The Role of Angiogenic Growth Factors in Arteriogenesis

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Key Words

Arteriogenesis • Growth factors • Shear stress •
Gene transfer • Hemodynamics • Receptors

Abstract

Background/Aims: Collateral vessels restore only about 40% of the maximum dilatory reserve after femoral artery occlusion, whereas complete normalization is reached by increased fluid shear stress (FSS). We studied the role of known potent angiogenic growth factors (separately or in combination) in arteriogenesis by determining their expression in FSS-stimulated collaterals and close-to-collateral infusion of growth factor peptides in a rabbit model of femoral artery occlusion. **Methods:** Values of maximum collateral conductance ($C_{\max}$) and post mortem angiograms were compared to those achievable by high FSS. mRNA levels of growth factor ligands and receptors were determined in FSS-stimulated collaterals. **Results:** Seven days after vessel occlusion, FSS-stimulated legs showed a $C_{\max}$ not significantly different from that of not occluded femoral arteries. Arteriogenesis was significantly less enhanced after growth factor treatment (MCP-1 86%, Ad5.1-FGF-4 75%, bFGF 72%, PDGF 64%, VEGF 50% of $C_{\max}$ after FSS stimulation). RT-PCR showed no differential expression of FGF receptors, but an up-regulation of VEGF-receptor-2. **Conclusion:** The most potent known

angiogenic growth factors at high pharmacological doses reach only a fraction of the maximum conductance obtained by high FSS. Arteriogenesis differs from angiogenesis, so the main focus to markedly improve arteriogenesis should be put on the underlying mechanisms of shear stress.

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Introduction

Despite efforts to design new and efficient treatments for occlusive vascular diseases, they remain the most important cause for death and morbidity in industrialized societies. An important field of research concentrates on stimulating self-cure mechanisms [i.e., the growth of pre-existent arterioles (collateral vessels) bypassing the obstruction], which is named arteriogenesis [1–3]. Although growing collateral arteries can potentially restore blood flow after coronary, cerebral or peripheral artery occlusions [4, 5], the adaptation remains incomplete in experimental (and most clinical) settings, even if arteriogenesis was stimulated by different growth factors or cyto-

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kines [5–9]. Angiogenesis and arteriogenesis differ in some important aspects; the most obvious difference is the dependence of angiogenesis on hypoxia and hypoxia-inducible factor, which is crucial for angiogenesis, but not for arteriogenesis [10]. Collateral vessels never become hypoxic because they are always perfused by arterial blood and are embedded in normoxic tissue. Arteriogenesis depends on the proliferation of smooth-muscle cells following different principles than endothelial cells in angiogenesis. Furthermore, arteriogenesis is dependent on an inflammatory environment and invasion of monocytes [11], which play a role in angiogenesis only in special circumstances (wound healing), but not in hypoxic growth.

That exogenously applied angiogenic growth factors are active in arteriogenesis is not new, but the enhancements in blood flow recovery were either relatively small or the endpoints (i.e., normalization of normal maximum flow) were not defined. The relatively minor improvements of flow recovery under the influence of angiogenic growth factors could lead to the hypothesis that anatomical restrictions might make therapeutic strategies useless because of the increased length and tortuosity of collateral vessels, which increases their resistance. However, we have recently shown that imperfect adaptation by collaterals (only 40% of maximum dilatory reserve) can be surpassed by creating a situation of chronically increased fluid shear stress (FSS) [12]. A fistula between the distal stump of the occluded artery and the accompanying vein stimulated collateral growth to such an extent that normal maximal flow could be reached after only 1 week of elevated shear stress [12]. This means that no structural factors restrict the growth of collateral vessels and the new benchmark for therapeutic interventions is to reach normal maximal flow.

Using that new endpoint, we tested known potent angiogenic growth factors separately or in combination by external infusion of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF)-AB, monocyte chemoattractant protein (MCP)-1, MCP-1/bFGF, MCP-1/VEGF, bFGF/PDGF-AB and intra-collateral adenoviral gene transfer of FGF-4 and compared their activity with the level reached by increased FSS. In addition, we tested the transcriptional activity of these growth factors and their receptors by RT-PCR and genome-wide screening on microarrays to answer the question whether angiogenic growth factors and their receptors play a role in maximal growth stimulated collateral arteries obtained by high FSS.

Materials and Methods

Animal Models

The present study was performed with the permission of the government of Hesse, according to section 8 of the German law for the protection of animals. The investigation conforms to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Sixty male New Zealand white rabbits (3.0 ± 0.3 kg body weight; Charles River, Sulzfeld, Germany) were randomly assigned to 1 of 8 groups receiving a single growth factor, recombinant human FGF-4 adenoviral vector (Ad.FGF), albumin or control vector ($n = 6$ each), or to 1 of 3 groups receiving growth factor combinations MCP-1/bFGF, MCP-1/VEGF, bFGF/PDGF ($n = 3$ each). Surgical procedures were carried out under anesthesia with ketamine hydrochloride (40 mg/kg body weight) and xylazine (4 mg/kg) administered i.m. To prevent pain, buprenorphin (50 μ g/kg) was applied.

For microarray analysis, 4 male Sprague-Dawley rats underwent arteriovenous shunt treatment as described below. Anesthesia was performed with ketamine hydrochloride (100 mg/kg body weight) and xylazine (4 mg/kg body weight).

Growth Factor Treatment

Before treatment, we determined the maximum tolerable growth factor dose that did not cause toxicity (VEGF by scrotal edema, bFGF by hematological toxicity [13]). For MCP-1, high doses were already tested in previous studies [14].

After ligation of the femoral artery, 4 groups received single growth factor infusions, 3 groups received infusions of high pharmacological doses of growth factor combinations locally into the collateral circulation via osmotic minipumps for 1 week: MCP-1 (0.5 μ g/kg/day); VEGF-165 (6 μ g/kg/day); bFGF (3.5 μ g/kg/day); PDGF-AB (2 μ g/kg/day); MCP-1/bFGF (0.5 μ g + 3.5 μ g/kg/day); MCP-1/VEGF-165 (0.5 μ g + 6 μ g/kg/day), and bFGF/PDGF-AB (3.5 μ g + 2 μ g/kg/day) (all from Reprotech, Rocky Hill, N.J., USA). A control group received 1-mg/ml albumin in isotonic buffer.

Intravascular Gene Transfer

A temporary occlusion above and a ligation of the femoral artery below the feeding arteries of the collateral system were applied (fig. 1a). A plastic catheter was inserted into the femoral artery pointing upstream and 2 ml of 0.9% NaCl solution were infused to remove blood from the collateral arteries. Thereafter Ad.5.1FGF-4 (2.87×10^{10} PFU; Bayer-Schering, Leverkusen, Germany) was injected into the pre-existent collateral network. An adenoviral construct coding for green fluorescent protein (Ad.GFP; purified with Adenopack100; Sartorius, Göttingen, Germany) was used as a control. After 30 min of incubation, the temporal occlusions were removed and the femoral artery was permanently occluded.

Arteriovenous Shunt

Arteriovenous shunts (fig. 1b) were prepared as previously described [12, 15]. Briefly, an arteriovenous fistula was created side-to-side between the distal stump of the occluded femoral artery and the accompanying vein to increase blood flow and thereby maintain FSS within the collateral arteries.

Hemodynamic Measurements

Seven days after ligation of the femoral artery, rabbits were anesthetized again and in those with an arteriovenous shunt the fistula was closed. Maximum collateral conductance ($C_{\max}$) was quantified as described earlier [5]. In short, central blood pressure in the carotid artery, peripheral pressures in both saphenous arteries and blood flows in both iliac arteries were measured during maximum adenosine induced dilatation. $C_{\max}$ was calculated using the following equation:

$$C_{\max} [\text{ml/min}/100 \text{ mm Hg}] = \frac{\text{blood flow} [\text{ml/min}] \cdot 100}{\text{CP} [\text{mm Hg}] - \text{PP} [\text{mm Hg}]}$$

where CP is the central pressure and PP is the peripheral pressure.

Histological Analysis

Histological analyses after transfection with Ad.GFP were performed. Hind limbs were perfused with physiological NaCl solution and afterwards with pFA (4%). Both musculus quadriceps intermedius were harvested and cryopreserved. Tissues were cut into sections (6 μm thick). After postfixation with formaline (4%), sections were washed in PBS and stained at 37°C with TRITC-BSI-Lectin (45 min; 1:30) followed by a nuclear staining with DAPI (10 min; 1:1,000). Pictures were taken with a Leica DC 200 microscope.

Postmortem Angiograms and Isolation of Collateral Vessels

To perform postmortem angiograms or to visualize collateral arteries intended to be isolated, hind limbs of euthanized rabbits were perfused with a gelatine- and barium-based contrast medium. Angiographically visible collateral arteries were counted according to the Longland classification [16].

RNA Isolation

PCR analyses were performed to verify the local restriction of adenoviral transduction of collateral arteries. Total RNA was isolated from different tissues of a rabbit 5 days after transduction with Ad5.1FGF-4 using RNeasy Minikit (Qiagen, Hilden, Germany). 300 ng of total RNA was transcribed using the superscript II system (Invitrogen, Karlsruhe, Germany). Human FGF-4 specific primers were used to amplify cDNA.

Changes in RNA expression of different collateral arteries after 7 days of increased FSS treatment (shunt) and ligation ($n = 3$) of different growth factor receptors, namely VEGFR (flk), FGFR-1 IIIc, FGFR-2 IIIb, FGFR-2 IIIc, were investigated by RT-PCR (semiquantitative PCR).

Microarray Analyses

For microarray analysis, collateral arteries were harvested from rat hind limbs 5 days after arteriovenous shunt and sham surgery, as described above. RNA of collateral arteries was isolated after treatment with DNase-I (Turbo DNase; Applied Biosystems/Ambion, Darmstadt, Germany). RNA samples (arteriovenous shunt and sham) were linearly amplified for 2 cycles to average yields of 11 μg cRNA (average 260/280 ratio 1.96) and subsequently labeled with Cy3- and Cy5 dyes (Amino Allyl MessageAmp™ aRNA Kit; Ambion) for microarray analysis. Each sample pair of arteriovenous shunt and control collaterals (1 μg cRNA per Cy-dye) was hybridized in duplicate, according to the

Fig. 1. a Intra-collateral gene transfer: the viral solution was infused into the segment of the femoral artery between the ligation and temporary occlusion. Via the feeding artery of the collateral system, the solution charged the collateral arteries and was incubated over a period of 30 min. **b** Scheme of the shunt model showing surgical procedures and resulting changes in arterial and venous blood flow and pressure. Because of the direct connection to the vein, resistance in the femoral artery distal to the ligation falls, while blood flow and FSS within collateral arteries strongly increase. In turn, increased FSS leads to a strong stimulation of collateral artery growth.

dye-swap design, for 16 h at 40°C (25% de-ionized formamid, 20 mg yeast tRNA, Hyb buffer; Amersham). Microarray analysis was performed essentially as described [17].

Statistical Analyses

All values are expressed as mean $\pm$ SEM. One-way ANOVA (Prism; GraphPad Software Inc., La Jolla, Calif., USA) was performed. $p < 0.05$ was considered statistically significant.

Results

Maximum Conductances after Growth Factor Treatment Do Not Reach Shunt Level

In rabbits with control occluded femoral arteries, $C_{\max}$ showed values of $133 \pm 12 \text{ ml/min}/100 \text{ mm Hg}$. Af-

Fig. 2. $C_{\max}$ (in ml/min/100 mm Hg) is shown after 1 week of treatment with different growth factors in comparison to controls and shunt treatment. The result after shunt treatment (increased fluid shear stress) is significantly higher compared to growth factor treatment. * $p < 0.03$; ** $p < 0.01$.

Fig. 3. Representative postmortem angiographies of variously treated rabbit hind limbs, 1 week after femoral artery ligation. Arrows show typical collateral arteries. **a** Control occluded femoral artery, few collaterals are visible. **b** After adenoviral gene transfer with FGF-4, collaterals are more numerous than in control angiographies. **c** Infusion of MCP-1 increased the collateral count. **d** Collateral arteries are multiplied and enlarged in diameter after shunt treatment.

ter ligation of the femoral artery with additional shunt treatment to increase FSS, $C_{\max}$ was 312 ± 10 ml/min/100 mm Hg. This result was significantly higher than that of the controls and closely approached the maximum conductance of unoccluded femoral arteries (347 ± 14 ml/min/100 mm Hg; $p = \text{n.s.}$).

To compare the growth stimulating effect of high FSS with that of an established pro-arteriogenic factor, we in-

fused MCP-1, which increased collateral blood flow to the highest observed rate in the growth factor group (269 ± 9 ml/min/100 mm Hg), followed by bFGF (226 ± 8 ml/min/100 mm Hg), PDGF (201 ± 19 ml/min/100 mm Hg) and VEGF (157 ± 12 ml/min/100 mm Hg), as shown in figure 2. Although these values, with the exception of VEGF, differed significantly from control (1% albumin 140 ± 12 ml/min/100 mm Hg), they did not reach

Fig. 4. Diagram shows collateral artery count for different groups. Collateral count after growth factor treatment is significantly higher compared to control treatments, but significantly lower compared to shunt treatment. * $p < 0.01$.

the level of shunt-treated ligatures ($p < 0.03$). $C_{\max}$ can be expressed as a percent of shunt values: MCP-1 86%, bFGF 72%, PDGF 64% and VEGF 50%. None of the tested growth factor combinations of MCP-1/bFGF, MCP-1/VEGF or bFGF/PDGF led to additional effects compared to single MCP-1 or bFGF infusion (data not shown).

After intracollateral gene transfer of Ad5.1FGF-4, $C_{\max}$ was 234 ± 24 ml/min/100 mm Hg (vs. control transduction 144 ± 10 ml/min/100 mm Hg, $p < 0.01$), which was 75% of conductance after shunt treatment ($p < 0.03$).

Collateral Count after Shunt Treatment Had Doubled Compared to Growth Factor Treatment

To further correlate hemodynamic results with morphometric parameters, numbers of visible collateral arteries in the different groups were counted (fig. 3). Compared to control groups (albumin 12 ± 1 and control vector 14 ± 1), collateral numbers in MCP-1 (20 ± 1), bFGF (22 ± 1), VEGF (20 ± 2), PDGF (19 ± 3), and Ad5.1FGF-4 (18 ± 1) were significantly increased. Compared to shunt-treatment (43 ± 5), collateral counts after growth factor therapy were significantly lower ($p < 0.01$; fig. 4).

Adenoviral Gene Transduction Is Locally Restricted to the Site of Transduction

A transgene-specific PCR of human FGF-4 revealed a locally restricted transduction of Ad5.1FGF-4 in collateral arteries of the virus-treated hind limb. An accumulation of excessive virus was detected in the spleen and, to a lesser extent, in the liver (fig. 5c).

Histological results after transduction of Ad.GFP aligned with RT-PCR analysis, green fluorescence was

only found in collateral arteries of transduced hind limbs (fig. 5a, b).

Most Growth Factor Receptors Are Not Up-Regulated after Increased FSS

Microarray analyses showed that angiogenic factors (PDGF, FGF-2, FGF-4) and their receptors were not up-regulated after 5 days of increased FSS.

Semiquantitative RT-PCR analyses of control-occluded collateral arteries or after 7 days of increased FSS were performed in order to detect a potential differential abundance of selected growth factor receptor transcripts. The content of mRNA of FGFR-1 IIIc and FGFR-2 IIIb was not increased in FSS-stimulated collateral arteries, and mRNA of FGFR-2 IIIc could not be detected (fig. 6). mRNA of flk-1 (VEGFR-2) was up-regulated after 7 days of increased FSS.

Discussion

Independent of localization in the various organs (coronary, cerebral, renal or peripheral), collateral vessels are easily identifiable by their tortuous course. Tortuosity means also an increase in length, and both add to the hemodynamic resistance and cause energy loss. Resistance in curvature flow is difficult to predict, but follows in principle the Dean equation, which means that tight turns of the vessels increase the Dean number and resistance [18, 19]. Since the development of collateral vessels after arterial occlusion stops prematurely and reaches only about 40% of the functional capacity of the artery before occlusion, it was assumed that compensation by

Fig. 5. Verification of single-sided local gene transfer (**a**, **b**). Histological investigations of collateral arteries after single-sided intracollateral transfection with Ad.GFP. **a** Collateral artery of the not transfected leg. Magnification $\times 40$. **b** Collateral arteries of the transfected leg. The green color indicates presence of GFP. Magnification $\times 40$. **c** Results after PCR of human FGF-4 in different tissues after single-sided transfection with Ad5.1FGF-4. Only collateral arteries of the transduced leg (3), spleen (11) and liver (12) showed PCR products.

collaterals is a self-limiting process that is resistant to stimulation. In a recent study, we could show that the cause for the premature halt in collateral development is not anatomical constraint, but rather the premature fall in FSS, which is related to the cube root of the expanding radius [12]. When the FSS is prevented from becoming normalized by the growth process through creation of a fistula between the peripheral stump of the occluded artery and the accompanying vein, draining most of the collateral flow into the venous system, complete normalization of the full vasodilatory reserve could be achieved [12].

Without anatomical constraint, growth factors should be able to significantly improve the function of collateral vessels after femoral artery occlusion. In the present study, we investigated the question of whether angiogenic growth factors are able to mimic the effects of high

shear stress when exogenously applied and whether they and their cognate receptors are involved in FSS-stimulated collateral artery growth. Growth factor studies have shown that those stimulating angiogenesis are also able, after exogenous application, to stimulate arteriogenesis. However, it remained unclear what the endpoints of these studies were and how close the effect was to the physiological situation. In the present study, we show that complete normalization can be reached, but that angiogenic growth factors in high pharmacological doses do not achieve the same level as FSS does.

In various studies, it was shown that growth factors and cytokines, at least under experimental conditions, are potent stimulators of arteriogenesis, capable of reducing signs of critical ischemia and improving collateral conductance [4, 5, 11, 20–22]. Although most of these studies showed differences compared to untreated controls, the

degree of compensation with regard to the maximum was not known because no maximal endpoint could be defined until now. A few studies [23] compared the effects of treatment with the maximum normal flow under vasodilatation, but it remained unknown whether the growth factor treatment was reaching the maximum achievable level.

Nevertheless, even under experimental settings it could not be shown at any time that the collateral circulation was able to completely compensate for the occluded artery after growth factor treatment, whereas we could show that increased FSS is able to rapidly stimulate arteriogenesis. In our experiment, FSS was enhanced and maintained within the collateral circulation by a surgical intervention, an arteriovenous shunt, connecting the femoral artery to the femoral vein [12, 15]. Because of the direct connection to the venous system, a pressure gradient along pre-existing collateral vessels was generated. The decreased resistance induced a strong and sustained increase in blood flow, and hence FSS, in the collateral arteries. Although the complete molecular mechanisms of the growth-regulating effect of increased FSS on collateral arteries are not known, it was shown that FSS activates the Ras-ERK and Rho pathways [12]. Despite the fact that the Ras-ERK pathway is strongly involved in the transmission of growth factor-initiated signals to cell proliferation [24], none of the angiogenic growth factors applied in this study is capable of matching the effect of increased FSS on arteriogenesis.

Gene Transfer Studies

Gene transduction of vascular smooth-muscle cells in vivo remains a problem, although different catheters and methods are available for vascular transfection [25, 26]. Nevertheless, none of these methods are suitable for the transduction of pre-existing collateral arteries because of their small dimensions. As well as the size of the collateral arteries, which leads to the problem of local delivery, barrier properties of the vascular wall also reduce the transduction efficiency of adenoviral vectors for vascular tissue [27, 28].

In some studies, intramuscular transfection was used to stimulate arteriogenesis [29–31]. However, although collateral artery growth can be enhanced, this method is not able to specifically target collateral arteries. It is still unclear whether transfection of surrounding skeletal muscle cells may influence the remodeling process within the collateral vessel wall. Therefore, a new specific method of intracollateral gene transfer was required to analyze the potency of adenoviral vectors in enhancing collateral growth.

Fig. 6. Semi-quantitative analyses of mRNA expression of growth factor receptors in ligature collaterals (LC) and shunt collaterals (SC). FGFR-IIIc was not detected. FGFR-1 IIIc and FGFR-IIIb showed no differential expression, whereas an up-regulation of VEGFR-2 could be shown.

We developed a model which assures that the applied virus can sufficiently interact with the collateral wall cells, hence increasing transduction efficiency [27, 32].

PCR analyses after intracollateral transduction revealed human FGF-4 transcripts only in collateral arteries of the transduced leg, in the spleen and, to a lesser extent, in the liver. This indicated a high specificity of this gene transfer technique because over-expression is restricted to transduced collateral arteries, which was confirmed by histology.

Other arteries did not show FGF-4 transcripts. The fact that we did not find endogenous rabbit mRNA of FGF-4 may be due to the observation that FGF-4 expression is restricted to embryogenesis and some types of cancer [33].

The Role of Angiogenic Growth Factors in Arteriogenesis

The differences between growth factor- and FSS-stimulated arteriogenesis caused us to investigate the expression of growth factor receptors in strongly growing FSS-stimulated collateral arteries to test the hypothesis that growth factor-stimulated arteriogenesis is limited by the availability of growth factor receptors. We performed PCR analyses for rabbit gene sequences of FGFR-1 III, FGFR-2 IIIb and FGFR-2 IIIc and VEGFR-2 in FSS-stim-

ulated collateral arteries. The rabbit gene sequence for PDGFR is presently not available. It is known that FGFR-1 is upregulated and activated in the early phases of arteriogenesis [34, 35]. Even though arteriogenesis is not self-limiting in our shunt model, expression of FGF-receptors is not upregulated under conditions of elevated FSS. Our observation that VEGFR-2 is up-regulated in FSS stimulated collaterals is consistent with other studies demonstrating that VEGFR-2, together with PECAM-1 and VE-cadherin, is part of a mechanosensory complex, and that 2 methods of VEGFR-2 activation exist: ligand-dependent and ligand-independent activation by FSS as a mechanotransducer for eNOS activity [35–37]. Additionally, we and others showed that inhibition of NO-synthesis blocked the effect of increased FSS [12, 38].

In our hemodynamic measurements, we confirmed that VEGF does not belong to the most powerful growth factors stimulating arteriogenesis [5]. However, external application of VEGF increased markedly the collateral count, as shown in postmortem angiograms. The discrepancy between functional and morphological results reflects earlier reports that VEGF induces growth of immature vessels with inferior blood conducting function [39].

For application, we used continuous intravascular infusion of high pharmacological doses of angiogenic growth factors or adenoviral gene transduction. Local intra-arterial delivery has proven to be superior to all other models of administration (e.g. intravenous, intramuscular, intrapericardial) because it allows prolonged exposure of the vasculature itself to therapeutic growth factors [40]. Hemodynamic measurements and collateral counts showed that all growth factors markedly promoted the growth of collateral arteries, though none of them reached the maximum conductance of nonoccluded femoral arteries, as shunt treatment did. The strongest recovery was achieved by MCP-1 followed by FGF. These factors activate and stimulate adhesion of monocytes and macrophages which are known to play an important role in collateral growth, partly also as producers of FGF-1 and FGF-2 [7, 14, 34, 40–42].

We further tested the hypothesis that growth factor combinations are needed to improve results. Surprisingly, no growth factor combination used in this study led to additional effects compared to single factor application. This is not in contradiction to other studies because growth factors separately or in combination are very potent in stimulating arteriogenesis in combination with angiogenesis in ischemic hind limb models [43–46], but they were never able to completely restore the conductance capacity of a larger artery. Alternatively, we used a hind limb

model where the region of collateral growth is not ischemic. For stimulating arteriogenesis by itself, known endogenous growth factors do not play the leading part, as shown in this study. We cannot exclude that there might be other combinations that are more efficient. However, their efficiency was shown only for angiogenesis.

Our previous studies with different methods of gene expression including genome-wide profiling with microarrays showed no increased transcription of the mentioned angiogenic growth factors (VEGFA, FGF-1, FGF-2, PDGF) or their receptors in growing collaterals, with the exception of MCP-1. On the other hand, there is evidence that growth-promoting factors, especially FGFs, are supplied by monocytes in a paracrine manner [34]. It is therefore not surprising that high pharmacological doses of growth factors are able to mimic parts of the natural process of arteriogenesis in an indirect way.

Nevertheless, chronic elevation of FSS by the shunt model is the only force that can completely restore (and overshoot) the physiological function of an occluded femoral artery. In shunt-treated animals, collateral count doubled compared to growth factor treatment and maximum conductances reached values of nonoccluded arteries at day 7, increasing further to double compared to nonoccluded arteries at 4 weeks [12]. The reason for the apparent discrepancy between the shunt effect on the collateral score (doubling compared to growth factor treatment) and on maximum conductance (e.g. MCP-1 reached 86% of shunt values) at day 7 is the higher degree of tortuosity of collaterals after shunt treatment, leading to a higher Dean number and resistance [18, 19]. However, tortuosity is not a limiting factor for the development of collaterals [12].

To our knowledge, this is the first study comparing the influences of shear stress with that of growth factor application on restoration of dilatory reserve capacity in a direct manner. There are some limitations to this study. With all growth factors, we used the maximum tolerable dose and did not test if submaximal doses or other application modalities had any different effects. To quantify arteriogenesis, we measured collateral conductance during maximum adenosine-driven vasodilatation, as exercise-induced vasodilatation is not feasible in rabbits. Of course, shunt treatment is not applicable in patients. But against the background of the negative outcome of clinical growth factor trials [40], it appears worthwhile to unravel the molecular events that occur with high shear stress to markedly improve the situation of patients with occlusive vascular diseases.

We conclude that increased FSS has the strongest stimulating effect on arteriogenesis because collateral ar-

teries can completely restore the maximum normal conductance within 1 week after femoral artery ligation, when FSS is sufficiently elevated. In contrast to this, growth factors applied at high pharmacological doses reach only a fraction of the maximum.

This highlights the fact that angiogenesis and arteriogenesis are differently regulated processes. Our studies with increased FSS define an endpoint for further studies employing new growth factors or pharmacological agents.

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