Therapeutic arteriogenesis: from experimental observations towards clinical application [cum laude]
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INVASIVE AND NON-INVASIVE EVALUATION OF SPONTANEOUS ARTERIOGENESIS IN A NOVEL PORCINE MODEL FOR PERIPHERAL ARTERIAL OBSTRUCTIVE DISEASE

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Abstract

Background: Our current knowledge regarding the efficacy of factors stimulating collateral artery growth in the peripheral circulation primarily stems from models in small animals. However, experimental models in large sized animals are a prerequisite for extrapolation of growth factor therapy to patients with peripheral atherosclerotic obstructive disease. Therefore we have developed a novel porcine femoral artery ligation model using non-invasive and invasive evaluation techniques.

Methods and Results: In 12 young farm pigs and 9 older minipigs a ligation of the superficial femoral artery was performed. Using an intra-arterial catheter, phosphate buffered saline (PBS) was administered with a first-pass over the collateral vascular bed. Directly after ligation as well as after 2 weeks of continuous infusion of PBS, perfusion of the leg was measured using various flow and pressure parameters. Using a pump driven extracorporal system, collateral conductance was determined under maximal vasodilatation. Conductance decreased after acute ligation to similar levels in both young farm pigs as well as the older minipigs (both 9.3% of normal perfusion) and recovered after two weeks to a higher value in farm pigs compared to minipigs (22.4% versus 12.7% of normal; \( P < 0.05 \)). Angiography using both X-ray and magnetic resonance imaging was performed to visualize the formed collateral arteries.

Conclusions: To the best of our knowledge this is the first in vivo pig model for hemodynamic assessment of growth of collateral arteries in the peripheral circulation that is suitable for evaluation of arteriogenic effects of growth factors or genes.
Introduction
In the last decades, the treatment of patients with obstructive coronary and/or peripheral artery disease has made significant improvement using novel selective pharmacological and invasive techniques such as angioplasty and stenting as well as bypass surgery. Nevertheless angioplasty and bypass surgery, the primary interventional invasive therapies for the treatment of atherosclerosis, are itself limited by the development over time of native vessel restenoses and graft occlusions. Moreover these therapies are not options for a significant number of patients with diffuse atherosclerotic disease.

The human circulation has a preexisting collateral vascular system, which in case of slowly progressive atherosclerotic narrowing may circumvent the stenosis and ensure blood flow to endangered ischemic territories. The growth of these small arterioles, that are only partially recruited under resting conditions, can be therapeutically enhanced. This process of active proliferation is termed arteriogenesis and results, in contrast to angiogenesis (the sprouting of capillaries) in true functional arteries. Several cytokines such as basic fibroblast-growth-factor, monocyte chemoattractant protein-1, granulocyte and macrophage colony stimulating factor and transforming growth factor beta are known for their stimulatory effect on arteriogenesis. The vast majority of experimental angi- or arteriogenesis data is based on studies in small animal species such as rabbits or mice. These data only partially reflect the situation in humans, since the total amount of new tissue necessary for the morphogenesis of developing collateral arteries is an order of magnitude smaller in mice versus man. This suggests that the time interval needed to transform a recently recruited collateral into a functional artery will take much longer in larger species. Thus, larger animal models are needed to study the time course of arteriogenesis anticipated in humans.

Furthermore, animal models for pre-clinical testing of growth factors should provide a similar collateral vascular growth as compared to the human situation. The dog heart for instance provides a very well developed collateral circulation, whereas the pig and rabbit heart are only poorly equipped with an efficient collateral circulatory system, similar to the human condition.

Since current animal models for peripheral atherosclerotic obstructive disease are limited to the mouse, rat or rabbit hindlimb, and peripheral vascular data from larger species are not available, we now tested the hypothesis, whether the pig hindlimb might provide a model for peripheral collateral artery growth that is more suitable for extrapolation to the human situation with peripheral vascular disease.

Materials and Methods
Preparation of animals
All animal procedures described in this study were approved by the Bioethical Committee of the District of Baden-Württemberg, Regierungspräsidium Stuttgart and Freiburg. The animals were handled in accordance with the American Physiological Society guidelines for animal welfare. Animals were housed in standard cages and fed water and chow ad libitum.
Arterial ligation

For this study, 12 farm pigs (Winter, Assmannshardt, Germany) weighing 25-35 kg with a mean age of 3 ± 1 months, and a second group of 9 Goettinger Minipigs (Ellegaard, Dalmose, Denmark) weighing 20-25 kg, with a mean age of 19 ± 3 months were used. After sedation with 5 ml azaperon (40 mg/ml), 3 ml Dormicum and 2 ml ketamine hydrochloride (100 mg/ml), the pigs were intubated and ventilated with a respirator (Engström 300, Engström Medical AB, Solna, Sweden). General anesthesia was maintained using isoflurane (1 vol% in O₂). Using sterile surgical technique, the femoral artery was exposed and ligated just distal from the bifurcation with the deep femoral artery. A double ligation was performed, considering a 4-cm distance between the two ligation sites. Furthermore, the lateral femoral circumflex artery was ligated.

Pump-driven infusion

Four groups of animals were chosen in total: acute ligation in farm pigs (n = 7) and minipigs (n = 4); and 2 weeks of ligation in farm pigs (n = 5) and minipigs (n = 5). In animals that underwent a chronic 2 weeks treatment with phosphate buffered saline (PBS), the femoral artery was canulated with a 1.6 mm silicon catheter. The tip of the catheter was placed in the occluded femoral artery, 5-10 mm distal to the bifurcation of the superficial and deep femoral artery. Hereafter, the catheter was subcutaneously tunneled to the animal’s back and connected to a portable elastomeric infusion system (Multiday Infusor 2.0 ml/hour; Baxter Healthcare Corp., Deerfield). This infusion pump was placed at the back of the pig and secured with an elastic cotton jacket. After suture of the overlying musculature, the skin was closed. Hereafter, the skin was cleaned with cod-liver oil and Nobecutan spray®. A depot of 3 ml penicillin / streptomycin was injected intramuscular as an antibiotic prophylaxis. All 21 pigs were operated following this protocol.

Hemodynamic measurements

The pigs were given premedication following the same protocol as described above and anesthesia was subsequently maintained using pentobarbital sodium 10 mg/kg/hour intravenously. The animals were monitored during the experiment using electrocardiography and non-invasive measurement of heart rate and arterial oxygenation using a transcutaneous tail probe. Mean systemic arterial pressure, distal pressure and perfusion pressure were measured using an amplifier filter (IFD, Mülheim/Ruhr, Germany). For the continuous measurement of systemic arterial pressure, a solid-state pressure gauge manometer was placed in the left carotid artery. The mesenteric artery was canulated - close to the level of the aorta - with a polyethylene heparinized catheter (1.0 mm) for the measurement of the perfusion pressure after installation of the extracorporal system. The saphenous arteries were exposed at the level of the ankle and cannulated with a polyethylene catheter (0.58 mm). These tubings were connected to a pressure transducer for the measurement of distal arterial pressure. For the measurement of volume flow to the region of interest, flow probes (Transonic Systems Inc., Ithaca, NY) were placed from an abdominal
approach just proximal from the bifurcation of the superficial and deep femoral artery. Continuous hemodynamic recordings were made using the data acquirement software Notocord-Hem 3.3.1.97 (Notocord systems SA, Croissy, France). To achieve maximum vasodilatation, papaverine (Sigma clinical co., St Louis, MO) 10 mg/ml was infused with a pump at 120 ml/hour (see figure 1).

Experimental protocol
At the different time-points the animals were anesthetized again according to the above-described protocol and monitored with electrocardiography and systemic pressure measurement. After cannulation of the saphenous arteries, the abdomen was opened. Both external iliac arteries were exposed just proximal to the ligation site and flow probes were applied on both sides. The mesenteric artery was cannulated with a heparin filled polyethylene catheter for measurement of perfusion pressure. Consequently, the abdominal aorta was exposed and the lumbosacral spinal arteries were ligated to prevent retrograde bleeding. After systemic infusion of 10.000 IE of heparin, proximal (distal from the renal artery) and distal (just proximal from the mesenteric artery) atraumatic clamps were inserted. Hereafter, two cannulas were interposed in the abdominal aorta for the appliance of a pump driven extracorporal circulation as depicted in Figure 1. The cannulas inserted in the abdominal aorta were connected to a tubing pump (MCP V5.10, Ismatec, Glattbrugg-Zürich, Switzerland) to control perfusion pressures. After stabilization of the animal, the pump speed was adjusted to systemic pressure. Subsequently, perfusion pressure was enhanced in four-six steps to under maximal vasodilatation using a continuous infusion of papaverine. Simultaneously, both femoral artery flow and peripheral pressures of the ligated and unligated leg were assessed.

Post-mortem angiography
After performance of the hemodynamic measurements, the animals were sacrificed using 10 ml potassium chloride intravenously. Subsequently, a buffer of sodium chloride with papaverine was infused into the peripheral circulation to achieve maximal vasodilatation. Hereafter, a contrast medium consisting of a solution of 60mg barium sulfate (Riedel-de Haën laboratory chemicals, Seelze, Germany) and 10 mg gelatin in 100 ml aquadest (18.2MO) was infused at a pressure of approximately 50 mmHg to preclude pressure derived vascular damage. The angiograms were obtained immediately after the experiment in 11 animals using a radiography apparatus (DX-15HF; Acoma) at a distance of 110 cm at 74 kV and 3.2 mAS. A Kodak XDA plus film (30 x 40 cm; Eastman Kodak Company, Rochester, NY) was used.

In vivo angiography
Animals were anaesthetized as described above and monitored with electrocardiography and systemic pressure measurement. Angiographies were obtained using MRA technique or clinical angiography.
**In vivo magnetic resonance angiography**

The purpose of these scans was to visualize the femoral artery architecture and to obtain a cross-sectional view perpendicular to the direction of flow. This pilot experiment was performed in two minipigs after two weeks of PBS infusion. Animals were anaesthetized as described above and monitored with electrocardiography and systemic pressure measurement. Magnetic resonance imaging (MRI) was performed on a 1.5-T whole-body imaging system (Sonata, Siemens). A standard quadrature head coil (20 x 26 cm) was used for signal detection. After placement of ECG monitoring leads the subject was imaged in supine position with its hindlimbs placed inside the head coil. Scout views were acquired in axial, coronal, and sagittal orientation to localize the arteries of interest. For selective depiction of the arteries we applied a contrast-enhanced magnetic resonance angiography (MRA) technique. MRA was performed during the first-pass of a gadolinium-based contrast agent (Magnevist, Schering, Berlin) in combination with a high resolution, T1-weighted gradient echo sequence. The total acquisition time for the 3D scan was 25 – 30 s using a repetition time of TR = 3.1 ms, an echo time of TE = 1.2 ms, and a flip angle of 20°. The in-plane resolution was 1.2 mm x 1.1 mm at a slice thickness of 1.6 mm. The final images were reconstructed from the 3D data set using a double oblique, targeted maximum intensity projection.

**Percutaneous angiography**

In a total of 3 pigs, a 7F or an 8F sheath was inserted directly into the right carotid artery. Electrocardiography was monitored and recorded. A 7F diagnostic catheter was positioned in the left proximal part of the femoral artery, and a single 20-50 ml bolus of nonionic contrast agent (Imeron, Byk Gulden) was selectively injected in either the proximal stump of the superficial artery or the proximal profound femoral artery.

**Calculation of conductance indices**

In the present model, pre-existing arterioles develop after occlusion of the (superficial) femoral artery from the deep femoral artery (stem region) into mature collateral vessels (mid region) connecting distally to the sural communicating artery (arteria suralis) and the medial saphenous artery (re-entrant region). Perfusion pressure \( P_{\text{perfusion}} \), measured in the distal aorta, and peripheral pressure \( P_{\text{distal}} \), measured in the saphenous artery are considered equal to the pressures in the stem- and re-entrant region, respectively. The change of flow to the region of interest, measured at the level of the external iliac artery \( Q_{\text{distal vascular bed}} \), is considered to be the result of a change in flow over the developed collateral vessels. Resistance of the distal and collateral artery network was defined as the following equation:

\[
R_{\text{distal vascular bed}} = \frac{(P_{\text{perfusion}} - P_{\text{distal}})Q_{\text{distal vascular bed}}}{Q_{\text{distal vascular bed}}}
\]

Conductance is defined as the reciprocal value of the vascular resistance. All conductance indices were calculated from the equation of the pressure-flow relation.
as the flow level of the distal vascular bed at a pressure difference ($P_{\text{perfusion}} - P_{\text{distal}}$) of 100 mmHg.

**Histology**

After intraoperative identification of collateral vessels – based on identification of the stem, midzone and reentry region and a corkscrew appearance - samples of arteriolar conduits were taken and frozen at -60°C in 4 pigs. Sections were immunohistochemically evaluated for morphological appearance (HE staining). Furthermore, in 4 Balb-C Mice the femoral artery was ligated. Directly after ligation, in 2 mice a postmortem angiography with a bismuth-gelatine mixture was performed and the midzones of small-preexisting visible collaterals was excised and transferred to further histological analysis (see above). In the 2 other mice, collaterals were excised after 14 days and identically prepared as ascribed above.

**Statistical analysis**

Continuous variables are expressed as mean ± standard deviation. A 2-tailed unpaired $t$ test was used to assess differences in continuous variables. Data analysis was performed using the SPSS 11.0 software package for Windows (SPSS Inc., 1999, Arlington, Virginia). For all tests, a p-value <0.05 was considered statistically significant.

**Results**

**General observation**

Overall, no macroscopic necrosis was observed. No signs of clinical infection could be observed (increased body temperature, wound swelling or redness). All animals survived the initial surgery and the follow-up period.

**Mean arterial blood pressures**

Systemic arterial pressures remained unchanged in both farm pigs and minipigs throughout the study (Table 1). However, mean arterial blood pressure was 10-20 mmHg lower in Gottinger minipigs than in the farm pigs. Blood pressures in the unligated right hindlimb were similar to systemic pressures. Peripheral blood pressure as measured in the saphenous arteries (Fig. 1) showed a reduction after occlusion that was higher in farm pigs (pressure fall: -73.4%) as compared to minipigs (pressure fall: -52.1% of normal blood flow; $P < 0.05$). Two weeks after ligation of the femoral artery blood pressure restoration reached a value of 64.9% of initial blood pressure in farm pigs as compared to 69.0% in minipigs (Fig. 2A; $P = \text{NS}$).

**Volume blood flow**

The reduction in blood flow in the iliac artery following occlusion of the femoral artery was higher in minipigs (-53.4%) versus farm pigs (-34.1%, $P < 0.05$). Two
weeks after femoral artery ligation, iliac blood flow returned to a value of 83.6% of the normal value in farm pigs and 72.5% in minipigs; (Fig. 2B).

The difference in the arteriogenic response was also reflected via the ankle-brachial index, which in the experimental setting was calculated from the ratio between the peripheral postocclusive pressure and the systemic (pump driven) pressure. In farm pigs this ratio was reduced by 75% (1.0 to 0.25) as compared to minipigs with a ratio-fall of 52%. The ratio of recovery of this index in farm pigs was significantly higher (46%) as compared to minipigs with 19% ($P < 0.05$) at the 2 weeks time period.

**Conductance**

The calculation of maximal conductance values revealed, that despite significant differences in change of blood flow and blood pressure, the overall initial reduction in conductance acutely after femoral occlusion was the same in farm- and minipigs. However there was a significant difference in the degree of restoration of perfusion (ml/min/100mmHg) at two weeks: farm pigs reached a conductance level of 22.4% as compared to minipigs, reaching a conductance level of 12.7% (Fig. 4). A moderate correlation was found between the collateral conductance and the ratio of the peripheral / systemic pressure in the ligated leg in both the farm pigs and the minipigs ($r^2 = 0.37; P = 0.006$; Fig. 5).

**Visualization of collateral arteries via angiography**

Directly after occlusion of the femoral artery interconnecting collateral vessels could rarely be observed in the farm pigs between the arteria profunda femoris and the distal part of the superficial femoral artery (see fig. 6A and 6B). Only collateral connections consisting of a stem, midzone and a re-entry (using Longland’s definition of collateral arteries) were considered to be “true” collaterals. Two weeks after occlusion typical corkscrew collaterals could be observed in the aforementioned region (fig. 6B-D). Selective injection into the proximal arteria profunda femoris with contrast agent however visualized several interconnecting collateral vessels between the profound and superficial femoral artery (see fig. 6C and 6D). After selective injection of contrast agent into the proximal stump of the femoral artery a second group of collateral vessels could be made visible, directly bypassing the site of occlusion and thereby bridging from the proximal superficial arteria femoralis to the distal part. Using magnetic resonance angiography collateral arteries could be visualized in minipigs, two weeks after femoral artery ligation (fig. 7).

**Histology mice and pigs**

Histological examination of non-recruited collaterals, after intraoperative excision revealed, that the initial size of the arteriolar conduits is between 10-30 μm in Balb-C mice as well as minipigs. However the increase in diameter and the number of smooth muscle cell layers were significantly different. The inner diameter of mature mouse collaterals showed values of $160 \pm 18 \text{ μm}$ (fig. 8A) versus $620 \pm 150 \text{ μm}$ in
Figure 1. Schematic drawing of the extracorporal circulation. Sites of volume flow and intravascular pressure measurements are depicted.

Table 1: Hemodynamic data in minipigs and farm pigs. * P < 0.05 compared to value before ligation. Values are mean ± SD.
Figure 2: Changes in peripheral blood pressures (A) and blood flows (B) in farm pigs and minipigs.

Figure 3: Examples of mean flow / Δ pressure (P_{perfusion} - P_{peripheral}) relationship per group.

Figure 4: Absolute values of conductance directly after ligation and after 2 weeks of administration of PBS. Relative conductance (left to right in percentages) are noted above the bars.
Figure 5: Correlation between conductance and peripheral/systemic pressure gradient.

Figure 6: Postmortem angiographies without ligation (A). Postmortem angiographies with femoral ligation (B). In vivo angiography. Selective injection into the profound arteria femoralis (C). Selective injection into the superficial arteria femoralis (D).
Figure 7: *in vivo* angiography – NMR technique.

- early phase after intravenous contrast application
  - double ligation
  - collateral artery

Figure 8: Histology of collateral arteries in mice (A) and minipigs (B).

- non-recruited collateral artery (mouse hindlimb) after angiographic dissection of the midzone
- mature collateral artery after 14 days (mouse hindlimb) after angiographical dissection of the midzone

- non-recruited collateral artery (pig hindlimb) after angiographic dissection of the midzone
- mature collateral artery after 14 days (pig hindlimb) after angiographical dissection of the midzone
minipigs (fig. 8B).

Discussion
The present study describes for the first time, a femoral arterial ligation model in the pig, that is suitable for evaluation of arteriogenesis using both non-invasive and invasive evaluation techniques.

Mechanism of arteriogenesis
According to Poiseuille's law, large conductance arteries are a prerequisite for tissue perfusion making blood flow restoration to ischemic tissues dependent on the development of large conductance vessels rather than new capillary networks. Since vascular resistance is inversely proportional to the fourth power of the radius, small changes in collateral diameter can be responsible for large changes in collateral conductance. When a hemodynamically relevant stenosis develops in a main artery, it causes a fall in intravascular pressure in the dependent vasculature. Consequently, blood flow is re-distributed from a neighboring artery through pre-existing arterioles interconnecting the two vascular territories. Shear stress in these arterioles increases significantly, which in turn leads to the activation and morphological change of the endothelium, upregulation of cell adhesion molecules, invasion of circulating monocytes and their precursors and, finally, into formation of functional arteries. This process, termed arteriogenesis, is an efficient rescue system to bypass the site of stenosis or occlusion and thus restore blood flow to ischemic tissues.

Importance of animal size and species
Numerous experimental techniques have been developed to assess the effects of arteriogenesis on tissue perfusion. Experimental models in small animals such as in mice 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 collaterals in larger species. Therefore, these small animal models may not represent accurately the process of arteriogenesis in human subjects. Since 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. Due to the larger size of the pig compared to the previous animal models, proliferating collaterals can be visualized more easily using clinical diagnostic techniques such as magnetic resonance imaging and percutaneous transluminal angiography. Furthermore the pig hindlimb model provides a broad spectrum of functional hemodynamic parameters and allows the assessment of vascular conductance under conditions of maximal vasodilation. As shown in previous studies in the heart, the pig has a very limited innate coronary collateral circulation, with only sparse endocardial connections, whereas the dog is endowed with numerous, generally epicardial, innate anastomoses, that are thought to have greater potential for development than those of the pig. This apparent
difference has been responsible for generally greater acceptance of the pig as an animal to study angiogenic/arteriogenic interventions in the heart, and for criticism of the dog model. For the hindlimb collateral circulation, these potential differences between species has not been elucidated up till now. A coincidental finding of the present study was that farm pigs showed a significantly faster recovery of hemodynamics as compared to minipigs. This may be explained by the differences in animal strain and / or age, which have shown to be influential on the arteriogenic response after femoral artery ligation \textsuperscript{27-29}. However, we could not detect proliferation activity of the growing collateral vessels with Ki-67 (data not shown). This could be due to the fact, that our histological observation was performed two weeks after femoral ligation, while the highest proliferative status may already be reached already 2-5 days after femoral ligation.

For the peripheral circulation only limited data exist, that adequately reflect the situation in human vascular disease. The porcine hindlimb provides a similar anatomy as compared to humans, as shown by our study. In case of stenosis or occlusion of the arteria femoralis, preexisting collateral vessels are being recruited from the deep femoral artery (arteria femoralis profunda), connecting to the distal zone of the superficial femoral artery (arteria femoralis superficialis).

\textit{Hemodynamics of arteriogenesis}

The described technique of a pump driven shunt in this study allows a pressure and flow controlled perfusion of the vascular bed under conditions of maximal vasodilation (continuous administration of papaverine). Moreover, our conductance measurements revealed the typical positive pressure intercept. Using this pressure intercept at different pressure gradients, maximal collateral flow and thus collateral conductance (i.e. reciprocal value of resistance) of the collateral vessels can be assessed \textsuperscript{30}. The resistance of the collateral vessels will decrease over time, resulting in a lower pressure gradient. When the collateral resistance becomes very small, indicating that collaterals become now nearly as efficient as the native arterial system before the ligation, the peripheral pressure will equal aortic pressure and the ratio \( P_{\text{aorta}} / P_{\text{periphery}} \) becomes close to the value of 1, as also shown in the present study. After femoral occlusion this ratio decreased to values of 0.2-0.4. However, these ratios only moderately corresponded with collateral vascular perfusion as reflected by the collateral conductance. Furthermore, the hemodynamic data of this study show, that an increase or decrease in resting blood flow must not necessarily correlate with the conductance per se. Our flow parameters revealed restoration of more than 30% of resting blood flow after two weeks in both pig strains, whereas the conductance value only showed a very low improvement in minipigs as compared to a significant increase in farm pigs. Hence, a reliable evaluation of changes in the arteriogenic response depends on a complete vasodilation of the collateral circulation and, secondly, should include blood flow values plus the corresponding blood pressure ratios at different pressure levels expressed as conductance.

In conclusion, our study provides a basis for future efforts to evaluate the
arteriogenic effects of different growth factors or genes in a large animal model that is better suitable for extrapolation to the human situation of patients with peripheral arterial obstructive disease than the existing small animal models.

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