Granulocyte-macrophage colony-stimulating factor stimulates arteriogenesis in a pig model of peripheral artery disease using clinically applicable infusion pumps

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Background: **A growing number of patients suffer from peripheral artery disease (PAD). Current therapies are often limited by the extent of vascular pathology and the occurrence of restenosis after angioplasty. The stimulatory effect of growth factor administration on collateral vessel formation (arteriogenesis) has evolved as a potential new treatment for this patient group. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was shown to stimulate arteriogenesis in small-animal models and in a pilot study in patients with coronary artery disease. Although a recent clinical study demonstrated disappointing results after subcutaneous GM-CSF application in patients with PAD, we hypothesized that intra-arterial cytokine application using implantable infusion pumps might well stimulate arteriogenesis in a large-species model of peripheral vascular disease. We also aimed to compare continuous and intermittent infusion regimens and to validate experimental and clinically available measurements of collateral artery growth.**

Methods: **Twenty-four pigs underwent unilateral occlusion of the right femoral artery and received either GM-CSF continuously, GM-CSF intermittently, or phosphate-buffered saline (PBS). After 1 week, collateral conductance was determined under maximal vasodilatation with adenosine and by using a pump-driven extracorporal shunt system.**

Results: **Conductance showed a significant stimulatory effect of GM-CSF on arteriogenesis (collateral conductance [mL/min/mm Hg]: PBS, 37.7 ± 5.4; GM-CSF continuous, 69.2 ± 12.5; GM-CSF intermittent, 71.5 ± 11.1). Flow measurements under reactive hyperemia were consistent with these results (flow occluded/non-occluded hind limb: PBS, 40.5% ± 9.1%; GM-CSF continuous, 48.9% ± 3.9%; GM-CSF intermittent, 48.7% ± 4.4%). Measurements of ankle/brachial indices were not sensitive enough to detect the differences in collateral growth between the three groups.**

Conclusion: **These results demonstrate the proarteriogenic properties of GM-CSF in larger animal species, revealing comparable efficacy of continuous and intermittent intra-arterial infusion. Furthermore, we provide evidence that implantable pumps offer a possible means for the intra-arterial application of growth factors. Intra-arterial application of GM-CSF might be a future treatment option for vascular occlusive disease. Finally, we show that in the peripheral circulation, pressure measurements alone have a low sensitivity to determine the effects of proarteriogenic therapy compared with flow or combined flow-pressure measurements. (J Vasc Surg 2006;43:1263-69.)**

Clinical Relevance: **The stimulation of collateral artery growth (arteriogenesis) with granulocyte-macrophage colony-stimulating factor (GM-CSF) has evolved as a promising treatment strategy for patients with coronary artery disease, but a recent study in patients with peripheral artery disease showed negative results after subcutaneous GM-CSF therapy. The current experimental study in a porcine peripheral artery disease model now elucidates the efficacy of continuous intra-arterial GM-CSF application in a large-animal model of peripheral artery disease. A programmable pump-system was implemented that might overcome the delivery problem that was encountered during the patient trial. Clinical and experimental end points were applied to compare a continuous with an intermittent treatment regimen.**

Peripheral artery disease (PAD) is estimated to symptomatically affect 3% to 6% of men aged >60 years. Despite the progress in diagnosis and prevention, roughly 25% of all patients with intermittent claudication have significant disease progression, many to the point of developing critical limb ischemia.[1] Current interventional treatment options include endovascular or surgical revascularization; however, not all atherosclerotic occlusions can be revascularized by these approaches. Furthermore, many PAD patients have an increased operative risk.

The pharmacologic stimulation of true collateral artery growth (arteriogenesis) is a promising alternative therapeutic modality.[2] In contrast to angiogenesis, which describes the formation of capillary networks through endothelial

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sprouting, arteriogenesis refers to the growth of larger arteries from small pre-existing anastomoses.[3] One promising candidate for therapeutic stimulation of arteriogenesis is granulocyte-macrophage colony-stimulating factor (GM-CSF), which was shown to promote collateral artery growth by prolongation of monocyte survival in a rabbit hindlimb model of arteriogenesis.[4]

In a clinical pilot trial, GM-CSF increased collateral flow in patients with coronary artery disease,[5] whereas in the recently conducted START trial, no significant effects were seen in PAD patients.[6] In contrast to the experimental study and the coronary artery disease trial, in the Stimulation of Arteriogenesis (START) trial GM-CSF, was applied solely via a subcutaneous route, which might well be an explanation for the negative outcome of the study.

We hypothesized that arteriogenesis can be stimulated by GM-CSF application in a large-animal model of PAD if the cytokine is infused locally into the collateral circulation over a prolonged time period. The substance was applied via clinically available programmable implantable infusion pumps. Because GM-CSF has previously been shown to downregulate the expression of its own receptor (GM-CSFRα) on circulating cells and thereby decrease the efficacy of the compound in a tachyphylactic reaction,[7] we also compared a continuous with an intermittent intra-arterial treatment regimen. To measure efficacy, we applied the clinically applicable end points of ankle/brachial-index (ABI) and flow under reactive hyperemia, as well as the experimental end point of collateral conductance.

MATERIAL AND METHODS

Operational procedure. The current study was performed in conformity with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996), after appropriate institutional approvals were secured.

Twenty-four German Landrace pigs with a mean body weight of 75 ± 3 kg received an intramuscular injection of flunitrazepam (0.2 mg/kg body weight) and 10 to 20 mg/kg of ketamine. Anesthesia was intravenously induced with 0.001mg/kg of fentanyl and 2 to 4 mg/kg of propofol. After muscular relaxation had been induced with 0.08 mg/kg of vecuronium, the animals were ventilated with N_2O:O_2 in a ratio of 2:1. Anesthesia was maintained by continuous intravenous infusion of 0.06 mg/kg/h of vecuronium, 0.001 mg/kg/h of fentanyl, and 5 mg/kg/h of propofol.

Femoral artery ligation. All animals underwent unilateral occlusion of the right femoral artery, 3 cm distal from the branching of the circumflex femoral artery. The circumflex femoral artery was also ligated to further reduce blood flow to the distal hindlimb. A sterile catheter was inserted and fixed upstream in the proximal stump of the occluded femoral artery, ensuring delivery of the substances directly into the collateral circulation. The catheter was tunneled subcutaneously and connected to an electronic pump device (Synchromed EL, Medtronic, Inc, Minn), which was implanted in the subcutaneous tissue of the lower abdomen.

Observation period. During the observation period, the animals were allowed to move freely, with access to water and chow. Eight animals received human recombinant GM-CSF (Sargramostin, Berlex, Inc, Montville, NJ) in a 0.05% solution of phosphate-buffered saline (PBS) via the implantable pump device at a pump rate of 750 μL/d continuously over the whole observation period of 7 days. Another group of eight animals received GM-CSF in an intermittent fashion (1-hour infusion, 7-hour break, three cycles per day). Both groups received a total of 5 μg/kg GM-CSF per day intra-arterially in correspondence with the clinical trials that used subcutaneous injections for proarteriogenic therapy and with the clinically used doses for stem cell mobilization. The third group received PBS only via continuous infusion.

Hemodynamic measurements. With some adaptations, measurements were performed as previously described.[8] Animals were anesthetized and heparinized. For the ABI index measurements, the right radial artery was dissected and cannulated for continuous pressure monitoring. The abdomen was incised along the median and the peritoneum was opened. Afterwards, the abdominal aorta was carefully dissected from the iliac bifurcation to the branching of the renal arteries. The caudal mesenteric artery was dissected and cannulated for monitoring and measurement of the prestenotic blood pressure during the collateral conductance measurements. Two ultrasound flow-probes (Transonic Systems, Inc, Ithaca, NY) were placed around the common iliac arteries, one on each side. For the monitoring and measurements of the poststenotic blood pressures, the very distal posterior tibial arteries were dissected and cannulated with a sterile polyethylene catheter. For all hemodynamic measurements, the catheters of the mesenteric artery, the radial artery, and both posterior tibial arteries were connected to pressure transducers.

ABI and iliac artery flow at rest and during reactive hyperemia. The ratio of posterior tibial artery pressure and radial artery pressure served as the ABI. For the induction of reactive hyperemia, the abdominal aorta was clamped for 1 minute between the mesenteric and renal arteries. After the clamp was released, maximal iliac blood flow and accompanying blood pressure were measured. The mean of three measurements (with a minimum of 5 minutes between each measurement) was used for data analysis.

Maximal iliac artery flow after clamp release was expressed as a ratio of occluded/nonoccluded hindlimb. In addition we measured flow/pressure-deficit ratio (FPDR = iliac artery flow/Δp) as previously described.[9] FPDR corresponds to the collateral conductance, but whereas true conductance is derived from the slope of the curve of several flow/pressure relations using an extracorporal circulation, FPDR is derived from a single measurement under reactive hyperemia. FPDR of the ligated hindlimb is expressed as an absolute value in mL/min/mm Hg, as well as the ratio of the occluded vs nonoccluded hindlimb to correct for differences in systemic pressure.

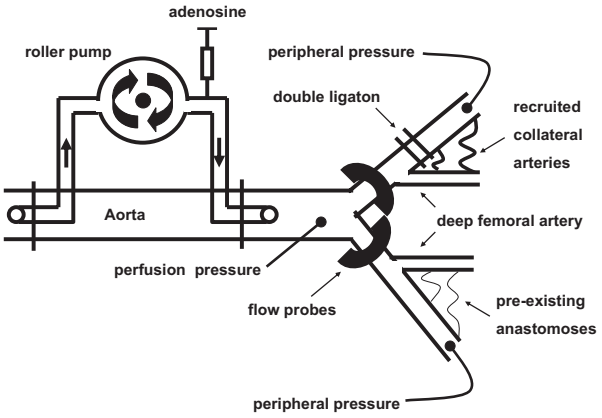

Fig 1. Experimental setup for assessment of collateral conductance. The establishment of an extra-corporal circulation allows measurements under maximum vasodilatation and at different flow/pressure levels.

Collateral conductance measurements. After completing the reactive hyperemia measurements, an extracorporal circulation was created to allow assessment of collateral conductance in a standardized manner. The abdominal aorta was cannulated with the tip of the catheter pointing upstream and another catheter was inserted with the tip pointing downstream. The tip of the distal catheter was placed 2 cm proximal to the branching of the caudal mesenteric artery. This shunt system was driven by a roller-pump, and a bubble trap/arterial filter was inserted into the shunt system to prevent arterial embolism. This setup allows the installment of different perfusion levels (Fig 1).

Both hindlimbs were then perfused at different pressure/flow levels under complete vasodilatation via continuous infusion of adenosine (1 mg/kg/min). At each pressure/flow level, blood pressure in the caudal mesenteric artery (prestenotic pressure) and the posterior tibial arteries (poststenotic pressure), as well as flow in the common iliac artery, was measured continuously.

For each level of perfusion pressure, the pressure gradient ($\Delta p = p_{pre\text{-}stenotic} - p_{poststenotic}$) was plotted against the corresponding blood flow in the common iliac artery (mL/min). Collateral conductance was then calculated from the slope of the resulting curve and expressed as mL/min/mm Hg. Collateral conductance is the reciprocal value of the vascular resistance of the collateral arterial network and the most accurate measurement to express the capacity of a collateral network.[10] In the pig hindlimb, conductance of the unrecruited collateral circulation has previously been shown to be relatively independent of interindividual variation in animals of the same size and weight.[11]

Statistical analysis. All results are expressed as mean ± standard deviation. Intergroup comparisons were performed using a one-way analysis of variance and the Bonferroni correction. Values of $P < .05$ were considered to be statistically significant.

Table I. Collateral conductance of the occluded hindlimb 7 days after femoral artery occlusion (raw data)*

Animal	*PBS*	*GM-CSF continuous*	*GM-CSF intermittent*
1	47.1	65.6	69.4
2	38.7	76.3	64.1
3	29.2	47.7	87.5
4	35.4	67.7	84.1
5	40.8	79.2	63.6
6	41.0	59.8	76.1
7	34.4	88.4	54.2
8	35.2	68.7	72.9
Mean ± SD	37.7 ± 5.4	69.2 ± 12.5	71.5 ± 11.1

PBS, Phosphate buffered saline; *GM-CSF,* granulocyte-macrophage colony-stimulating factor.
*Units: mL/min/mm Hg.

RESULTS

No animal died during or after the initial operation or had loss of hindlimb function, necrosis, or gangrene. Postoperatively, all animals appeared clinically healthy and showed normal behavior regarding food and water uptake. There was no significant difference in body weight between the treatment groups at baseline or after the treatment period. The implantation of the programmable electronic pumps did not impair the animals or lead to infection. One week after femoral artery occlusion and pump implantation, hemodynamic parameters were assessed (Table I and II.)

ABI and iliac artery flow at rest and during reactive hyperemia. At resting conditions, no statistically significant difference in the ABI between control and treated groups was found (n = 8 for each group). Although ABI decreased strongly in all groups during reactive hyperemia after temporary clamping of the aorta, this decrease did not differ significantly between the groups (Fig 2). Resting flow also did not differ between groups (data not shown). During reactive hyperemia, a significantly increased iliac artery flow and an increase in FPDR were observed in both groups of GM-CSF treated animals compared with control animals (Fig 3).

Collateral conductance measurements. Collateral conductance measurements under pump-controlled pressure and flow conditions during extracorporal circulation confirmed the stimulatory effect of GM-CSF (Fig 4). No significant difference was observed for any of the above-mentioned parameters between continuous and intermittent infusion of GM-CSF.

DISCUSSION

We report that the proarteriogenic properties of GM-CSF are preserved in a large animal species when applied intra-arterially. Furthermore, we suggest the applicability of clinically available infusion pumps as a platform for the intra-arterial delivery of growth factors. Intermittent intra-arterial application did not further increase efficacy. Finally, we show that combined flow/pressure measurements are

Table II. Hemodynamic results 7 days after femoral artery occlusion

	PBS	*GM-CSF continuous*	*GM-CSF intermittent*	P
ABI at rest	0.70 ± 0.05	0.70 ± 0.03	0.69 ± 0.07	NS
ABI during hyperemia	0.28 ± 0.03	0.30 ± 0.07	0.29 ± 0.08	NS
Flow right vs left (%)	40.5 ± 9.1	48.9 ± 3.9	48.7 ± 4.4	<0.05*
FPDR occluded limb†	23.3 ± 2.8	28.6 ± 3.0	29.6 ± 5.0	<0.05*
FPDR right vs left (%)	14.9 ± 3.4	19.1 ± 1.4	18.8 ± 1.4	<0.05*
Conductance†	37.7 ± 5.4	69.2 ± 12.5	71.5 ± 11.1	<0.05*

PBS, Phosphate buffered saline; *GM-CSF,* granulocyte-macrophage colony-stimulating factor; *ABI,* ankle/brachial index; *NS,* not significant; *FPDR,* flow/pressure-deficit ratio.
*For both GM-CSF groups vs PBS.
†Units: mL/min/mm Hg.

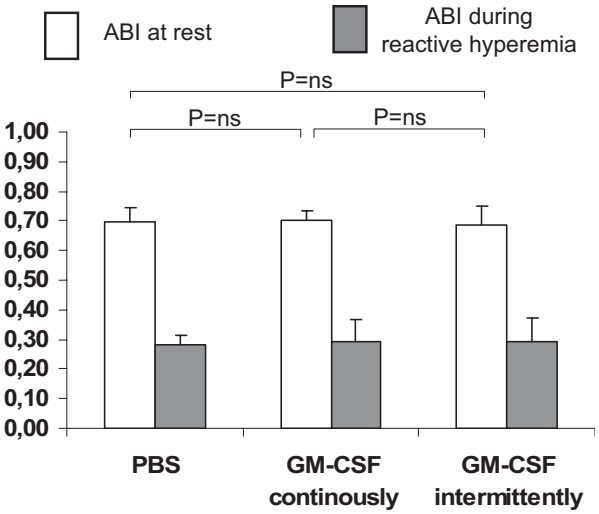

Fig 2. Ankle/brachial index *(ABI).* Assessment of ABI as a clinically available parameter of collateralization at rest as well as during reactive hyperemia failed to detect significant differences between the treatment groups. Data shown are mean ± standard deviation. *PBS,* Phosphate-buffered saline; *GM-CSF,* granulocyte-macrophage colony-stimulating factor.

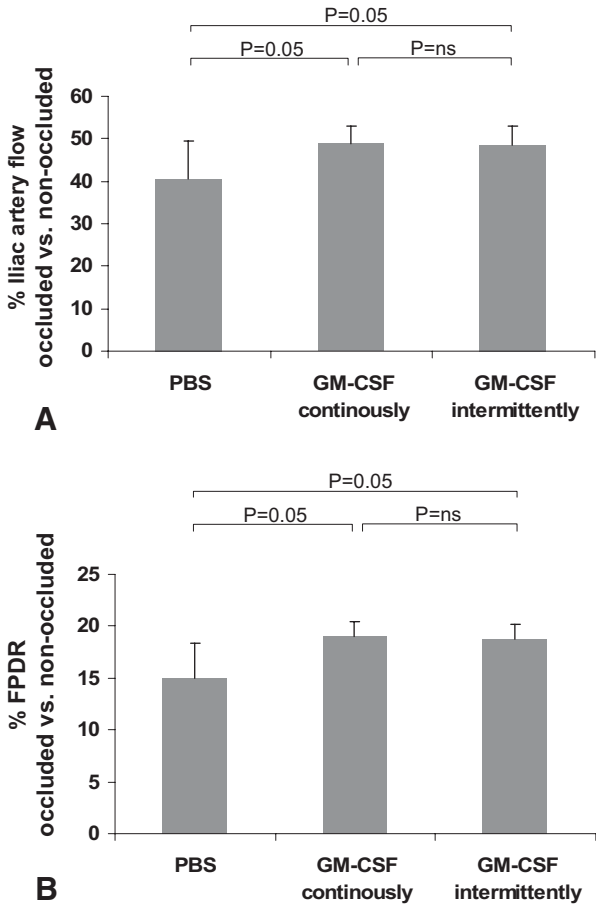

Fig 3. Hemodynamic assessment of iliac artery flow and flow/pressure-deficit ratio *(FPDR)* as a relative value of occluded/nonoccluded hindlimb during reactive hyperemia. Measurement of (**A**) iliac artery flow and (**B**) FPDR after temporary occlusion of the abdominal aorta showed a significant increase in iliac artery flow during reactive hyperemia in the granulocyte-macrophage colony-stimulating factor *(GM-CSF)* treated groups. Data shown are mean ± standard deviation. *PBS,* Phosphate-buffered saline.

more sensitive to detect stimulation of arteriogenesis than pressure measurements alone.

Numerous experimental techniques have been developed to assess the effects of arteriogenesis on tissue perfusion. Experimental models in small animals are useful to study molecular aspects of arteriogenesis, for example, in genetic knockouts, but may not be sufficient to predict the time course of the arteriogenic response of collateral arteries in larger species. Therefore, these small-animal models may not accurately reflect the process of arteriogenesis in human subjects. Because collateral vessels in mice require only a few cell cycles of smooth muscle and endothelial cell proliferation to reach their final effective diameter, collateral arterioles in larger-sized animals such as the pig closely approximate the growth dynamics to be expected in humans.

Because of the larger size of the pig compared with the previous animal models, the pig hindlimb model provides a broad spectrum of functional hemodynamic parameters

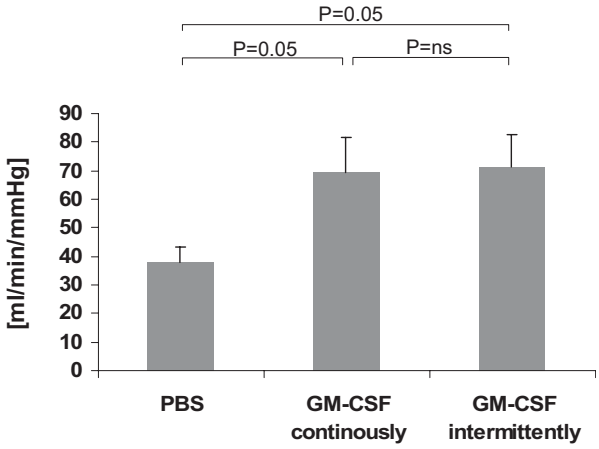

Fig 4. Collateral conductance measurements under complete and maximal vasodilatation achieved by continuous adenosine infusion and extra-corporal circulation. Collateral conductance (mL/min/mm Hg) as a parameter of flow-increase per increase in blood pressure gradient was significantly higher in the granulocyte-macrophage colony-stimulating factor *(GM-CSF)* treated groups, although there was no significant difference between continuous and intermittent drug application. Data shown are mean ± standard deviation. *PBS,* Phosphate-buffered saline.

and allows the assessment of vascular conductance under conditions of maximal vasodilatation. We chose a 1-week observation period because the natural time course of arteriogenesis in the otherwise healthy animals tends to mask stimulatory treatment effects at long time points.[12]

In this study we evaluated the effect of GM-CSF to enhance arteriogenesis. Previous studies have shown that the intra-arterial application of GM-CSF into the collateral circulation of the rabbit hindlimb leads to a significant increase in collateral conductance. Although the mechanisms by which GM-CSF enhances arteriogenesis remain partially unclear, increased survival of infiltrating monocytes via inhibition of apoptosis seems to play a role.[4] Infiltrating monocytes are important mediators of collateral artery growth,[13] accumulating around proliferating blood vessels and contributing to the arterial remodeling process via expression of matrix metalloproteinases, growth factors, and cytokines with known involvement in arteriogenesis such as tumor necrosis factor-α,[14] transforming growth factor-β, fibroblast growth factor-2, and GM-CSF itself. However, after transmigration into the perivascular tissue, the life span of macrophages is relatively short and depends on specific growth factors.[15]

Walsh and Sata[16,17] propose that apoptosis might be already initiated during transendothelial migration via Fas-ligand-receptor binding. This might be counteracted by GM-CSF application and therefore prolong monocyte/macrophage survival. Another potential mechanism of action might be the release of pluripotent cell populations from the bone marrow, which might act as stimulators of collateral artery growth.[18]

Granulocyte-colony stimulating factor (G-CSF) is another growth factor acting on the myeloid lineage that has recently been found to improve myocardial perfusion in combination with the intracoronary delivery of progenitor cells.[19] G-CSF has fewer side effects compared with GM-CSF and is therefore probably more easily implemented in clinical trials. However, no data are available yet on the specific effects of G-CSF on arteriogenesis.

The mode of administration is an important issue in the development of clinically feasible proarteriogenic therapies. In our previously mentioned rabbit hindlimb model, we applied GM-CSF intra-arterially, directly into the collateral circulation.[4] Seiler et al[5] showed in a small pilot study in 20 patients with coronary heart disease that GM-CSF treatment also induces an increase of collateral flow in humans.[5] In this study, GM-CSF was given as an intra-coronary bolus followed by long-term subcutaneous application.

In the recently published START trial, GM-CSF was applied solely subcutaneously in 40 patients with PAD.[6,20] This was done because intra-arterial application was thought to be too cumbersome in the setting of PAD. No significant difference was detected in this study between the control and the treatment group for our primary end point walking distance. It can be postulated that the mode of application negatively influenced the outcome of the START study.

The present study for the first time proves the proarteriogenic capacity of GM-CSF in a large-animal model of PAD, showing almost a doubling of collateral conductance in treated animals compared with control animals. We do realize that this by no means guarantees the success of the eventual intra-arterial application of GM-CSF in PAD patients (vs the subcutaneous application in the START-study). It is an encouraging result though, especially taking into account the positive outcome previously reported for patients with coronary artery disease, and it justifies further studies on intra-arterial application of proarteriogenic factors in PAD patients.

Because it was previously shown that GM-CSF exposure may downregulate the expression of its own receptor on circulating monocytes and progenitor cells,[7] we also compared an intermittent infusion with continuous infusion. Intermittent infusion did not further enhance the pro-arteriogenic efficacy of GM-CSF. Thus, the present study does not support the use of intermittent infusion of GM-CSF. It can be envisioned though that intermittent dosage regimens are beneficial when different growth factors (several growth factors are known to down regulate their own receptors) are used, or even more interestingly, when combination therapies are used.

CONCLUSIONS

The present study demonstrates the applicability of programmable and implantable perfusion pumps for such dosage regimens. It should be noted that these pump are designed for long-term subcutaneous implantation and can be reprogrammed and refilled if needed.

We used an acute ligation of the femoral artery in our model, whereas PAD-patients have a chronic, progressive

disease. Thus, their collateral circulation is chronically, albeit mostly insufficiently, recruited. A local and continuous administration as can be achieved with implantable pumps might be a prerequisite to induce arteriogenesis in these patients.

Recent clinical studies have raised safety concerns for the application of both GM-CSF and G-CSF for the stimulation of neovascularization. Patients receiving a high dose of growth factor appear to be at a higher risk of acute coronary events than the control group. Therefore, it has been advised that future trials of growth factor therapy for the stimulation vascular proliferation should be limited to patients with no other therapeutic option until the safety of these drugs is further investigated.[21]

Finally, the present study confirms the feasibility of combined flow/pressure measurements to assess the efficacy of collateral artery growth. Because of the strong dependency of flow on the diameter of the blood vessel, conventional imaging techniques such as angiographic scoring are not sensitive enough to detect the small differences in vascular anatomy that might result in significant functional improvement.[22] Although an improvement of patient symptoms such as pain and walking distance is the final aim of clinical studies to stimulate arteriogenesis, these parameters are subjective, and additional end points are needed. Under experimental conditions, an assessment of perfusion-dependent muscle function, as performed by Lee et al,[23] is more accurate. The invasive instrumentation at the site of collateral growth, however, makes such an assessment of muscle function difficult for arteriogenesis studies.

In our own study, iliac flow and the FPDR under reactive hyperemia was increased in GM-CSF treated animals compared with controls. This is important information for future clinical trials, because the measurement of true collateral conductance, as described in this study, will never be feasible in the clinical setting, whereas ABI, iliac flow, and the FPDR[20] can be obtained relatively easily in patients. ABI at rest and under reactive hyperemia did not differentiate between groups, whereas measurements of collateral conductance revealed a difference of almost 100% between treated and untreated animals. This stresses the need for combined pressure-flow measurements also in the clinical setting of peripheral artery disease.

AUTHOR CONTRIBUTIONS

Conception and design: SG, IEH, NVR
Analysis and interpretation: SG, IEH, NVR
Data collection: SG, SU, IEH
Writing the article: SG, NVR
Critical revision of the article: IEH, CB, JJP, IB, NVR
Final approval of the article: IEH, SU, CB, SO, JGT, JJP, IB, NVR
Statistical analysis: SG; IEH, JGT, NVR
Obtained funding: SO, IB
Overall responsibility: SG

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