Chapter 2

Murine Models of Limb Ischemia

V. van Weel, D. Eefting, P.H.A. Quax

Extracted and adapted from:
Murine Models of Myocardial and Limb Ischemia: Diagnostic Endpoints and Relevance to Clinical Problems.


Animal models of peripheral arterial occlusive disease

Experimental hind limb ischemia models have been developed in both small and large animals. In general, large-animal models benefit from the ease of accurate identification of the lower extremity inflow vessels and their branches, as well as a multitude of blood flow measures that can be used throughout the course of a study. Small-animal models benefit from the availability of transgenic lines and genetic tools for analyzing alterations in gene and protein expression.

Severity of peripheral ischemia

In models of peripheral arterial disease, the level of ischemia depends on the one hand on location and length of the artery occlusion and on the other hand on the capacity of collateral and capillary development. Many research groups are using a model of hind limb ischemia by arterial occlusion in mice to mimic peripheral artery disease. The model of complete excision of the femoral artery and its side-branches originally developed in mice was based on a previously developed rabbit model. Unilateral hind limb ischemia was operatively induced as follows: vessel exposure was obtained by performing an incision in the skin overlying the middle portion of the left hind limb. The proximal end of the femoral artery and the distal portion of the saphenous artery were each ligated, and the arteries as well as all side-branches excised. This model permitted the use of the contra-lateral extremity as a control. The original series of investigations characterized and documented the operative reduction and subsequent endogenous restoration of hind limb blood flow in this animal model (demonstrated with laser-Doppler perfusion imaging). Neovascularization, as evidenced by increased endothelial cell proliferation and capillary density, was shown to develop in association with augmented expression of VEGF mRNA and protein. This reparative angiogenesis is dependent upon VEGF-A and FGFs upregulation, as confirmed by impaired neovascularization after administration of neutralizing antibody. Dissection of the complete femoral artery and its side-branches results in a deep distal ischemia. This is mainly the result of the fact that a collateral network cannot be formed since the pre-existing arterioles are disconnected from the vasculature. Alternative models differ from each other in that the level of vascular occlusion is the major determinant for the amount of ischemia and the magnitude of angiogenic response. We studied the consequences of ligature interruption of the arterial supply at various locations from the iliac artery, proximal to the inguinal ligament, down to the saphenous artery in the lower limb of mice (data not shown). Symptoms of ischemia, such as necrosis of the toes and limb paralysis, were more profound by placing the ligation more proximally, and were most evident when placed around the iliac artery. This phenomenon is also known from the clinic where patients with...
proximal occlusion(s) of the arterial tree of the limb, e.g. the iliac artery, suffer the most from profound ischemia. Therefore both the excision and the proximal ligature methods, especially if performed in the background of an impaired angiogenic and/or arteriogenic response, will result in reductions in blood flow to the ischemic limb even at rest and would therefore more closely replicate the clinical situation of patients with severe limb ischemia. Dissection of femoral artery and vein (and sometimes even the nerve) also results in severe ischemia, often leading to auto-amputation. The latter model, however, does not seem clinically relevant since the problem in patients with ischemic disease consists of arterial obstructions, rather than venous complications. Models more closely resembling ischemic disease in patients consist of ligation or electrocoagulation over a short arterial segment. Distal ligation leaves the arterial side-branches intact, thus resulting in a mild chronic ischemia at rest and reduced blood flow reserve unveiled by stress. Ethical concern regarding animal sufferance suggests application of these milder ischemia models, especially under experimental conditions in which underlying disease (diabetes or atherosclerosis) or immunosuppression may compromise post-ischemic healing.

Definitions of different events relevant to reparative neovascularization

Importantly, the type of hind limb ligation model determines which process of vascular growth can be best evaluated. The knowledge of the different variables (including time scale) relevant to these processes is crucial in the selection of follow-up time points and type of perfusion recovery parameters. Recently, it was shown that the sprouting of new capillaries (angiogenesis) is a process distinct from arterial formation (arteriogenesis). Capillary formation is mainly triggered by ischemia, thus the process will be more evident in, but not exclusive of, distal hind limb muscles. Accordingly, in the mild model of ligation, histology shows increased capillary density mainly in the gastrocnemius (calf) muscle. At variance, more profound ischemia, as triggered by dissection of the whole femoral artery, results in enhanced capillary density in adductor muscle as well as in other muscles of the thigh. Arteriole growth accompanies capillarization of ischemic muscles, suggesting that these collaterals originate from maturation of recently sprouted capillaries that had recruited smooth muscle cells and pericytes. The term of arteriogenesis, however, is also referred as to identify the remodeling of pre-existing arterial collaterals that interconnect the vascular networks lying up and downstream to the arterial obstruction. This remodeling process is seemingly independent from ischemia. In fact, enlarged collaterals are evident especially in the upper leg, at large distance from ischemic limb regions, precede the full development of reparative capillarization, and are crucial for hemodynamic recovery after femoral artery ligation. The main trigger for arterial collateralization appears to be the increased shear-stress in the preexisting vessels that are recruited to bypass blood flow around the occlusion of a main artery. Likewise, in patients with peripheral atherosclerosis, progressive
occlusion of conductance arteries causes blood flow redistribution through interconnecting arterioles, thereby significantly increasing shear stress. The cellular and molecular mechanisms involved in arteriogenesis were recently characterized in mice and rabbits. It was shown that endothelial cells respond to changes in local hemodynamics by increasing the transcription of a number of genes, including NO synthase and the adhesion molecules ICAM-1, VCAM-1, E-Selectin and MCP-1.\textsuperscript{18} The upregulation of chemokines leads to attachment of monocytes to the activated endothelium, invasion into arteriolar wall, and infiltration of the peri-adventitial space. Migrated monocytes express and release cytokines, growth factors, and proteases thus favoring the growth of all components of the elongating artery that invades extracellular matrix.\textsuperscript{19} The process is very rapid in otherwise healthy rodents, as indicated by angiographic evidence of collateral formation at 7 days after femoral artery ligation. In contrast, in atherosclerotic patients, the adaptive mechanism has a low progression and is influenced by underlying risk factors.

**End-points in hind limb ischemia models**

A series of end-points based on clinical, hemodynamic, and histological parameters has been established in hind limb ischemia models.

**Clinical end-points**

Calculation of a clinical score based on the number of necrotic toes is useful for evaluation of treatment effect on recovery from ischemia. In our experience, the measurement showed absolute inter-observer reproducibility. Monitoring clinical outcome enables the investigator to introduce adequate measures to avoid animals’ sufferance, including euthanasia of those with auto-amputation of the foot.

**Measurements of blood flow**

Measurement of lower extremity blood flow (micro-circulation) can be achieved with laser Doppler perfusion imaging (LDPI). LDPI is performed using a beam from a 2-mW helium-neon laser that sequentially scans a 12 X 12-cm tissue surface to a depth of a few hundred micrometers. According to the “Doppler shift” theory, moving blood will reflect back laser light at a wavelength different from the one that is transmitted. Detected changes in wavelength are converted into flux values based on the velocity of the moving blood. These flux values are then transformed into a color-coded image representing the microvascular blood flow distribution. The method is limited by penetration depth of the Laser beam (1-2 mm), thus allowing measurement of superficial skin perfusion only. Functional perfusion in both paws is generally obtained at baseline, immediately after surgery, and serially over 4 weeks during recovery. The influence of external factors, including ambient light and temperature, on laser Doppler blood flow is minimized by performing the measurements in a dark
room with anesthetized mice placed on a heating pad or double-glassed vessel filled with water at a constant temperature of 37°C. Although Doppler flow measurements are useful for identifying flow deficits relative to a non-occluded contra-lateral limb, they may be less useful in identifying subtle changes in flow. Previous studies established that laser Doppler flow velocity correlates with capillary density in the ischemic limb in C57BL/6 mice.\(^1\)\(^{14}\)\(^{20}\) In a comparative study with angiography, we demonstrated that laser Doppler paw perfusion recovery and upper limb collateral artery growth follow an identical time course after femoral artery occlusion (see Chapter 5). Furthermore, laser Doppler perfusion was locally increased around the surgical wound in the upper limb, suggesting wound angiogenesis (data not shown). To avoid measuring perfusion near the wound, it is preferable to exclusively select the paws, more distally, as a region of interest for image analysis. An advantage of laser Doppler analysis over post-mortem angiography is that the former, being non-invasive, can be sequentially repeated over time. Another means of assessing the function of microcirculation is to measure collateral-dependent blood flow. This could be achieved by measuring blood flow to the distal hind limb with the use of microspheres. Regional blood flow is proportional to the number of microspheres trapped in the area of interest. However, tissue blood flow has to be determined with the following equation: sample flow (ml/min) / radioactivity (fluorescence) in reference sample = tissue flow / radioactivity (fluorescence) in organ, meaning that microsphere injections have to be monitored.\(^1\)\(^{15}\) Flow probes can be surgically and temporarily placed over a vessel (a. iliaca or a. saphena) to measure blood flow and peripheral vascular resistances, under basal conditions as well as in response to vasodilators (e.g., adenosine or papaverine). Unfortunately, the sites where blood flow is measured for this purpose are also a source for blood supply to regions other than the collateral-dependent tissue of the distal hind limb. Thus, when a vasodilator is administered, blood flow markedly increases to these regions because vascular resistance of normal tissue can decrease far more than the resistance of the collateral circuit. As a result, the measurement of blood flow only partially represents flow delivered to the collateral-dependent tissue.

**Evaluation of collateral formation by angiography**

Angiography is used to visualize collateral vessels in rodents by employing adapted contrast agent and micro radiographic equipment.\(^7\) However, the complexity and multiple potential sources of the collaterals and the lack of sufficient radiographic spatial resolution limit this method’s ability to accurately measure collateral blood flow. We used post-mortem angiography to assess collateral formation in mice submitted to unilateral coagulation of the left common femoral artery proximal to the bifurcation of superficial and deep femoral artery.\(^4\) To this aim, papaverin (2 mg/ml) was injected into the aorta to induce vasodilation, followed by polyacrylamidebismuth contrast medium at constant pressure (100 mmHg). Post-mortem angiographic images were acquired by röntgenographic exposure using a Faxitron X-ray machine.
Images of the non-operated limb served as a control to verify whether the angiographic technique was successful. Grading of collateral filling was performed in a single blinded fashion and was based on the Rentrop classification.\textsuperscript{21} Grading was as follows: 0=no filling of collaterals, 1=filling of collaterals only, 2=partial filling of distal femoral artery, 3=complete filling of distal femoral artery. Angiography showed a rapid increase of collateral vessel development in C57BL/6 WT mice with visible pre-mature collaterals within 3 days, followed by filling of the distal femoral artery with contrast medium via collaterals already at 7 days after femoral artery occlusion (see Chapter 5). The appearance of typical corkscrew collateral arteries and a gradual increase of their diameter were observed from 7 through 28 days after surgery. Collateral arteries were located in both quadriceps muscle and adductor muscle compartment. Since the occlusion was performed proximal to the bifurcation of superficial and deep femoral artery, collaterals in the adductor region did mainly originate from pelvic branches of the iliac artery, bridging to the sural artery and saphenous artery. However, collaterals were also apparent originating from the deep femoral artery, thus distally to the occlusion. This suggests that not only a local increase of shear-stress, but also systemic changes, e.g. circulating ischemia-induced growth factors or activated inflammatory cells, may be a trigger for the development of collateral arteries. In the quadriceps region, collaterals mainly originated from branches of the iliac artery bridging to the popliteal artery, from which the tibial artery originates.

**Other methods for assessment of collateral formation**

Micro CT scan (Figure 1) is a promising approach for 3D anatomic study of neovascularization development after ischemia\textsuperscript{22}, albeit spatial resolution is still a limiting parameter. Magnetic resonance imaging (MRI) is a rapidly gaining importance for regional blood flow assessments, as well as assessments of blood vessel function and integrity. Contrast-enhanced MRI, with first-pass gadolinium-based contrast agent, can be used to visualize (collateral) arteries and to obtain regional and muscle-specific perfusion measures.\textsuperscript{23,24} Both methods could provide, in the near future, a number of data points to help researchers understand and quantitatively measure lower extremity perfusion. One special problem of X-ray angiography consists of its invasiveness. In addition, traditional angiography provides 2-D projection images and exposes to the risk of allergic reaction or kidney toxicity due to the use of iodinated contrast agents.
Histological measurements of angiogenesis

Capillary density remains the most commonly assessed histological measure of angiogenesis. The analysis of capillary density does not preclude additional assessment of perfusion changes in this model, although the correlation between both was demonstrated to be good in multiple experimental conditions.\textsuperscript{1,7,14} Endothelial cells are quantified with the use of immunohistochemical techniques (anti CD-31, anti von Willebrand factor antibodies), and the capillary density of ischemic limb is counted, using contra-lateral side for normalization with the caveat that the latter might be influenced by endocrine angiogenic factors which are released from the ischemic muscle (a phenomenon referred as remote preconditioning). In the murine hind limb model, capillary density typically increases in hind limb muscles reaching a plateau between 14 and 21 days post-ischemia (see Chapter 5). To study vascular cell proliferation, bromodeoxyuridine (BrdU, that incorporates in replicating DNA) can be infused either continuously after induction of ischemia or over specific time windows in order to determine the temporal pattern of proliferation. Double immunolabeling for BrdU and CD-31 established that endothelial cell proliferation temporally coincides with the increase in capillary density.\textsuperscript{1} Then, the newly formed capillaries tend to regress spontaneously, due to complete maturation of arterial collaterals that enable reperfusion downstream to the arterial occlusion. The cycling fraction of endothelial cells can also be determined by evaluating the expression of minichromosome maintenance protein-2 (MCM-2) or similar markers with immunohistochemistry, thus avoiding the need of BrdU infusion. Immunohistochemistry is also used for the detection of inflammatory cells infiltrating the ischemic area and participating to the neovascularization process.\textsuperscript{13}
Discussion

Animal models of limb ischemia seem suitable to study different forms of neovascularization and their effects on hemodynamics. In this thesis, we used a mouse model in which we applied a short occlusion of the proximal femoral artery, leaving arterial side-branches intact, thereby allowing collateral arteries to develop. Collateral artery growth was quantified by angiography and immunohistochemistry, whereas its hemodynamic effects were studied with laser-doppler perfusion imaging. Although stimulation of neovascularization using vascular growth factors was successful in many previous animal studies, placebo-controlled studies in patients were less beneficial. This may be explained by that “healthy” animals are used that upon arterial occlusion demonstrate acute ischemia, whereas vascular disease in patients is more complex with chronic ischemia and endothelial dysfunction. Moreover, differences in expression patterns of endogenous VEGF in ischemic muscle were reported for ischemic muscle either derived from rabbits after femoral artery occlusion as compared to human amputation material.\textsuperscript{25} To induce more chronic ischemia, animal models with more “patient-like” profiles can be used, for example with hyperlipidemia or diabetes. In addition, these models provide the advantage of having a wider therapeutic window to test vascular growth factors by retardation of collateral artery growth (see Chapter 5). An interesting finding is that major differences in blood vessel formation exist between mouse strains resembling differences in collateral formation between patients.\textsuperscript{26} Corresponding differences in lymphocyte-related immune response between these strains let us to a role for lymphocytes, such as T-cells and natural killer cells, in arteriogenesis (see Chapter 3). Finally, animal models facilitate evaluation of post-ischemic gene expression profiles by differential gene expression analysis using micro-array techniques at various time points after femoral artery occlusion.\textsuperscript{27}

In conclusion, although ischemic hind limb models in animals are not an exact representation of ischemic disease in patients, they are highly suitable to study the cellular and molecular mechanisms of angiogenesis, vasculogenesis and arteriogenesis by modulating the surgical procedure and careful interpretation of the various evaluation techniques, possibly leading to novel therapies to treat ischemic disease in patients.
Chapter 2

### References
