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Modulation of vascular remodeling : a role for the immune system, growth factors, and transcriptional regulation

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Variations in surgical procedures for hind limb ischemia mouse models result in differences in collateral formation

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Abstract

Objective: To identify the optimal mouse model for hind limb ischemia which offers a therapeutic window that is large enough to detect improvements of blood flow recovery e.g. using cell therapies.

Materials and Methods: Different surgical approaches were performed: single coagulation of femoral and iliac artery, total excision of femoral artery and double coagulation of femoral and iliac artery. Blood flow restoration was analyzed with Laser Doppler Perfusion Imaging (LDPI). Immunohistochemical stainings, angiography and micro-CT-scans were performed for visualization of collaterals in the mouse.

Results: Significant differences in flow restoration were observed depending on the surgical procedure. After single coagulation, blood flow already restored 100% in 7 days, in contrast to a significant delayed flow restoration after double coagulation (54% after 28 days, $P < 0.001$). After total excision, blood flow was 100% recovered within 28 days. Compared to total excision, double coagulation displayed more pronounced corkscrew phenotype of the vessels typical for collateral arteries on angiographs.

Conclusion: The extent of the arterial injury is associated with different patterns of perfusion restoration. The double coagulation mouse model is in our hands the best model for studying new therapeutic approaches since it offers a therapeutic window in which improvements can be monitored efficiently.

Introduction

Symptoms of ischemia in patients with peripheral arterial disease (PAD) are dependent on several factors. For instance the extent and level of stenosis or occlusion are important. Also factors affecting the development of collaterals like hemodynamic factors as good antegrade flow and peripheral runoff vessels play a role [1]. These factors among others make it challenging to develop a good animal model for studying collateral formation in PAD.

Animal models of hind limb ischemia have been developed in mice [2-10], rats [11, 12] and rabbits [2]. Ischemia-induced collateral artery formation has been mostly studied in mouse models. Surgical procedures range from a single ligation of the femoral- or iliac artery [8, 10] to a complete excision of the artery [5, 13] and sometimes even the vein and nerve are dissected too [14, 15]. Besides, the level of vascular occlusion, which is a determinant of the amount of ischemia, ranges from a proximal ligation of the iliac artery [12] to a distal ligation just proximal to the bifurcation of the saphenous artery and the popliteal artery of the lower limb of mice [6]. These variations hamper the comparison of the outcomes of hind limb ischemia induction. Another problem is that mice rapidly form collaterals, which limits the therapeutic window for potential arteriogenic agents. The aim of this study was to develop a hind limb ischemia mouse model with a therapeutic window large enough for testing new therapeutic approaches like cell therapy. The effect of different surgical techniques and levels of vascular occlusion was compared for repair of blood flow, collateral artery formation and capillary formation in the ischemic hind limb.

First, a single electro-coagulation of the femoral artery in C57Bl6 mice, which is the most traditional model of hind limb ischemia, is discussed. Secondly, a more proximal electrocoagulation was studied. Thirdly, a total excision of the femoral artery with all their side branches as an often used model of hind limb ischemia was studied [5, 13]. Finally, an alternative model of double electro-coagulation of both femoral artery and iliac artery, more closely resembling multilevel PAD, was developed.

Materials and methods

Experimental animals

For testing different surgical approaches to induce hind limb ischemia, male C57Bl6 mice (Jackson) were used, aged 10-12 weeks. In addition, we performed a double coagulation in immune-deficient NOD-scid IL2Rgamma(null) mice. Experiments were approved by the committee on animal welfare of our institute.

General aspects of the surgical procedures

Before surgery, mice were anesthetized with an intraperitoneal injection of a combination of Midazolam (5mg/kg, Roche), Medetomidine (0.5mg/kg, Orion) and Fentanyl (0.05 mg/kg, Janssen). In all models, the femoral vein and nerve were preserved. After surgery the skin was closed with 6-0 Ethilon sutures.

Technical details of different surgical procedures for inducing hind limb ischemia (Different surgical procedures are also illustrated in figure 1)

- **Single electro-coagulation of femoral artery:**
A small skin incision was made in the left inguinal region. Directly after incision, the subcutaneous fat pad in the thigh was visible. It was not necessary to cleave the fat pad, just pull it distally. After dissection of the artery from the nerve and vein, ischemia was induced by electro-coagulation of the left femoral artery, proximal to the superficial epigastric artery. Electro-coagulation resulted in complete transaction of the artery. After electro-coagulation, the proximal end of the artery is moving proximally into the surrounding tissue and the distal end is moving distally, so there is a distance of a few millimeters between both ends after the surgical procedure.
- **Single electro-coagulation of iliac artery:**
A bigger skin incision in the inguinal region is made now. Again there is no need to cleave the fat pad. For exposure of the iliac artery, we used a retroperitoneal approach. By carefully moving the peritoneum proximally with a cotton swab, a good exposure of the iliac artery was possible. Again preparation of the artery from the vein was necessary. The internal iliac artery serves as a landmark; direct proximally of the internal iliac artery was an electrocoagulation of the common iliac artery performed.
- **Total excision of femoral artery:**
After incision of the skin from the inguinal region till the knee, we cleaved the subcutaneous fat pad for a better exposure. First preparation of the common femoral artery took place (proximal excision site). Two 8-0 ties were placed around the artery, in direction of the inguinal ligament as much as possible. Then dissection of the whole artery from the vein and nerve in distal direction was performed. All side branches of the artery were carefully dissected free and coagulated. Before excision, preparation of the distal level was performed and again two 8-0 ties were placed around the artery. The distal ligation level is at the popliteal artery level, just distal from the bifurcation of the saphenous artery and the popliteal artery. After cutting the artery between the two ligatures proximal and distal, the whole artery was removed from the surrounding tissue.

- Double electro-coagulation of both femoral artery and iliac artery:

For a double coagulation model, both common iliac artery and femoral artery were electrocoagulated. First an electro-coagulation of the common iliac artery was performed and directly afterwards an electro-coagulation of the femoral artery. These coagulations are at the same anatomical levels used in the single electro-coagulation procedures of the femoral artery and the iliac artery. Same techniques were used as described above.

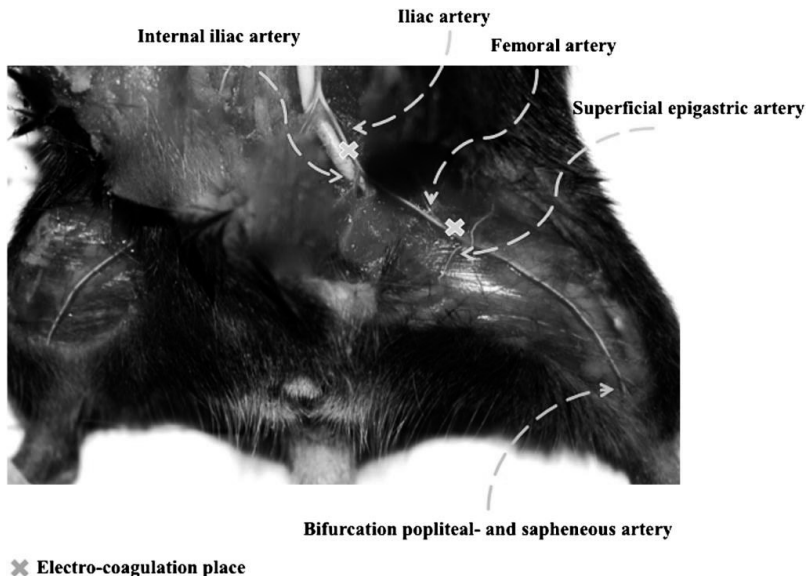


Figure 1. Illustration of the anatomical levels of electro-coagulation places in different models of hind limb ischemia. Crosses represent electro-coagulation places. *See color figure on page 214.*

LDPI

Measurements of perfusion were performed of the mouse hind limb before, directly after and weekly over 4 weeks after the surgical procedure with Laser Doppler Perfusion Imaging (LDPI) (Moor Instruments). To control for temperature variability during measurements, all animals were kept in a double-glassed jar filled with 37°C water, keeping environment temperature at a constant level during the LDPI-measurements. Since LDPI-outcomes are sensitive for temperature changes, it is very important to control environment temperature during LDPI-measurements. Each animal served as its own control. Eventually, perfusion was expressed as a ratio of the left (ischemic) to right (non-ischemic) paw. Before LDPI, mice were anesthetized with an intraperitoneal injection of Midazolam (5mg/kg, Roche) and Medetomidine (0.5mg/kg, Orion).

Imaging

Post mortem angiography of both hind limbs was performed using polyacrylamide-bismuth contrast (0.1gr/ml) [9]. After thoracotomy, contrast fluid was injected into the left ventricle of the mouse heart. Five minutes before contrast injection, mice were intravenously injected with papaverine (50mg/ml) for vasodilatation. The skin of both hind limbs was removed and X-rays were made. For CT-scans, the same contrast and injection procedures were used. A SkyScan 1076 micro-CT-scan with a resolution of 18 micron was used. Angiographs and CT-scans were solely used to illustrate collateral formation in the post-ischemic hind limb. Quantification of collaterals was performed using immunohistochemistry.

Immunohistochemistry

Five μm -thick paraffin-embedded sections of skeletal muscle fixed with 3.7% formaldehyde were used. These were re-hydrated and endogenous peroxidase activity was blocked for 20 minutes in methanol containing 0.3% hydrogen peroxide. For CD31 staining, sections were pre-incubated with trypsin for 30 minutes at 37°C and incubated overnight with primary antibody (rat anti-mouse CD31Ab, BD Biosciences, dilution 1:200). Anti-rat immunoglobulin antibody was used as secondary antibody (goat anti-rat, AbCam, dilution 1:300). For an anti- α smooth muscle actin staining (mouse anti-human, DAKO, dilution 1:800), no antigen retrieval was necessary. Rat anti-mouse HRP (rabbit anti-mouse, DAKO, dilution 1:300) was used as secondary antibody. Negative controls were performed by using isotype controls. Stainings were quantified from randomly photographed sections using image analysis (Qwin, Leica).

Statistical analysis

Results are expressed as mean \pm sem. Comparisons between means were performed using an independent T-test or One-Way Anova. P-values <0.05 were considered statistically significant. All calculations were performed in SPSS 16.0.

Results

Impact of two different anatomical levels of electro-coagulation on blood flow restoration.

After single electro-coagulation of the femoral artery and single electro-coagulation of iliac artery, the LDPI-ratio's were significantly decreased immediately after coagulation. For both procedures, blood flow dropped to $<10\%$. No significant differences in blood flow restoration were observed despite differences in the anatomical level of coagulation used to initiate ischemia measured with LDPI (Fig 2).

Different patterns of blood flow restoration after total excision of femoral artery.

Like a single electro-coagulation of the femoral artery, a total excision of the femoral artery resulted in a decline of blood flow perfusion. But perfusion in the mouse hind limb restored considerably slower after a total excision (Fig 2). After total excision of the femoral artery, C57Bl6 mice just had 100% recovery after 28 days, whereas C57Bl6 mice already had 100% blood flow recovery within 14 days after a single electro-coagulation of the femoral artery. Thus, total excision of the femoral artery in C57Bl6 mice showed a more attenuated blood flow recovery compared to a single electro-coagulation.

Magnitude of impaired blood flow recovery and paw necrosis after a double electrocoagulation approach.

After double electro-coagulation of both femoral and iliac artery, blood flow restoration was significantly impaired to 54% after 28 days compared to 100% blood flow restoration in 7 days after single electro-coagulation of femoral artery or iliac artery ($P < 0.001$) (Fig 2). Although this is an extensive ischemic model and there was slow blood flow recovery after the surgical procedure, only 3 out of 10 mice had necrosis of one or more toe nails. There was no necrosis of the foot or limb. After single electro-coagulation of the femoral artery or iliac artery, we hardly see any necrosis of toe nails.

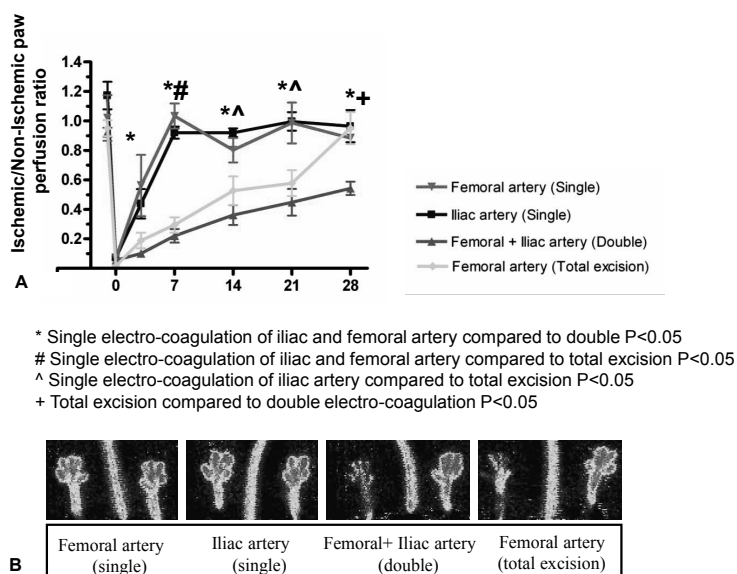


Figure 2. Blood flow restoration in hind limb of C57Bl6 mice. **A.** Blood flow recovery after a single femoral artery (distal anatomical level) electro-coagulation ($n=3$, green line) or a single iliac artery (proximal anatomical level) electro-coagulation ($n=9$, black line) or double electro-coagulation of both femoral artery and iliac artery ($n=9$, blue line) or total excision of the femoral artery ($n=6$, orange line) as monitored by Laser Doppler Perfusion Imaging (LPDI) and expressed as ratio between coagulated and non-coagulated limb. Data are presented as mean \pm sem. *#^+ $P < 0.05$ (ANOVA-test) **B.** LDPI images of the paws at day 7 after different surgical procedures. See color figure on page 214.

Imaging of collateral artery formation and capillaries in different surgical approaches of hind limb ischemia.

At 28 days after single electro-coagulation of the femoral artery or double electrocoagulation of both left femoral artery and iliac artery in C57Bl6 mice, angiographs showed normal arterial anatomy at the right side (non-operated side) and an increased number of collateral arteries in the left hind limb (operated side). Typical corkscrew-like collaterals can be observed in the (post-) ischemic hind limb (Fig 3A,B). Angiographs made 28 days after a total excision of the femoral artery in C57Bl6 mice showed also more neovascularization in the (post-) ischemic hind limb compared to the non-operated hind limb. However, vessels formed after total excision of the femoral artery seem to have a different aspect on angiographs, i.e. a very disturbed pattern of vasculature, with little or no typical corkscrew collaterals as we observed after single or double coagulation (Fig 3C).

The increase in collaterals in the (post-) ischemic hind limb was also confirmed by CT-scans of these mice made 28 days after coagulation of both femoral artery and iliac artery (Fig 3D,E,F). These CT-scans illustrate very nicely the formation of new vessels both on the iliac level after iliac coagulation as well as on the femoral level after femoral coagulation.

Increased collateral and capillary density in the ischemic muscle after double coagulation of femoral artery and iliac artery.

Collateral density in the adductor muscle of the (post-) ischemic hind limb was higher compared to the adductor of the non-ischemic hind limb, although not significant (respectively 1.73 and 0.97; $P=0.246$) (Fig 4A,B,C). In addition, in the lower limb, a significant increase in capillary density was observed in the ischemic as compared to non ischemic calf muscle 28 days after surgical procedure ($P=0.020$) (Fig 4D,E,F).

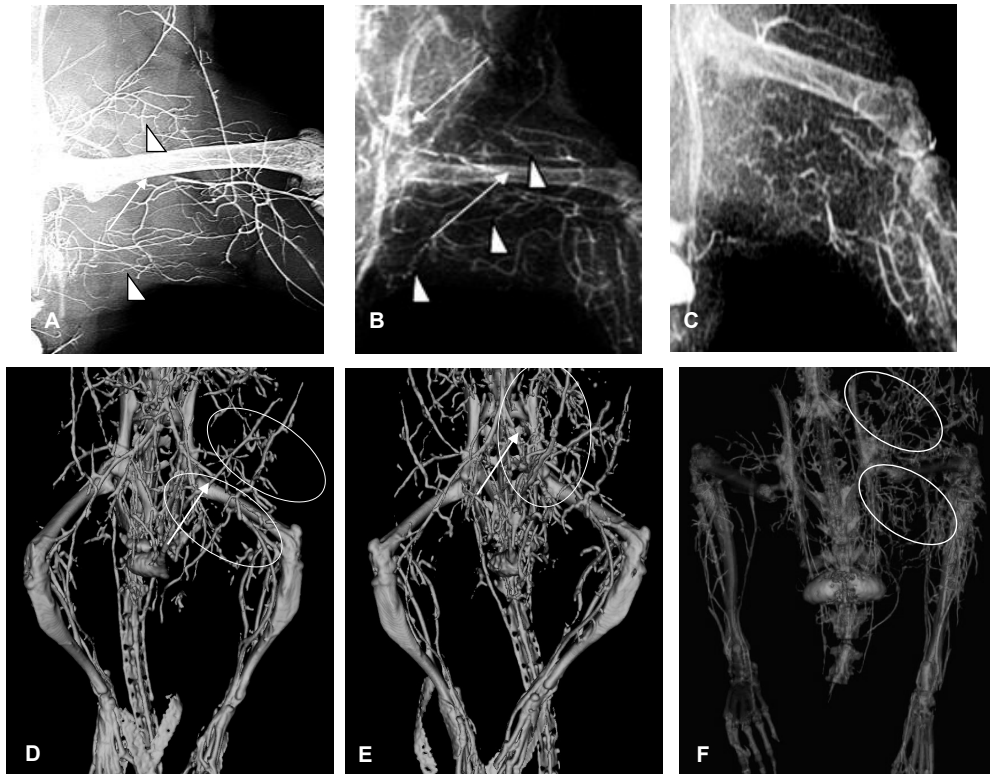


Figure 3. Angiographs of hind limbs of C57Bl6 mice made 28 days after induction of ischemia by different surgical procedures. Angiograph made after **A.** single electro-coagulation of femoral artery, **B.** double electro-coagulation of both femoral artery and iliac artery and **C.** total excision of the femoral artery. Arrows indicate electro-coagulation places in the single electro-coagulation model (note that the artery retracts after coagulation) and double electro-coagulation model. Arrow heads show numerous typical corkscrew collaterals formed in (post) ischemic hind limb. After a total excision of the femoral artery and all side branches, a disturbed pattern of small vessels are formed in the adductor muscle.

MicroCT-scans of hind limbs made 28 days after **D.** single electro-coagulation of the femoral artery, **E.** single electro-coagulation of the iliac artery, **F.** double electrocoagulation of both femoral artery and iliac artery. Numerous collateral arteries are formed around the iliac artery after single electro-coagulation of the iliac artery. Moreover, after single electro-coagulation of the femoral artery, collaterals are formed solely at femoral level. Double electro-coagulation showed numerous collaterals at both levels. Arrows indicate electro-coagulation places. Circles represent collateral zone. See color figure on page 215.

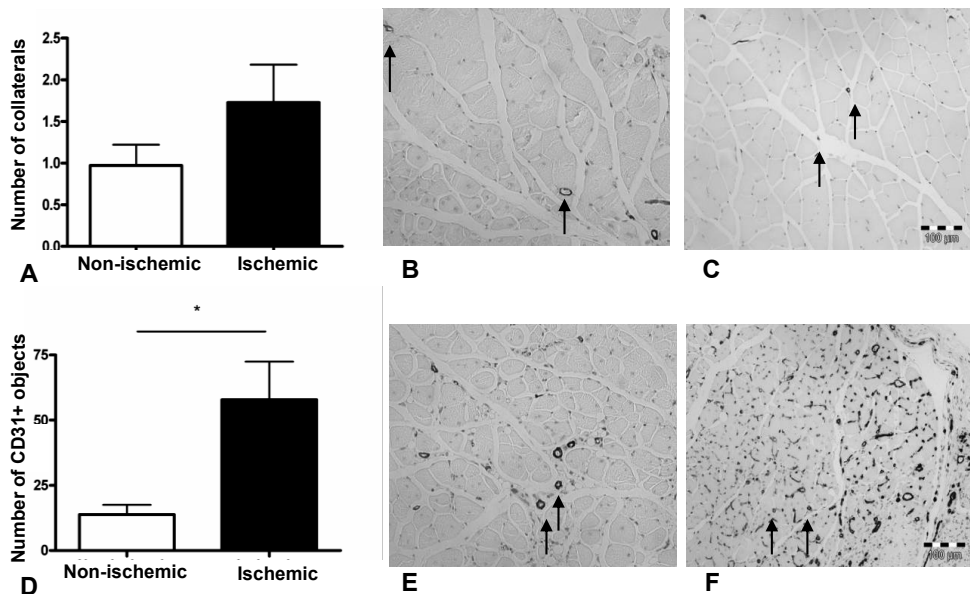


Figure 4. Immunohistochemical stainings of skeletal muscle 28 days after double coagulation with anti- α -smooth muscle actin antibody and anti-CD31 antibody for detection of collaterals and capillaries. **A.** Quantification of anti- α -smooth muscle actin stained adductor muscle sections comparing ischemic hind limb with non-ischemic hind limb (9 section per mouse were analyzed to get obtain the mean per animal, next the mean of $n=9$ animals was determined). Although the number in collaterals seems to increase, the differences between the number of collaterals is not statistically significant; $P=0.246$. Data were presented as mean \pm sem. Representative photographs of anti- α -smooth muscle actin stained **B.** non-ischemic adductor muscle sections and **C.** ischemic adductor sections. **D.** Quantification of anti-CD31 stained calf muscle sections comparing ischemic hind limb with non-ischemic hind limb (9 section per mouse were analyzed to get obtain the mean per animal, next the mean of $n=9$ animals was determined). Number of CD31+ blood vessels in ischemic hind limb differs significantly from non-ischemic hind limb. * $P=0.020$. Data were presented as mean \pm sem. Representative photographs of anti-CD31 stained calf muscle sections after double electro-coagulation of both femoral artery and iliac artery of the **E.** non-ischemic hind limb and **F.** ischemic hindlimb. See color figure on page 216.

Double electro-coagulation of both femoral artery and iliac artery in immune-deficient mice.

In order to validate the double electro-coagulation model of hind limb ischemia for testing human cell therapies, we performed a double coagulation in immune-deficient NOD-scid IL2Rgamma(null) mice. Similar to double electro-coagulation in C57Bl6 mice, blood flow restoration after double electro-coagulation in NOD-scid IL2Rgamma(null) mice was significantly decreased to 31% after 7 days compared to 104 % after a single electrocoagulation of the femoral artery ($P=0.002$) (Fig5A,B,C). Nine out of 10 mice had necrosis of one or more toe nails in this model of extensive ischemia. There was no necrosis of the paw or limb in these mice.

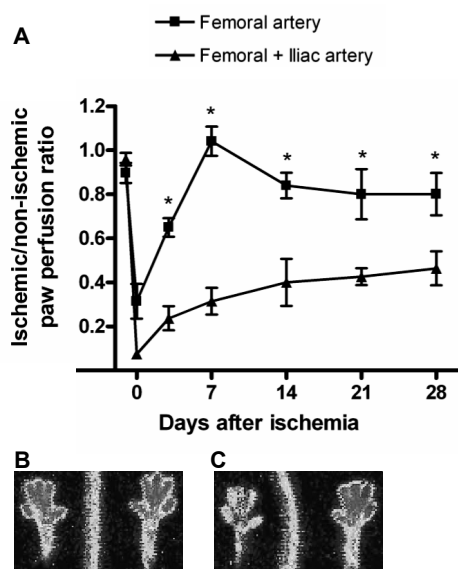


Figure 5. Blood flow restoration in hind limb of NOD-SCID IL2R γ (null) mice. **A.** Blood flow recovery after a single femoral artery electro-coagulation (n=5) or a double electro-coagulation of both femoral artery and iliac artery (n=10). After a double electro-coagulation of femoral artery and iliac artery, perfusion remained significantly impaired until 28 days after the surgical procedure. * $P < 0.014$. **B.** LDPI images of ischemic paw perfusion (left) and non-ischemic paw perfusion (right) 7 days after single electro-coagulation. **C.** LDPI images of ischemic paw perfusion (left) and non-ischemic paw perfusion (right) 7 days after double electro-coagulation. See color figure on page 217.

Discussion

In the present study, it is demonstrated that the extent of the arterial defect (single ligation of artery, total excision of artery or double ligation of artery) is associated with different patterns of perfusion restoration in the mouse hind limb. Blood flow recovery was substantially impaired in a mouse model of double electro-coagulation of both femoral artery and iliac artery compared to single electro-coagulation of one of these arteries. This results in an increase of the therapeutic window to study improved restoration of blood flow after experimental therapeutic approaches as cell therapy.

The anatomical level of occlusion of the artery (single electro-coagulation of femoral artery or iliac artery) had a similar effect on blood flow recovery in the hind limb ischemia mouse model. These results resemble studies of Shireman et al [16]. They showed similar patterns of blood flow recovery after transection of the proximal femoral artery, compared to transection of the distal femoral artery.

Angiographs made 28 days after total excision of the femoral artery showed a disturbed pattern of new small vessels formed in the (post-) ischemic hind limb. In contrast, angiographs made after single or double coagulation of the vascular tree showed more profound collateral arteries with the typical corkscrew phenotype in the (post-) ischemic hind limb. Some technical and physiological differences between these models could explain the disturbed pattern of vessels on angiographs after a total excision of the femoral artery.

First, after a single ligation of the femoral artery, all side branches of the artery were kept intact. However, after total excision of the femoral artery all the connections to the pre-existing collateral bed were likely to be disrupted completely. For restoration of the blood flow, not only pre-existing distant vessels need to enlarge their diameter to become collaterals, but also the disrupted connections need to be repaired in this model. Accordingly, in the profound ischemic model of total excision, it is not very likely that a process of solely arteriogenesis will appear. The process of angiogenesis will be most likely involved too, because all preexisting connections of arterioles to the vascular tree are disrupted and need to be repaired.

Sprouting of new capillaries (angiogenesis) [17] is a distinct process from collateral artery formation (arteriogenesis) [18]. Formation of new capillaries is mainly triggered by ischemia [17, 19, 20]. Arteriogenesis refers to the remodeling of pre-existent arterial collaterals that interconnects the vascular networks lying proximal and distal to the arterial obstruction and is triggered by increased shear-stress [21-23]. Despite the fact that a disturbed pattern of blood vessels is formed in the adductor muscle, mice though can restore blood flow restoration to 100% after total excision. Since all pre-existing connections of arterioles to the vascular tree are disrupted and need to be repaired (angiogenesis) in this model, blood flow restoration takes longer compared to single electro-coagulation of the artery. Oses et al. [24] recently demonstrated very elegantly significant differences in ischemia induced vascular growth mechanisms between the tight (mostly attributable to arteriogenesis) and the tibiofibular region (angiogenesis predominated in the tibiofibular region). So, the model of total excision of the femoral artery seemed not to be recommendable for studying arteriogenesis solely. One has to keep in mind that technical variations in hind limb ischemia mouse models do have physiological consequences, although the impact of these variations is often underestimated.

The impact of the use of technical variations in hind limb ischemia models on the outcome can be illustrated with conflicting outcomes of several experiments on VEGF-mediated gene therapy. Several research groups [25, 26] reported an enhanced revascularization after arterial gene transfer of VEGF in the ischemic hind limb models, whereas others did not see any effect [27]. Takeshita et al [25] showed a significant increase in angiographic

score of developed collaterals after VEGF administration. On the other hand, van Weel et al. [27] did not see any effect of VEGF in the hind limb ischemia model on angiographic rentrop score and blood flow restoration measured with LDPI. This difference in outcome could be explained by the fact that Takeshita et al. tested VEGF administration in a model of total excision of the femoral artery with all their side branches, whereas van Weel et al. used the model of single electro-coagulation of the femoral artery.

Models of hind limb ischemia in immune-deficient mice have been established to investigate the role of human cells in arteriogenesis. Kalka et al. [28] reported impaired blood flow restoration in nude mice after resection of the femoral artery. However, our results showed that after single electro-coagulation of the femoral artery of immune-deficient mice, blood flow recovery was 100% within 7 days. Once again, underscoring the impact of the different surgical procedures. The extremely fast blood flow restoration makes our model difficult for testing the potential stimulating role of different human cells in collateral artery formation. In this study, validation of the double electro-coagulation model was also performed in immune-deficient mice too. Although this is a more severe model of ischemia, blood flow gradually recovered after a double electro-coagulation and no abundant paw necrosis was developed in these mice. Furthermore, the therapeutic window for stimulation of blood flow restoration is considerably enlarged in a double coagulation hind limb ischemia model in immune-deficient mice (31% blood flow recovery within 7 days in NOD-scid IL2Rgamma(null) mice). This illustrates that the double coagulation model in immune deficient mice is a useful model for testing new human cell therapies for patients with PAD.

Although the study was designed to identify the most optimal model for testing strategies to improve blood flow restoration, we realize that there are some limitations. The first relates to the degree of ischemia that is inflicted. Since it is not possible for us to quantify the differences in ischemia that occurs after the surgery we can only assume that inducing the different extents of arterial defects (single electro-coagulation, total excision or double electro-coagulation) is associated with climbing amounts of ischemia. Therefore, we mainly focused our analyses on differences in collateral artery formation which is triggered by increased shear stress and not directly by ischemia. A second limitation is that we have performed our studies on healthy mice, whereas most patients with severe PAD have risk factors such as diabetes and hypercholesterolemia. To resemble clinical situation, one could consider to use hypercholesterolemic or diabetic mice for the hind limb ischemia model. However, for comparison of the surgical procedures we decided not to include these factors.

The double electro-coagulation model was only tested in immune-deficient mice, in which human cells can be evaluated as candidates for cell therapy.

In conclusion, there is a variety of surgical approaches for inducing ischemia in the mouse hind limb. The results of the present study show that the amount of injury to the vascular tree (single ligation of artery, total excision of artery or double ligation) does have consequences for the pattern of blood flow restoration, while the level of vascular occlusion (femoral or iliac) does not. For testing new therapeutic approaches for patients with PAD, the double coagulation model might be the optimal model, because it provides a substantial therapeutic window to stimulate blood flow restoration.

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