion as well as limiting of myocardial damage and subsequent remodeling after infarction. These findings support the possibility of a novel strategy using uPA to stimulate vessel growth in both ischemic cardiac and peripheral skeletal muscle tissues, perhaps via direct effects as well as by releasing and activating endogenous growth factors, proteases, and cytokines.

The ability to activate and liberate a protein milieu with multiple complementary functions that act in concert to promote stable/mature vessel formation distinguishes uPA from other molecules that have been used to promote therapeutic neo-angiogenesis. An inherent weakness of monotherapy strategies is that overexpression of individual bioactive molecules is capable of activating only specific signaling pathways (*e.g.*, VEGF and fibroblast growth factor 4) or transcriptional regulators that control sets of pathways normally induced by hypoxia (*e.g.*, hypoxia inducible factor 1α). The predominant mechanism(s) by which uPA overexpression leads to the *in vivo* effects demonstrated in the present study remains incompletely understood.

Among the many functions performed by uPA is activation of MMPs by proteolytic cleavage of polypeptide precursors. The protease activity of uPA is normally tightly regulated by specific inhibitors such as plasminogen activator inhibitor-1. Overexpression of uPA would potentially overwhelm this regulation, leading to enhanced levels of activated MMPs. In addition, uPA is a chemoattractant for circulating monocytes,[26,27] which play substantial roles in collateral vessel development.[28,29] Moreover, prior studies from our group have shown that binding of uPA to its receptor induces MMP-9 expression in monocytes.[30,31] It has been proposed that uPA overexpression enhances vessel growth by increasing interstitial ECM breakdown through activation of MMPs, which in turn enables neovascularization by providing access points for infiltrating vascular precursor cells and growth factor–secreting monocytes,[32,33] as well as by increasing tissue compliance for vessel expansion.[7,13,14,34]

Our data complement the results from previously published studies with uPA and uPA receptor knockout mice, which demonstrated a crucial role of uPA in physiological levels of post-natal revascularization of ischemic myocardium through angiogenesis and collateralization.[5] We observed a highly significant increase in the density of capillaries and smooth muscle cell containing arterioles in hearts treated with uPA plasmid. Increased neovascularization was confined to ischemic regions, suggesting that uPA

complemented the endogenous revascularization process and did not promote hyper-vascularization of non-injured normally perfused regions—an important safety consideration for any potential pro-angiogenic therapy.

We have shown previously that proliferation as well as migration of smooth muscle cells can be enhanced by applying exogenous uPA, leading to enhanced outward remodeling of vessels in a conduit artery injury model.[16,17] Collateral arteriogenesis has several features in common with injury-induced large artery remodeling: both processes are associated with monocyte extravasation and infiltration, inflammation, upregulation of growth factors and expression of adhesion molecules, and activation of smooth muscle cell proliferation and migration.[35–37] Thus, the collateralization may be at least in part responsible for the enhanced preservation of myocardial tissues in hearts receiving uPA after infarction.

Interestingly, protection from scar expansion in the myocardial infarction model was not equivalent in all groups, even though each of the treatments significantly enhanced vascular density in the regions surrounding the infarction. The combination of uPA and VEGF was not as effective at protecting myocardium as either plasmid alone. It is possible that this observation is a result of the reduced dose of the individual factors used in the combination. Confirmation of this explanation would require a dose-response analysis with the individual plasmids, which was not performed. It would be technically very challenging to obtain clear data over such a narrow dose range given the multiple variables affecting delivery of plasmids to tissues and variables of uptake and expression by individual cells.

In the absence of explanations based on dose, these data may indicate that, in addition to angiogenesis, uPA and VEGF influence fundamentally different cardioprotective mechanisms that are not synergistic and may even counteract each other. For instance, uPA promotes infiltration of monocytes, which may be activated by the hostile ischemic environment, and these macrophages may escape into the surrounding vessels through VEGF-induced vascular leakage. Such an occurrence could potentially induce damage to surrounding tissue and lessen the positive effects resulting from enhanced revascularization.

The effect of VEGF on myofibroblast accumulation in the peri-infarct area was less pronounced than the effect of uPA alone. However, the reduction in the transmural scarred area and the enhanced neovascularization were similar in the uPA and VEGF groups. Despite this decrease, uPA and VEGF plasmid treatment did not significantly affect the hypertrophy of the left ventricular wall and enlargement of the left ventricular cavity after myocardial infarction. Further studies are needed to clear up the precise mechanisms responsible for the reduction of infarct size by uPA and VEGF, to determine why these mechanisms are not complementary, and to clarify how uPA overexpression affects heart function and collagen accumulation after MI.

The clinical exploitation of VEGF-based therapeutic approaches has been limited with respect to gene dose by the development of local edema as a consequence of vascular leakiness.[38] Prior studies have shown that expression of a mixture of different VEGF isoforms,[39,40] or additional complementary factors such as angiopoietin-1,[41] can partly mitigate edema. The present study confirmed our hypothesis that uPA overexpression would not lead to the unwanted consequence of tissue edema. However, this study does not indicate whether the absence of uPA angiogenesis-associated edema is a consequence of a limited degree of secondary VEGF activation or rather of the activation and release of complementary vessel-stabilizing factors from the ECM. This question might be addressed in future studies directly exploring the local modulation of protein expression and activity by uPA.

In conclusion, gene therapy with uPA appears to be a viable new monogenic therapy for modulation of ischemic diseases in both the cardiac and skeletal systems. The absence of associated edema suggests that it may be tolerated at high doses, although any consequences of cellular infiltration have yet to be understood. It is tempting to speculate that some of the cells observed as monocytic cells represent vascular progenitors; this topic will form the basis for future investigations. Meanwhile, previous widespread clinical experience with uPA protein as a well-tolerated therapy for thrombolysis lends encouragement for further development of uPA-based approaches for the novel indication of therapeutic angiogenesis.

Translation of uPA to the clinic will require additional studies, including formulation, dose optimization, and optimization of delivery modality. In addition, it will be necessary to demonstrate that the observed enhancements in neovascularization and protection are reflected in improved mechanical function of the heart *in vivo* using echocardiography or other imaging modalities.

MATERIALS AND METHODS

Construction of plasmids. Full-length complementary DNAs (cDNAs) of human $VEGF_{165}$ and human and mouse uPA were inserted into a pcDNA3 vector (Invitrogen, Carlsbad, California) that utilized the cytomegalovirus promoter/enhancer. As controls, we used pcDNA3 vectors that did not contain any component cDNA or cDNA of β-galactosidase. Plasmids were grown in *Escherichia coli*, followed by column purification using the Qiagen Plasmid Purification Kit (Qiagen, Valencia, CA).

Animal studies. Animals were cared for in accordance with guidelines published by the National Institutes of Health, and all study procedures were approved by both the Russian Cardiology Research Center and the Indiana University School of Medicine Institutional Animal Care and Use Committees.

Transfection efficiency. The efficiency of transgene expression was assessed by visualization of β-galactosidase activity in the rat hearts at day 7 after MI by left anterior descending ligation, followed by direct injection of β-galactosidase-coding plasmid into the border zone of myocardium. The method is described in detail in the **Supplementary Materials and Methods**.

Measurement of human uPA and VEGF gene expression. Expression of human $VEGF_{165}$ and uPA was assessed by reverse transcription polymerase chain reaction, western blotting, and immunohistochemistry. The methods are described in detail in the **Supplementary Materials and Methods**.

Rat myocardial infarction model. A MI model was created in adult male Wistar rats (weighing 300–350 g) as previously reported.[42] The animals were anesthetized with ketamine (75 mg/kg, intraperitoneally), and left thoracotomy was performed through the fourth intercostal space. The pericardium was opened and the left anterior descending coronary artery was ligated permanently with a 3-0 silk suture at a site 3 mm distal to the edge of the left atrial appendage. Rats were randomly divided into five treatment groups (n = 6–8 rats/group for vascular density evaluation, and 10–13 rats/group for MI size assessment): (i) phosphate-buffered

saline, (ii) β-galactosidase plasmid, (iii) human uPA plasmid, (iv) human $VEGF_{165}$ plasmid, and (v) an equal mixture of human uPA and human $VEGF_{165}$ plasmids. For myocardial infarction size assessment, the group treated with β-galactosidase plasmid served as a control. Twenty minutes after ligation, 50 µl of plasmid suspension in phosphate-buffered saline (62.5 µg) was injected with a 30-G tuberculin needle directly into each of four sites of the myocardium of the left ventricular wall around the ischemic region, for a total dose of 250 µg of plasmid. For the group that received both uPA and VEGF plasmids, the dose of each plasmid was 125 µg (for a total dose of 250 µg of plasmid).

Mouse hind limb ischemia model and blood flow evaluation. Unilateral hind limb ischemia was created in C57BL/6J mice (8–12 weeks old) as previously described.[43] The animals were anesthetized with 2.5% avertin, after which an incision was made at the midline of the left hind limb. The femoral artery and vein and their branches were ligated from the inguinal ligament to the bifurcation of the saphenous and popliteal arteries, followed by excision of the region between the ligatures. Mice were randomized into four groups ($n = 6$ mice/group): (i) control (empty vector), (ii) murine uPA plasmid, (iii) human $VEGF_{165}$ plasmid, and (iv) an equal mixture of murine uPA and human $VEGF_{165}$ plasmid. One day after surgery, a total of 100 µg of plasmid was injected at five sites of the ischemic limb in a total volume of 100 µl. As in the case of myocardial infarction, for the group that received both the uPA and the $VEGF_{165}$ plasmids, each dose was cut in half for a total dose of 100 µg. The sites of injection were TA, one injection; gastrocnemius, two injections; and quadriceps, two injections. Blood reperfusion in both ischemic and normal hind limbs was evaluated at 5-day intervals for 20 days using laser Doppler imaging (Moor Instruments, Devon, UK) as previously described.[44] In brief, animals were anesthetized by isoflurane inhalation and placed on a heating pad set at 37 °C. The data were collected from the plantar surfaces of both limbs. To account for variability among measurements, including consequences of ambient light and temperature, the results are expressed as the ratio of perfusion in the ischemic (left) and the intact (right) limbs.

Vascular density evaluation. At the end of the studies (for MI at day 14 and for hind limb ischemia at day 20), animals were killed and hearts (from rats) or TA muscles (from mice) were harvested, mounted into OCT medium, and snap-frozen in liquid nitrogen. Frozen sections (6 µm) were used for vessel density assessment. Vessels were visualized by staining tissue sections for von Willebrand factor (rat hearts), α-SMA (rat hearts), or CD31 (mouse muscles). The methods used for vessel visualization are described in detail in the **Supplementary Materials and Methods**.

Infarct size evaluation. To prepare hearts for evaluation of infarct size, rats were killed by an intravenous injection of a saturated KCl solution, which led to cardiac arrest at diastole. The hearts were excised and sectioned transversely into three pieces between the apex and the site of the ligature (with thickness approximately 1.5–2 mm). Each piece was mounted into OCT medium and snap-frozen in liquid nitrogen, followed by preparation of 10-µm sections obtained at each of the three levels of the left ventricle. Sections were stained with Mallory trichrome reagent as previously described.[45] The images of the sections were acquired using a digital camera and subsequently analyzed with UTHSCSA Image Tool software (UTHSCSA Dental Diagnostic Science). The infarct size is expressed as the ratio of transmural scarred tissue area to left ventricular cross-sectional area measured in three sections from each of the three levels of the left ventricle. Data are presented as an averaged ratio ± SEM.

To assess overall cardiac hypertrophy/remodeling, measurements of the circumference of the left ventricular cavity as well as the left ventricular border zone wall thickness and interventricular septum thickness were performed. A transverse section representing the middle of the left ventricle at the region of broadest cavity dilatation was used for each heart. For border zone wall thickness, measurements were obtained on two separate areas for each section and averaged. A single investigator blinded to the treatment groups performed all measurements.

Evaluation of monocyte/macrophage accumulation. The extent of monocyte/macrophage accumulation in the infarcted left ventricle was evaluated immunohistologically by counting $CD68^+$ cells in the tissue at day 3 after surgery/plasmid injection. Rats were killed, and hearts were harvested and mounted in OCT medium, then snap-frozen in liquid nitrogen. Frozen sections (6 µm) were incubated with anti-rat CD68 immunoglobulin-Gs (Serotec, Raleigh, NC; catalog number MCA341R, dilution 1:100). The level of monocyte/macrophage infiltration was represented as the number of $CD68^+$ cells per field using a 40× objective. All available fields were measured within three distinct regions of the cross-section of left ventricle: (i) myocardial infarction area including border zone, (ii) remote non-infarcted subendocardium, and (iii) remote non-infarcted interventricular septum.

Evaluation of edema formation. Mice (C57BL/6J) were anesthetized; the skin covering the TA muscle was shaved and disinfected. Percutaneous injections of 20 µl of murine uPA– or human $VEGF_{165}$–carrying or empty plasmid (1 mg/ml in phosphate-buffered saline) were followed by electroporation. Electroporation was performed using an ECM 830 electroporation generator (BTX, Holliston, MA) with two-needle array electrodes (needles 5 mm apart) with the following settings: two pulses at 60 V for 50 msec, 500 msec apart. To evaluate edema development, 100 µl Evans blue solution (7.5 mg/ml) was injected intravenously 4 days after treatment. Thirty minutes after dye injection, mice were killed and the TA muscles were harvested, weighed, and dried at 55 °C for 24 hours. Dye was extracted by incubating tissues in 500 µl formamide for 24 hours at 55 °C. Dye concentration was measured by spectrophotometry at 610 nm.

Statistical analysis. Data are expressed as mean ± SEM where possible. The differences in the data between two groups were determined with a Student's *t*-test or the Mann–Whitney rank sum test. Comparisons of multiple groups were performed by analysis of variance with correction for multiple comparisons. All analyses were performed using Jandel SigmaStat.

ACKNOWLEDGMENTS

This work was supported by U.S. Civilian Research and Development Foundation grant RB1-2454-MO-02, AHA Scientist Development Grants grant 0430209N, and Russian Foundation for Fundamental Researches grants 05-04-49108A and 06-04-08138-OFR. This work is part of the PhD thesis of Z.I.T.

SUPPLEMENTARY MATERIAL

Figure S1. Immunohistochemical analysis of monocyte/macrophage accumulation in myocardial sections in the hearts from the groups treated with control or uPA plasmid.
Materials and Methods.

REFERENCES

1. Rajagopalan, S, Mohler, ER, Lederman, RJ, Mendelsohn, FO, Saucedo, JF, Goldman, CK *et al.* (2003). Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. *Circulation* **108**: 1933–1938.
2. Kim, HJ, Jang, SY, Park, JI, Byun, J, Kim, DI, Do, YS *et al.* (2004) Vascular endothelial growth factor-induced angiogenic gene therapy in patients with peripheral artery disease. *Exp Mol Med* **36**: 336–344.
3. Eppler, SM, Combs, DL, Henry, TD, Lopez, JJ, Ellis, SG, Yi, JH *et al.* (2002) A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. *Clin Pharmacol Ther* **72**: 20–32.
4. Deindl, E, Ziegelhoffer, T, Kanse, SM, Fernandez, B, Neubauer, E, Carmeliet, P *et al.* (2003) Receptor-independent role of the urokinase-type plasminogen activator during arteriogenesis. *FASEB J* **17**: 1174–1176.
5. Heymans, S, Luttun, A, Nuyens, D, Theilmeier, G, Creemers, E, Moons, L *et al.* (1999) Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac

rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nat Med* **5**: 1135–1142.
6. Wall, TC, Phillips, HR, Stack, RS, Mantell, S, Aronson, L, Boswick, J *et al.* (1990) Results of high dose intravenous urokinase for acute myocardial infarction. *Am J Cardiol* **65**: 124–131.
7. Blasi, F (1999) Proteolysis, cell adhesion, chemotaxis, and invasiveness are regulated by the u-PA-u-PAR-PAI-1 system. *Thromb Haemost* **82**: 298–304.
8. Naldini, L, Tamagnone, L, Vigna, E, Sachs, M, Hartmann, G, Birchmeier, W *et al.* (1992) Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. *EMBO J* **11**: 4825–4833.
9. Plouet, J, Moro, F, Bertagnolli, S, Coldeboeuf, N, Mazarguil, H, Clamens, S *et al.* (1997). Extracellular cleavage of the vascular endothelial growth factor 189-amino acid form by urokinase is required for its mitogenic effect. *J Biol Chem* **272**: 13390–13396.
10. Saksela, O and Rifkin, DB (1990) Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. *J Cell Biol* **110**: 767–775.
11. Dumler, I, Kopmann, A, Weis, A, Mayboroda, OA, Wagner, K, Gulba, DC *et al.* (1999) Urokinase activates the Jak/Stat signal transduction pathway in human vascular endothelial cells. *Arterioscler Thromb Vasc Biol* **19**: 290–297.
12. Goncharova, EA, Vorotnikov, AV, Gracheva, EO, Wang, CL, Panettieri, RA, Stepanova, VV *et al.* (2002) Activation of p38 MAP-kinase and caldesmon phosphorylation are essential for urokinase-induced human smooth muscle cell migration. *Biol Chem* **383**: 115–126.
13. Mukhina, S, Stepanova, V, Traktuev, D, Poliakov, A, Beabealashvilly, R, Gursky, Y *et al.* (2000) The chemotactic action of urokinase on smooth muscle cells is dependent on its kringle domain. Characterization of interactions and contribution to chemotaxis. *J Biol Chem* **275**: 16450–16458.
14. Gualandris, A, Lopez Conejo, T, Giunciuglio, D, Albini, A, Sabini, E, Rusnati, M *et al.* (1997) Urokinase-type plasminogen activator overexpression enhances the invasive capacity of endothelial cells. *Microvasc Res* **53**: 254–260.
15. Koolwijk, P, van Erck, MG, de Vree, WJ, Vermeer, MA, Weich, HA, Hanemaaijer, R *et al.* (1996) Cooperative effect of TNFalpha, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. *J Cell Biol* **132**: 1177–1188.
16. Plekhanova, O, Parfyonova, Y, Bibilashvily, R, Domogatskii, S, Stepanova, V, Gulba, DC *et al.* (2001) Urokinase plasminogen activator augments cell proliferation and neointima formation in injured arteries via proteolytic mechanisms. *Atherosclerosis* **159**: 297–306.
17. Parfyonova, YV, Plekhanova, O, Solomatina, M, Naumov, V, Bobik, A, Berk, B *et al.* (2004) Contrasting effects of urokinase and tissue-type plasminogen activators on neointima formation and vessel remodelling after arterial injury. *J Vasc Res* **41**: 268–276.
18. Weis, SM and Cheresh, DA (2005) Pathophysiological consequences of VEGF-induced vascular permeability. *Nature* **437**: 497–504.
19. Vicat, JM, Boisseau, S, Jourdes, P, Laine, M, Wion, D, Bouali-Benazzouz, R *et al.* (2000) Muscle transfection by electroporation with high-voltage and short-pulse currents provides high-level and long-lasting gene expression. *Hum Gene Ther* **11**: 909–916.
20. Fortuin, FD, Vale, P, Losordo, DW, Symes, J, DeLaria, GA, Tyner, JJ *et al.* (2003) One-year follow-up of direct myocardial gene transfer of vascular endothelial growth factor-2 using naked plasmid deoxyribonucleic acid by way of thoracotomy in no-option patients. *Am J Cardiol* **92**: 436–439.
21. Hedman, M, Hartikainen, J, Syvanne, M, Stjernvall, J, Hedman, A, Kivela, A *et al.* (2003). Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT). *Circulation* **107**: 2677–2683.
22. Mann, MJ (2000). Gene therapy for peripheral arterial disease. *Mol Med Today* **6**: 285–291.
23. Markkanen, JE, Rissanen, TT, Kivela, A and Yla-Herttuala, S (2005) Growth factor-induced therapeutic angiogenesis and arteriogenesis in the heart–gene therapy. *Cardiovasc Res* **65**: 656–664.
24. Cao, Y, Hong, A, Schulten, H and Post, MJ (2005) Update on therapeutic neovascularization. *Cardiovasc Res* **65**: 639–648.
25. Post, MJ and Simons, M (2003) The rational phase of therapeutic angiogenesis. *Minerva Cardioangiol* **51**: 421–432.
26. Gyetko, MR, Todd, RF, Wilkinson, CC and Sitrin, RG (1994). The urokinase receptor is required for human monocyte chemotaxis *in vitro*. *J Clin Invest* **93**: 1380–1387.
27. Poliakov, AA, Mukhina, SA, Traktuev, DO, Bibilashvily, RS, Gursky, YG, Minashkin, MM *et al.* (1999). Chemotactic effect of urokinase plasminogen activator: a major role for mechanisms independent of its proteolytic or growth factor domains. *J Recept Signal Transduct Res* **19**: 939–951.
28. Heil, M, Ziegelhoeffer, T, Pipp, F, Kostin, S, Martin, S, Clauss, M *et al.* (2002) Blood monocyte concentration is critical for enhancement of collateral artery growth. *Am J Physiol Heart Circ Physiol* **283**: H2411–H2419.
29. Ito, WD and Khmelevski, E (2003). Tissue macrophages: "satellite cells" for growing collateral vessels? A hypothesis. *Endothelium* **10**: 233–235.
30. Jadhav, U, Chigurupati, S, Lakka, SS and Mohanam, S (2004). Inhibition of matrix metalloproteinase-9 reduces *in vitro* invasion and angiogenesis in human microvascular endothelial cells. *Int J Oncol* **25**: 1407–1414.
31. Menshikov, M, Elizarova, E, Plakida, K, Timofeeva, A, Khaspekov, G, Beabealashvilli, R *et al.* (2002). Urokinase upregulates matrix metalloproteinase-9 expression in THP-1 monocytes via gene transcription and protein synthesis. *Biochem J* **367**: 833–839.
32. Carmeliet, P, Moons, L, Dewerchin, M, Rosenberg, S, Herbert, JM, Lupu, F *et al.* (1998) Receptor-independent role of urokinase-type plasminogen activator in pericellular plasmin and matrix metalloproteinase proteolysis during vascular wound healing in mice. *J Cell Biol* **140**: 233–245.
33. Carmeliet, P, Schoonjans, L, Kieckens, L, Ream, B, Degen, J, Bronson, R *et al.* (1994). Physiological consequences of loss of plasminogen activator gene function in mice. *Nature* **368**: 419–424.
34. Stefansson, S and Lawrence, DA (2003) Old dogs and new tricks: proteases, inhibitors, and cell migration. *Sci STKE* **2003**: pe24.
35. van Royen, N, Piek, JJ, Buschmann, I, Hoefer, I, Voskuil, M and Schaper, W (2001). Stimulation of arteriogenesis; a new concept for the treatment of arterial occlusive disease. *Cardiovasc Res* **49**: 543–553.
36. Sanchez-Margalet, V, Cubero, JM, Martin-Romero, C, Cubero, J, Cruz-Fernandez, JM and Goberna, R (2004). Expression of activation molecules in neutrophils, monocytes and lymphocytes from patients with unstable angina treated with stent implantation. *Clin Chem Lab Med* **42**: 273–278.
37. Davis, C, Fischer, J, Ley, K and Sarembock, IJ (2003). The role of inflammation in vascular injury and repair. *J Thromb Haemost* **1**: 1699–1709.
38. Dvorak, HF, Nagy, JA, Feng, D, Brown, LF and Dvorak, AM (1999). Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. *Curr Top Microbiol Immunol* **237**: 97–132.
39. Rebar, EJ, Huang, Y, Hickey, R, Nath, AK, Meoli, D, Nath, S *et al.* (2002). Induction of angiogenesis in a mouse model using engineered transcription factors. *Nat Med* **8**: 1427–1432.
40. Amano, H, Hackett, NR, Kaner, RJ, Whitlock, P, Rosengart, TK and Crystal, RG (2005). Alteration of splicing signals in a genomic/cDNA hybrid VEGF gene to modify the ratio of expressed VEGF isoforms enhances safety of angiogenic gene therapy. *Mol Ther* **12**: 716–724.
41. Yamauchi, A, Ito, Y, Morikawa, M, Kobune, M, Huang, J, Sasaki, K *et al.* (2003). Pre-administration of angiopoietin-1 followed by VEGF induces functional and mature vascular formation in a rabbit ischemic model. *J Gene Med* **5**: 994–1004.
42. Pfeffer, JM, Pfeffer, MA, Fletcher, PJ and Braunwald, E (1991). Progressive ventricular remodeling in rat with myocardial infarction. *Am J Physiol* **260**: H1406–H1414.
43. Scholz, D, Ziegelhoeffer, T, Helisch, A, Wagner, S, Friedrich, C, Podzuweit, T *et al.* (2002). Contribution of arteriogenesis and angiogenesis to postocclusive hindlimb perfusion in mice. *J Mol Cell Cardiol* **34**: 775–787.
44. Rehman, J, Traktuev, D, Li, J, Merfeld-Clauss, S, Temm-Grove, CJ, Bovenkerk, JE *et al.* (2004). Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* **109**: 1292–1298.
45. Mallory, GK, White, PD and Salcedo-Salgar, J (1939). The speed of healing of myocardial infarction: a study of the pathologic anatomy in 72 cases. *Am Heart J* **18**: 647–671.