

Increased Vascularity Detected by Digital Subtraction Angiography after VEGF Gene Transfer to Human Lower Limb Artery: A Randomized, Placebo-Controlled, Double-Blinded Phase II Study

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Vascular endothelial growth factor (VEGF) gene therapy may be useful for the treatment of lower-limb ischemia. The objectives of this study were to evaluate safety and angiographic and hemodynamic responses of local catheter-mediated VEGF gene therapy in ischemic lower-limb arteries after percutaneous transluminal angioplasty (PTA). For this study, we recruited patients with chronic lower-limb ischemia and atherosclerotic infrainguinal occlusion or stenosis suitable for PTA. In the study, 18 patients received 2×10^{10} plaque-forming units (pfu) VEGF-adenovirus (VEGF-Ad), 17 patients received VEGF-plasmid/liposome (VEGF-P/L; 2000 μ g of VEGF plasmid, 2000 μ l of DOTMA:DOPE), and 19 control patients received Ringer's lactate at the angioplasty site. Digital subtraction angiography (DSA) was used to evaluate vascularity before, immediately after, and 3 months after the PTA. Clinical follow-up data, basic laboratory tests, and ankle-brachial index (ABI) were evaluated. Primary endpoint was DSA analysis of vascularity, and secondary endpoints were restenosis rate, Rutherford class, and ABI after 3 months follow-up. No major gene transfer-related side effects or differences in laboratory tests were detected between the study groups. However, anti-adenovirus antibodies increased in 61% of the patients treated with VEGF-Ad. For the primary endpoint, follow-up DSA revealed increased vascularity in the VEGF-treated groups distally to the gene transfer site (VEGF-Ad $P = 0.03$, VEGF-P/L $P = 0.02$) and in the VEGF-Ad group in the region of the clinically most severe ischemia ($P = 0.01$). As for the secondary endpoints, mean Rutherford class and ABI showed statistically significant improvements in the VEGF-Ad and VEGF-P/L groups, but similar improvements were also seen in the control patients. We conclude that catheter-mediated VEGF gene therapy is safe and well tolerated. Angiography demonstrated that VEGF gene transfer increased vascularity after PTA in both VEGF-Ad- and VEGF-P/L-treated groups.

Key Words: angiogenesis, gene therapy, growth substances, peripheral vascular disease, angioplasty

INTRODUCTION

Gene therapy is a new approach for the treatment of peripheral arterial occlusive disease (PAOD). Experimental studies have demonstrated successful arterial gene transfer using plasmids, retroviruses, and adenoviruses [1–3]. We have earlier reported that replication-deficient adenoviruses and plasmid/liposomes can be used for gene transfer in human lower-limb arteries and coronary arteries after angioplasty using a perfusion coil balloon catheter [4,5]. The maximal gene transfer efficiency with

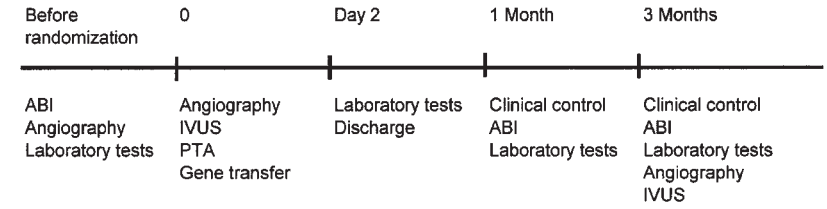
adenoviruses was ~ 5.0% of the arterial cells and was proportional to the virus titer [4]. Gene transfer vector is also released from the catheter to the bloodstream [6], causing transfection of distal vascular endothelium in the ischemic limb. From these findings we concluded that this is a feasible method for delivery of therapeutic genes into ischemic limbs during PTA procedures.

Vascular endothelial growth factor (VEGF) is an angiogenic factor that stimulates endothelial cell migration and proliferation, angiogenesis, and accelerated endothelial repair [7,8]. Animal studies also suggest that VEGF gene

transfer induces rapid production of nitric oxide (NO) and prostacyclin from endothelium, causing a vasculoprotective effect at the site of angioplasty and distally in the treated vessels [9,10]. Initial uncontrolled clinical trials have suggested promising therapeutic effects with naked VEGF plasmid gene transfer [11–13]. However, no data are available from randomized, double-blinded, placebo-controlled trials. In the present study we evaluated safety and hemodynamic and angiogenic responses of VEGF-Ad and VEGF-P/L gene therapy in ischemic lower limbs after PTA.

RESULTS

The study protocol is presented in Fig. 1. Demographics of the patients and drug treatments at the time of recruitment are shown in Table 1. On admission, 40 patients



ABI=Ankle-brachial index, IVUS=Intravascular ultrasound, PTA=Percutaneous transluminal angioplasty.

FIG. 1. Study protocol.

suffered from claudication and 14 patients had critical lower-limb ischemia. After PTA, patients in all study groups were put on statin therapy. Aspirin as a permanent medication and/or clopidogrel for 1 month were started in seven patients in the control group, eight patients in the VEGF-P/L group, and five patients in the VEGF-Ad group.

Safety

During the median follow-up period of 24 months (with a range of 4–36 months), three deaths were recorded (Table 2). These three patients had critical ischemia on admission, and they died because of acute myocardial infarction 16–20 months after the gene transfer. None of the deaths was related to gene therapy. Procedural complications occurred in two patients. One patient in the VEGF-Ad group suffered from distal embolization during a prolonged procedure and received thrombolysis, but the clinical outcome was satisfactory. Another patient developed a pseudoaneurysm at the site of femoral artery puncture.

In general, VEGF gene transfer was well tolerated. No changes in systolic or diastolic blood pressure were observed after the gene transfer. No marked lower-limb edema was present, and only mild edema was detected in a few patients in all study groups, and it was resolved without any specific

TABLE 1: Baseline characteristics of patients

	Control	VEGF-P/L	VEGF-AdV
Number of patients	19	17	18
Age (Range)	73 (61-86)	74 (55-84)	70 (53-86)
Men / Women	8/11	6/11	9/9
Claudication	15	11	14
Critical ischemia	4	6	4
Coronary artery disease	13	9	8
Previous TIA or stroke	6	5	5
Hypertension	13	12	14
Chronic pulmonary disease	5	2	3
Renal dysfunction	2	0	1
Diabetes			
Oral/insulin medication	6	4	3
Anticoagulation			
Aspirin	13	4	10
Warfarin	4	5	4
Beta-blocker	12	12	11
Calcium-blocker	2	3	6
ACE inhibitor	6	6	6
Nitrates	9	7	5
Statins	6	6	5
Gene transfer site			
Femoro-popliteal	15	14	13
Infrapopliteal	4	3	5

No significant differences were found in baseline characteristics (one-way ANOVA or chi-square/Fisher's exact test).

TABLE 2: Safety analysis after follow-up

	Control	VEGF-P/L	VEGF-AdV
Deaths	1	1	1
Major complications	0	0	2a
New cancers	0	0	0
Number of patients with an increase in anti-adenovirus antibody (% of patients)	0 (0%)	0 (0%)	11b (61%)
Number of patients with fever after procedure (% of patients)	0 (0%)	3 (18%)	1 (6%)

^aPeripheral embolisation, pseudoaneurysm.

^bP = 0.0001 between the study groups. No significant differences were found in other parameters (chi-square/Fisher's exact test).

TABLE 3: Laboratory parameters (mean \pm SD)

	Baseline			2 days			28 days			3 months		
	Control	VEGF-P/L	VEGF-AdV	Control	VEGF-P/L	VEGF-AdV	Control	VEGF-P/L	VEGF-AdV	Control	VEGF-P/L	VEGF-AdV
Hemoglobin (g/l)	136 \pm 15	131 \pm 15	136 \pm 16	125 \pm 16	122 \pm 12	129 \pm 16	135 \pm 13	128 \pm 15	137 \pm 20	135 \pm 18	132 \pm 14	137 \pm 19
Platelets (x109/l)	244 \pm 49	254 \pm 57	223 \pm 57	212 \pm 46	206 \pm 43	177 \pm 41	245 \pm 61	285 \pm 63	231 \pm 55	242 \pm 52	268 \pm 73	226 \pm 60
Leukocytes (x109/l)	8 \pm 3	7 \pm 2	9 \pm 6	8 \pm 2	7 \pm 2	7 \pm 7	8 \pm 2	7 \pm 2	9 \pm 8	8 \pm 2	8 \pm 2	8 \pm 2
CRP (mg/l)	<5	6 \pm 17	5 \pm 10	14 \pm 17	29 \pm 20	27 \pm 28	<5	5 \pm 14	<5	<5	<5	11 \pm 21
ALT (U/l)	22 \pm 13	24 \pm 14	23 \pm 17	19 \pm 10	28 \pm 15	24 \pm 15	23 \pm 12	21 \pm 13	28 \pm 18	20 \pm 9	22 \pm 13	25 \pm 23
APT (U/l)	152 \pm 46	173 \pm 101	157 \pm 34	140 \pm 39	158 \pm 117	138 \pm 34	161 \pm 49	157 \pm 72	155 \pm 36	154 \pm 52	151 \pm 51	160 \pm 46
Crea (μ mol/l)	99 \pm 24	104 \pm 20	100 \pm 31	95 \pm 21	98 \pm 13	96 \pm 27	96 \pm 16	101 \pm 15	98 \pm 26	95 \pm 18	106 \pm 24	100 \pm 23
LD (U/l)	382 \pm 113	400 \pm 68	351 \pm 54	351 \pm 116	400 \pm 120	351 \pm 64	385 \pm 109	379 \pm 73	390 \pm 76	379 \pm 112	389 \pm 70	367 \pm 69
VEGF (pg/ml)	276 \pm 147	383 \pm 168	198 \pm 112	280 \pm 191	371 \pm 258	133 \pm 28	309 \pm 247	562 \pm 396	210 \pm 130	238 \pm 145	386 \pm 327	429 \pm 314
CEA (μ g/l)	1.7 \pm 1.0	2.7 \pm 2.5	2.3 \pm 2.1									
PSA (μ g/l)	1.1 \pm 2.3	0.5 \pm 1.0	0.5 \pm 0.8									

CRP, C-reactive protein; ALT, alanine aminotransferase; APT, alkaline phosphatase; Crea, creatinine; LD, lactate dehydrogenase; VEGF, vascular endothelial growth factor; CEA, carcinoembryonal antigen; PSA, prostate specific antigen.

No significant differences were found between the study groups (Kruskall-Wallis test or one-way ANOVA). Within the study groups all groups showed an increase in CRP ($P < 0.01$) and a decrease in platelet count ($P < 0.01$) 2 days after the procedure (Wilcoxon signed rank test).

treatments. No tumors or changes in visual acuity were observed. An increase in anti-adenovirus antibodies was observed in 11 VEGF-Ad patients. One of them had a transient fever reaction. In addition, three VEGF-P/L patients had a transient fever reaction. The basic laboratory tests did not reveal any marked differences among the study groups (Table 3). An increase in C-reactive protein values and a decrease in platelet counts were found in all study groups 2

days after the procedure. No changes were found in plasma VEGF levels after the gene transfer. No VEGF-Ad or VEGF-P/L was found in blood or urine at day 2 after PTA.

Procedural Outcome and Angiography

All endovascular interventions were technically successful. The primary angiographic success rate ($< 30\%$ residual diameter stenosis) for all patients was 87% (91% after

TABLE 4: Restenosis rates and angiographic evidence of new vessel formation at the gene transfer site, at the most ischemic/symptomatic area, and distal to the gene transfer site

	Control	VEGF-P/L	VEGF-AdV	P^a between all groups	P^b between VEGF-P/L and control	P^b between VEGF-AdV and control
Restenosis						
Yes	5	3	6	0.43	1.0	0.53
No	12	13	9			
New vessels at the gene transfer site						
Yes	8	9	12	0.20	0.72	0.08
No	8	7	3			
New vessels at the most ischemic/symptomatic area						
Yes	3	8	10	0.02	0.10	0.01
No	14	8	5			
New vessels distal to the gene transfer site						
Yes	3	10	9	0.02	0.02	0.03
No	14	6	6			

^aChi-square test (2-sided).

^bChi-square/Fisher's exact test, Bonferroni's method (2-sided).

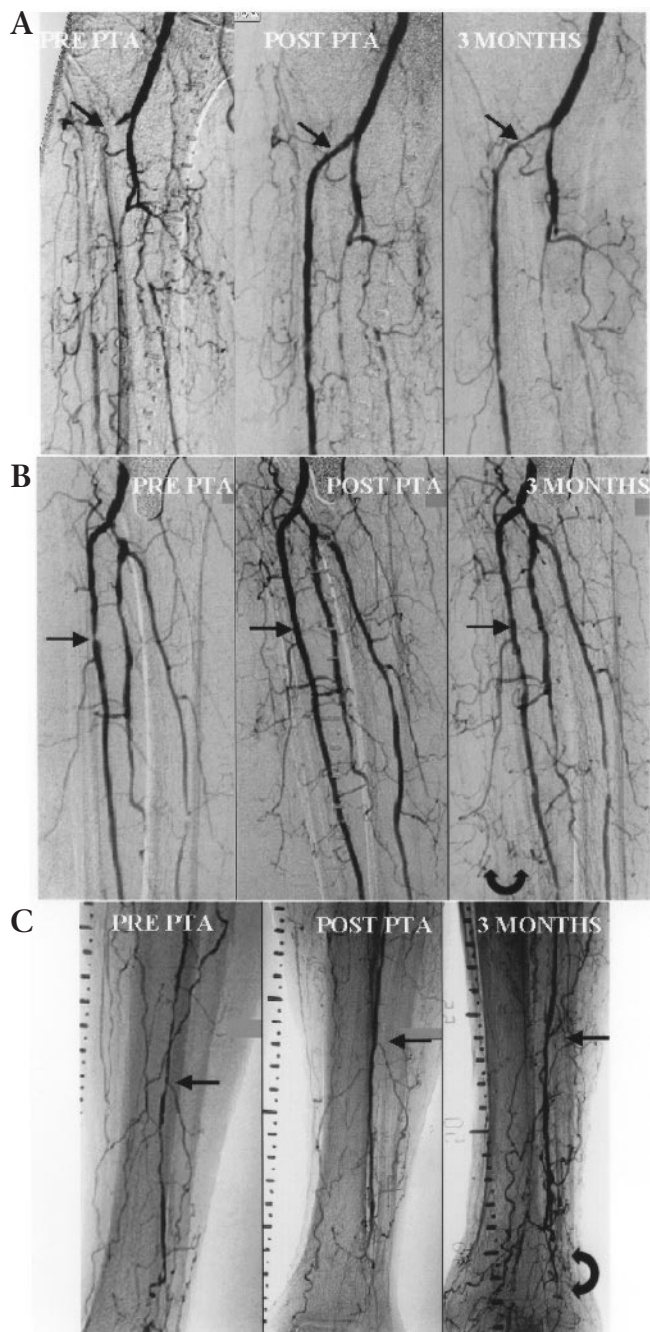


FIG. 2. Representative DSA at 3 months follow-up. (A) Control patient. PTA of anterior tibial artery occlusion. DSA demonstrates continued patency at the angioplasty site (arrow). There was no detectable increase in peripheral vascularity at follow-up. (B) VEGF-Ad patient. Infrapopliteal PTA. Follow-up DSA demonstrates continued patency of the gene-delivery site at proximal anterior tibial artery (arrow) and increased peripheral vascularity in comparison with post-PTA DSA. (C) VEGF-P/L patient. PTA of peroneal artery stenosis. Continued patency at the gene-delivery site (arrow) and increased peripheral vascularity in comparison with post-PTA DSA.

VEGF-treated groups at the entire limb distal to the gene transfer site in comparison to the control group (VEGF-Ad, $P = 0.03$; VEGF-P/L, $P = 0.02$; Table 4 and Fig. 2). Also, the VEGF-Ad group showed increased vascularity in the area of the most severe ischemia ($P = 0.01$). Angiographic restenosis rate did not differ among the groups (Table 4). Restenosis developed in five, three, and six patients in the control, VEGF-P/L, and VEGF-Ad groups, respectively. In these patients, increased vascularity at or distal to the gene transfer site was detected in one, one, and three patients, respectively.

Clinical Outcome and Hemodynamic Studies

No statistically significant differences were observed between the groups in major amputations, ulcer healing, or resolution of the rest pain in the treated legs (Table 5). Of the secondary endpoints, mean Rutherford class and ABI showed statistically significant improvements within the VEGF-Ad and VEGF-P/L groups, but similar improvements were also seen in the control patients; furthermore, intergroup comparison of ABI and Rutherford class did not reach statistical significance (Table 5).

DISCUSSION

We evaluated safety and angiographic and hemodynamic responses of VEGF-Ad and VEGF-P/L gene therapy in the treatment of PAOD. Genes were delivered by catheter during PTA procedure in a randomized, placebo-controlled, double-blinded study. Both VEGF-Ad and VEGF-P/L transfers improved the primary angiographic endpoint 3 months after the procedure. Of the secondary endpoints, improvements were found in ABI and Rutherford class in all study groups.

VEGF was chosen as a treatment gene because it is an angiogenic growth factor that stimulates endothelial migration, proliferation, angiogenesis, and repair of endothelial injuries [7,8]. VEGF also has a strong vasculo-protective effect through the stimulation of NO and prostacyclin production in vascular cells [9,10]. Catheter-mediated gene delivery was chosen as a means of administration during PTA because its efficacy has been demonstrated in a similar clinical situation with a *lacZ* marker gene [4]. The rationale for the VEGF gene therapy was as follows: At the site of PTA VEGF could have a vasculo-protective effect. In addition, gene transfer vector released

femoropopliteal and 75% after infrapopliteal procedures). A total of 11 patients received a stent (5 in control group, 3 in the VEGF-P/L, and 3 in the VEGF-Ad group). Peripheral runoff after PTA was poor in 69% of the treated limbs (0–1 patent calf arteries), whereas the corresponding figure before PTA was 76%.

The primary endpoint of the study was the DSA analysis of vascularity 3 months after the procedure as compared with the post-PTA angiograms. Visually assessed peripheral vascularity increased significantly in both

into distal circulation [6] could have angiogenic and vasculoprotective effects in the ischemic limb. It is conceivable that a more widespread distribution of the transgene will be achieved as compared with intramuscular delivery, because PTA will open the vascular bed and increase flow to the ischemic limb. We cannot at the moment determine whether the effect of the VEGF gene transfer on the vascularity is due to angiogenesis, arteriogenesis, and/or an increased vasculoprotective effect. Increased vascularity at the site of PTA was mostly composed of muscle branches and not bridging collaterals,

while some bridging collaterals were also found more distally. It is possible that VEGF transfection helped to preserve flow in the treated limb within a few weeks after PTA. Continuous blood flow could stabilize hemodynamics even though expression of the transgene is lost during 2–3 weeks after the gene transfer [4,6]. It should be pointed out that obstructed infrapopliteal arteries were present in most patients even after PTA, and that some of the stenoses were at the same or even above the level of the angioplasty. Thus, ischemia and the need for new vessels usually remained even at the level of a patent PTA in the infrapopliteal region. On the other hand, we found no effect on restenosis, although both VEGF-A and VEGF-C adenoviruses have shown efficacy in animal studies [14]. It is possible that severely atherosclerotic human arteries behave differently than arteries tested in animal models.

Even though adenovirus is usually the most efficient gene transfer vector [10], it was not superior to plasmid/liposomes. This may be related to the fast inactivation of adenovirus in blood. The safety profile of the VEGF gene transfer with both vector systems did not reveal any major problems. VEGF-Ad gene transfer has been reported to cause a dose-dependent edema, tissue necrosis, and hypotension in experimental animals after intramuscular (i.m.) delivery [15]. In humans, spider angiomas and edema have been reported after i.m. plasmid delivery [11,12]. We saw only a mild transient peripheral edema in a few patients, but this is most likely due to successful revascularization because similar edema was also seen in a few control patients. It is likely that intraarterial (i.a.) administration will diminish the likelihood of edema as compared to i.m. administration. During the median follow-up of 24 months, only three deaths were recorded. These were due to myocardial infarction and were dis-

TABLE 5: Clinical and haemodynamic results after follow-up

	Control		VEGF-P/L		VEGF-AdV	
Number of major amputations	0		0		1	
Healing of ischemic ulcers	2		3		1	
Resolution of rest pain	1		0		1	
Rutherford grade (mean±SD)						
Baseline	2.3±1.0	<i>P</i> ^a =0.01	3.2±1.5	<i>P</i> ^a =0.004	2.5±0.7	<i>P</i> ^a =0.007
After follow-up	1.2±1.1		1.7±1.7		1.2±1.0	
ABI (mean±SD)						
Baseline	0.64±0.2	<i>P</i> ^a =0.02	0.62±0.2	<i>P</i> ^a =0.004	0.54±0.2	<i>P</i> ^a =0.006
After follow-up	0.77±0.2		0.77±0.2		0.80±0.2	
Subjective improvement in clinical situation (% of patients)	84%		88%		78%	

^a*P* values when baseline and follow-up were compared within each group (Wilcoxon signed rank test).
No significant differences were found between the parameters in intergroup comparisons (Kruskal-Wallis test or chi-square/Fisher's exact test).

tributed evenly among the study groups 16–20 months after the operation. No new cancers were diagnosed during the followup. Incidence of new cancers in the study population is 3400–4300/100,000/year [16]. Thus, we could anticipate 3–4 new cancers/100 patients/year. It is evident that long-term followup is required to determine cancer incidence after VEGF gene therapy.

We found no statistically significant differences in the clinical outcome or hemodynamic studies among the groups. The ABI index, however, may not be an optimal measurement to reflect physiological changes. The reason is that this index is based on the blood pressure in the distal posterior or anterior tibial artery and an increase in ABI reflects an improvement in the patency of inflow (that is, patency of the main arteries proximal to the measurement site), whereas increased vascularity was mostly composed of small side branches, in many cases proximal to the ankle level, and thus less effective in terms of increasing ABI. In the future, transcutaneous oxymetry or magnetic resonance perfusion imaging might be more sensitive measurements. It is also possible that catheter-mediated delivery of the vectors is not optimal and that i.m. or combined i.a. and i.m. administrations could lead to different outcomes of the treated limbs [10].

Previous uncontrolled VEGF gene therapy trials for therapeutic angiogenesis have shown favorable effects [12,13,17,18], whereas local delivery of recombinant VEGF protein was not effective after 60 and 120 days of follow-up [19]. The current study suggests that VEGF-Ad and VEGF-P/L gene therapy leads to increased vascularity in the treated limbs. We conclude that further randomized, double-blinded, placebo-controlled studies are justified for the treatment of PAOD with both adenoviral and plasmid/liposome vectors.

MATERIALS AND METHODS

Patients suffering from PAOD were evaluated for the study. Inclusion criteria were angiographically proven atherosclerotic infrainguinal stenosis or occlusion suitable for PTA. Exclusion criteria were type I diabetes, malignancy, osteomyelitis, fertile women, age < 50 years, signs of active inflammation, abnormal prostate-specific antigen (PSA) or carcinoembryonic antigen (CEA) values, and poor cooperation. Written informed consent was obtained from every patient. Patients were randomized into one of the three study groups: 1) VEGF-Ad gene transfer; 2) VEGF-P/L gene transfer; and 3) Ringer's lactate control group. Randomization was done before the beginning of the study in the blocks of nine patients using a procedure based on random digits. The trial, conducted on an intention-to-treat basis, originally aimed at 20 patients per group, but 6 patients were excluded either because of last-minute laboratory results and signs of acute infection or because they canceled their participation just before the operation. The treatment and followup were made in a double-blinded manner. Dropouts at the 3-month follow-up visit were due to the following reasons: patients refused control angiography because of a good clinical result (one VEGF-Ad patient and one VEGF-P/L patient), pseudoaneurysm and groin hemorrhage (VEGF-Ad patient), technical problems with control imaging procedure (VEGF-Ad patient), unstable angina pectoris (control patient), and a poor general condition because of severe angina and asthma (control patient). The dropouts came from all study groups and were considered not to affect the study results significantly. All studies were approved by the Ethical Committee of the University of Kuopio and the Finnish Agency for Medicinal Products.

VEGF-P/L was made as follows: 2000 µg pCMV-hVEGF₁₆₅ (GenBank accession no. AB021221) plasmid was complexed with 2000 µl of DOTMA:DOPE (1 mg/ml, 1:1; Valentis Inc.) and mixed with 1000 µl Ringer's solution. Human clinical-grade plasmids were manufactured under GMP as described [5]. Plasmids were free of any microbiological or endotoxin contamination (< 200 EU/dose, Whittaker, USA). Replication-deficient human clinical-grade E1- partial E3-deleted first-generation adenoviruses were used [14]. An adenovirus containing the same VEGF expression cassette as in the VEGF-P/L was produced under GMP in 293 cells as described [4]. Adenoviruses were analyzed for the integrity and absence of replication-competent adenoviruses using E1- and E2-specific PCRs, Southern blot analysis, and a cytopathic effect assay on A549 cells [4,20,21]. Virus particles were determined by optical density at 260 nm. Viral preparations were free of microbiological contaminants and endotoxin (< 20 EU/dose, Whittaker, USA).

Preceding PTA, a selective DSA (Siemens Polytron Top, Erlangen, Germany) and intravascular ultrasound (IVUS) (Sonos Intravascular, Hewlett-Packard, Andover, USA) with a 3.5F 30-MHz imaging catheter (Medi-tech/Boston Scientific, Watertown, USA) were conducted. PTA was carried out as described [22], after which i.a. gene transfer was done at the site of PTA using an infusion-perfusion coil-balloon catheter (Dispatch catheter, diameter 2.5–3.5 mm, length 20 mm; Boston Scientific) for infrapopliteal lesions and a channeled-balloon catheter (diameter 4–5 mm, length 40 mm; Remedy, Boston Scientific/Medi-tech) for femoropopliteal lesions [4]. Gene transfer was done during a 10-minute infusion (0.5 ml/minute). The completion angiogram was done at a frame rate of 3/s using power injection of 8 ml contrast media (Omnipaque 300 mg/ml; Nycomed, Norway) at 8 ml/s. Postprocessing of the DSA included a standardized digital image integration to demonstrate a complete contrast filling during the arterial phase up to the early capillary flush. Quantitation of the diameter stenosis before and after PTA was done using the software of the equipment.

Study protocol. Patients were followed at hospital for 2 days. A careful clinical status examination was carried out by one vascular surgeon (K.M.) before the treatment, at discharge, and at clinical controls 1 and 3 months after the procedure. Selective i.a. DSA was carried out 3 months after the primary PTA by using strictly identical parameters. Basic laboratory tests including blood count, CRP, ALT, AFOS, LD, creatinine and anti-adenovirus antibodies were analyzed at the accredited Kuopio University Hospital Central Laboratory according to ISO9002 standards. Plasma VEGF levels were analyzed using a specific ELISA test (Quantikine; R & D Systems, Minneapolis, MN). Assays were done from plasma instead of serum to

avoid variation due to endogenous VEGF released from platelets. The presence of gene transfer vectors in blood and urine samples was analyzed by PCR as described [6]. The ischemic status of the limbs was assessed according to Rutherford classification [23]. ABI was measured before and 1 and 3 months after the gene transfer. Assessment of the clinical response was based on anamnesis, clinical status, Rutherford classification, and ABI.

Classification of the angiographic findings at the pre-, post-, and followup DSA was done according to standardized criteria [24,25]. Image readings were done independently by two radiologists (H.M., P.M.) who did not have access to followup laboratory or clinical information. Evaluation was done in a blinded manner using the following criteria: 1) stenosis exceeding 50% at the PTA site indicated restenosis [24]; and 2) visual comparison of the vasculature was done between post-PTA and followup angiograms [25]. In the assessment of the vasculature the number and abundance of the smallest arterial branches in each anatomical region was registered. For individual arterial branches recognizable in both the post-PTA DSA and the follow-up DSA, the visualized lengths between the angiographies were compared. In statistical analysis a binary classification (unchanged/decreased versus increased vasculature) obtained as a mean of the ratings of the two observations was used. Findings were reported separately from the gene transfer site, from the area of the most severe ischemia, which was located by trophic changes and clinical information, and from the entire limb distal to the gene transfer site, which may have some overlap with the most ischemic area. To assess intraobserver agreement, 15 randomly selected angiograms were re-evaluated by one radiologist (P.M.) [26]. Time elapsed between the two readings was ≥ 6 months. The evaluation demonstrated excellent intraobserver agreement (kappa 0.87–1.0, agreement 93–100%). Interobserver association was good to excellent (kappa 0.60–0.88, agreement 80–94%).

Significance of the results was calculated with the SPSS 9.0 program (SPSS Inc., Chicago, Ill) using tests indicated in each table.

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