HIGHLIGHTED TOPIC | Skeletal and Cardiac Muscle Blood Flow

Preclinical models of human peripheral arterial occlusive disease: implications for investigation of therapeutic agents

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Waters, Richard E., Ronald L. Terjung, Kevin G. Peters, and Brian H. Annex. Preclinical models of human peripheral arterial occlusive disease: implications for investigation of therapeutic agents. J Appl Physiol 97: 773–780, 2004. First published April 23, 2004; 10.1152/japplphysiol.00107.2004.—Peripheral arterial occlusive disease (PAOD) is now recognized as a combination of clinical syndromes that are associated with significant morbidity and mortality. The primary pathophysiology of PAOD is impaired perfusion to the lower extremity. Effective pharmacotherapy designed to increase perfusion in PAOD is lacking, and revascularization options are suboptimal. New and more efficacious therapies that improve blood flow are definitely needed, and thus designing, describing, and validating these new therapies in preclinical PAOD models will be essential. This study describes the various preclinical PAOD models presently in use, correlates the models to human PAOD, and reviews the available end points that can be used to detect a response to therapy.

angiogenesis; endothelial cells; growth factors; perfusion; vascular surgery

SCOPE OF CLINICAL PROBLEM

Atherosclerosis is the leading cause of morbidity and mortality in the Western world. Peripheral arterial occlusive disease (PAOD) due to atherosclerosis encompasses a spectrum of clinical syndromes with an incidence and prevalence nearly equal to coronary artery disease (18). Approximately 15% of adults over the age of 55 have detectable hemodynamic impairments attributed to PAOD, and the number of patients with PAOD can be expected to increase as the population ages (13, 42). The two major clinical presentations of PAOD are intermittent claudication and critical limb ischemia (Table 1). In patients with intermittent claudication, arterial occlusive disease is manifested by insufficient blood flow during exercise. In patients with critical limb ischemia, blood flow is inadequate to meet the resting demands of the limb. Even in its mildest form, and in the absence of “classic” symptoms of leg pain, PAOD is associated with marked disability and impairment in a patient’s quality of life (39).

The primary pathophysiology of PAOD is impaired perfusion to the lower extremity. Interestingly, the standard treatments for PAOD (antiplatelet agents, angiotensin-cascade antagonists, and cholesterol-lowering therapy) are targeting general atherosclerotic risk-factor reduction, but none is designed to improve tissue perfusion (23, 25, 48). Life style modifications such as cessation of smoking are clearly valuable in limiting limb loss in patients with critical limb ischemia. Exercise training is commonly regarded as the most effective therapy in patients with intermittent claudication but has only been validated in small, selected patient populations subjected to intense supervision (18). Pharmacological therapies in PAOD are limited. Pentoxifylline, although widely prescribed in the United States, has little or no clinical benefit (46). Cilostazol, a phosphodiesterase inhibitor, offers some clinical efficacy; however, its safety in patients with ventricular dysfunction remains in question, and the FDA has issued a black box warning for patients with any degree of heart failure (4, 40). Mechanical revascularization, either surgically or percutaneously, can be beneficial, especially in patients with focal or “single-segment” atherosclerotic obstruction confined to aortoiliac levels. Unfortunately, obstruction at multiple levels is common, and thus complete revascularization in patients with PAOD is unusual (24). Surgical therapy carries the risk of perioperative morbidity and mortality, loss of conduit, and graft attrition. Percutaneous treatment is feasible in many patients with PAOD; however, intermediate-term (6–12 mo) recurrent obstruction is common, especially in the large proportion of patients with diffuse or distal disease (20).

New and more efficacious treatment options for PAOD are definitely needed. Numerous preclinical PAOD models exist, and the purpose of this study is to compare and contrast the various preclinical models of PAOD because the next generation of PAOD therapy will benefit from designing, describing, and validating approaches in preclinical PAOD models. “Preclinical” will be used to refer to any nonhuman in vivo or in vitro PAOD study with the exception of those studies performed exclusively to define the toxicity of an agent. Various models will be discussed in regard to their correlation to the human disease state (Fig. 1) and to the measures (i.e., end points) used to detect changes in perfusion that occur in response to therapy.

ESTABLISHING A PRECLINICAL MODEL OF PAOD

In the 1950s, Longland (36) described an attempt at establishing a preclinical model of chronic ischemia with the injec-
of thrombin and a sclerosant into the femoral artery of rabbits. This model was associated with a high incidence of experimental animal loss, but it did provide the conceptual framework for future chronic lower-extremity ischemia models. Since that time, experimental hindlimb 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 (i.e., mice) and genetic tools for analyzing alterations in gene and protein expression.

Species-to-species variations in myocardial injury and blood flow recovery after coronary artery ligation have been recognized for many decades (28). The same issue exists with lower-extremity blood flow, where species-to-species variations and genetic strains within the same species may be important. For example, Sunder-Plassman et al. (55) described multiple lower-extremity collateral pathways at baseline in the canine, necessitating the identification and occlusion of as many as 14 branches of the iliac, common femoral, and profunda femoris arteries to significantly alter flow. Likewise, Seifert et al. (53) described an abundant collateral network at baseline in the rat, which is due to the physiological absence of visible collateral vessels, fails to produce chronic ischemia at the profunda femoris artery. The limited baseline collateral network in the mouse, rabbit, and pig more closely resembles human vascular anatomy.

In addition to species and genetic background, it is also important to consider the nature of the vascular injury. The tourniquet technique is a well-characterized and technically simple preclinical model that has been used in cats, dogs, rats, rabbits, various species of primates, and even humans (2). By placing an external tourniquet on the proximal part of the extremity and rapidly inflating to high pressure, investigators can achieve prompt and virtually complete interruption of arterial and venous flow. If the procedure is performed quickly, problems with venous engorgement are avoided. However, the tourniquet method also occludes any potential collateral vessels, and widespread muscle necrosis distal to the occlusion occurs after as little as 6 h of ischemia, making this method unsuitable for studying chronic occlusive disease. Moreover, physiological adaptations to the ischemic insult are limited by the complete isolation of the limb from the systemic circulation. This model may involve direct compression and injury to myocytes, as well as peripheral nerves. Therefore, this approach is more suited for studying acute ischemia and reperfusion injury that occurs with the reestablishment of flow after acute arterial occlusion.

Surgical ligation of the lower extremity inflow vessels, although still a form of acute injury, has also been employed in an attempt to create states of chronic impaired flow. Vessel location, degree of side branch and collateral vessel occlusion, and species variability all contribute to the degree of ischemia seen after ligation (Figs. 1 and 2). Occlusions closer to the aorta (e.g., common iliac artery) and occlusions performed with the ligation of potential collateral sources produce more profound effects than simple distal occlusions (e.g., common femoral). Ligations alone, however, often do not result in a significant or sustainable reduction in resting blood flow. Lower extremity blood flow remains unchanged at rest after simple femoral artery (8) or common iliac artery (30) ligation in rats. A more extensive two-stage arterial ligation model in the rat, in which the common iliac artery and all its branches are ligated, does exhibit some flow reduction at rest but only out to 5 days (53). Hendricks et al. (21) reported a slightly more prolonged reduction in resting blood flow after common iliac artery ligation in rabbits, with flow recovery lengthened to ~2 wk. A porcine femoral artery ligation model with reductions in resting ischemic limb blood pressure and blood flow by internal flow probe lasting 2 wk has also been described (7). Although surgical ligation, even when performed as proximal as the common iliac artery and with accompanying ligation of visible collateral vessels, fails to produce chronic ischemia at

### Table 1. Comparison of the two major PAOD syndromes

<table>
<thead>
<tr>
<th></th>
<th>Intermittent Claudication</th>
<th>Critical Limb Ischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative abundance</td>
<td>Common</td>
<td>Less common</td>
</tr>
<tr>
<td>Defining symptoms</td>
<td>Pain with walking</td>
<td>Rest pain, nonhealing ulcers, gangrene</td>
</tr>
<tr>
<td>Frequency of comorbid disease</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Blood flow</td>
<td>Moderately impaired</td>
<td>Severely impaired</td>
</tr>
<tr>
<td>Effective therapy</td>
<td>Revasc (minority); exercise; atherosclerosis therapy</td>
<td>Revasc (majority)</td>
</tr>
<tr>
<td>Goal of therapy</td>
<td>↑ PWT, ↑ QOL</td>
<td>Limb salvage, mortality reduction</td>
</tr>
</tbody>
</table>

PAOD, peripheral arterial occlusive disease; Revasc, revascularization; PWT, peak walking time on treadmill exercise protocol; QOL, quality of life as assessed by validated questionnaires; ↑, increased.
rest, it does appear to produce a reduction in blood flow reserve with stress. Yang et al. (61) demonstrated a significant reduction in the degree of active hyperemia in response to treadmill exercise that persisted for at least 8 wk in rats after femoral artery ligation.

In humans, PAOD is largely a bilateral disease (23, 24). Models of bilateral common iliac artery ligation and simultaneous arterial and venous ligation have also been evaluated. Cheboun and Martins (9) reported gangrene in one or both legs in four of seven rats and three deaths within the first week after bilateral common iliac artery ligation. In addition to creating an acute insult that was too extensive to permit evaluation of chronic ischemia, this model prohibited use of the contralateral extremity as a control. Similarly, simultaneous ligation of the iliac artery and vein on one side resulted in all rats exhibiting gangrene (4 of 7 rats died in 4–6 days) and tissue loss of at least the foot (9). It is likely that the added venous insufficiency exacerbated tissue ischemia in this model (32). In general, in contrast to unilateral arterial ligations, bilateral arterial ligation models, especially if associated with venous ligation, have a substantial morbidity and mortality following the acute insult.

CURRENT PRECLINICAL MODELS OF CHRONIC HINDLIMB ISCHEMIA

In 1992, Pu et al. (45) described a preclinical hindlimb ischemia model that did produce persistent ischemia at rest. In contrast to ligation models, their model incorporated ligation and complete excision of the lower extremity inflow arteries.

Table 2. Measures of impaired arterial perfusion in rabbit hindlimb ischemia models of ligation and excision

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf BP ratio (I/N)</td>
<td>NA</td>
<td>0.19</td>
<td>0.36</td>
<td>0.45</td>
<td>NA</td>
<td>0.63</td>
</tr>
<tr>
<td>Calf radioisotopic perfusion ratio (I/N)</td>
<td>1.01</td>
<td>NA</td>
<td>0.72</td>
<td>0.76</td>
<td>0.83</td>
<td>NA</td>
</tr>
<tr>
<td>Femoral venous lactate levels (I/N)</td>
<td>0.98</td>
<td>1.31</td>
<td>NA</td>
<td>NA</td>
<td>1.15</td>
<td>1.02</td>
</tr>
</tbody>
</table>

NA, exact value not available. BP, blood pressure; I/N, ischemic to normal ratio.

markedly reducing the possibility of any recanalization or short bridging-collateral formation. The technique was initially applied in New Zealand White rabbits in which the distal external iliac artery (just above the level of the inguinal ligament), inferior epigastric, profunda femoral, circumflex femoral, and superior epigastric arteries were ligated proximally and in which the popliteal and saphenous arteries were ligated distally (Fig. 2). The entire femoral artery was then excised from just above the inguinal ligament to the level of the proximal popliteal and saphenous arteries. As a consequence, blood flow to the ischemic limb became completely dependent on collaterals issuing from the internal iliac artery (Fig. 2). Clinically, all animals had noticeable limping of their ischemic hindlimb on postoperative day 1. After day 10, the majority had either mild hindlimb limping or a functionally normal leg but noticeable muscular atrophy. Some animals developed superficial tissue necrosis in the foot, a few had a nonfunctional hindlimb, and six died before study termination but with no deaths attributable to the ischemia-inducing procedure. Angiograms showed slow filling of the arterial tree beyond the excised femoral artery with few thigh collaterals that persisted through 90 days. Assessments performed preoperatively and on postoperative days 1, 10, 20, 30, 40, and 90 showed that arterial perfusion was impaired, as demonstrated by calf blood pressure ratio and calf radioisotopic perfusion scanning as well as by increased femoral venous lactate levels, which were persistent throughout the study period (Table 2). Qualitatively similar results have been described in the same rabbit model by other investigators (26).

In the previously described models, arterial ligation had been followed by an endogenous response that limited the extent of the ischemia in the distal tissue. There is a large range in the endogenous angiogenic/arteriogenic response in humans. Children subsequent to Blalock-Taussig operations and adults with coarctation or aplasia of large vessels develop a significant collateral vascular tree (31, 51, 59), thus showing that humans, given an appropriate stimulus and a healthy vascular bed, can mount an angiogenic response that can prevent ischemia. However, a number of demographic and clinical predictors, highlighted in Table 3, can clearly impair this response (5, 58). To be able to integrate many of the items found in Table 3 into the preclinical models, Couffinhal et al. (11) and Murohara et al. (41) performed a similar ligation and excision of the femoral arteries in mice and demonstrated a reduction in hindlimb blood flow with laser-Doppler perfusion imaging (LDPI) that reached a plateau at ~28 days but persisted through 35 days. Further experiments demonstrated a more extensive and sustainable ischemia in diabetic (50), hypercholesterolemic (12, 15, 56), hyperhomocysteinemic (16), and

Table 3. Factors associated with an impaired angiogenic response to ischemia in humans and their preclinical correlate

<table>
<thead>
<tr>
<th>Clinical Factor</th>
<th>Preclinical Model</th>
<th>Magnitude of Impairment</th>
</tr>
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<tbody>
<tr>
<td>Hyperhomocysteinemia</td>
<td>Duan et al. (16)</td>
<td>+</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>Couffinhal et al. (12), Duan et al. (15), Van Belle et al. (56)</td>
<td>++</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Rivard et al. (50)</td>
<td>+ +</td>
</tr>
<tr>
<td>Advancing age</td>
<td>Rivard et al. (49)</td>
<td>+ + +</td>
</tr>
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Aged (49) mice. For example (Fig. 3), femoral artery ligation and excision in nonobese diabetic mice, which develop a form of diabetes having clinical features similar to humans with Type 1 diabetes mellitus, results in an LDPI ratio of 0.5 at 35 days. The same procedure in apoE−/− mice results in a LDPI ratio of 0.6 at 35 days (12, 50).

As described in Table 1, patients with PAOD have two major and distinct clinical manifestations. Patients with intermittent claudication have normal or slightly diminished lower extremity blood flow at rest but an inability to adequately increase blood flow with exercise (23, 24). Therefore, the arterial ligation model or the ligation with limited excision model, with an intact capacity to produce an angiogenic and/or arteriogenic response, will exhibit near normal flows at rest but impaired limb blood flow capacity with stress (e.g., exercise); thus it is well suited for the study of intermittent claudication in PAOD (60–64). The ligation and excision method, 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 critical limb ischemia.

END POINTS FOR ASSESSING CHANGES IN PERFUSION IN HINDLimb ISCHEMIA MODELS

A major therapeutic goal in PAOD is to increase perfusion, and preclinical models can be used to assess the potential utility of therapeutic modalities that might augment perfusion to the ischemic limb. In the use of preclinical models, the timing of the perfusion assessments is critical. Because all models are associated with some degree of perfusion recovery, interventions or agents tested at the time of surgery, or shortly after, will measure the capacity of an agent to improve on the degree of recovery. At later time points after surgery (typically more than 1 wk), the models are stable and there will be far less in the degree of catch up of the ischemic limb to the nonischemic limb. The downside is that at later time points perfusion in the ischemic limb will be closer to perfusion in the nonischemic limb, and thus the ability to detect the “efficacy” of an intervention or agent may be less. Studies to date have utilized a variety of techniques to evaluate changes in perfusion, and a standardized protocol needs to be established to enable comparison among studies and identification of therapies that warrant clinical trials.

Histological Measurements of Angiogenesis

In preclinical models, the assessments of changes in perfusion made on a histological level are by themselves not valuable. The correlation of the histological measures to human disease is also uncertain. For example, in humans, in the absence of pathological disease states, capillary density is directionally correlated with the oxidative capacity and endurance of a muscle group (27). Interestingly, although patients with PAOD (i.e., intermittent claudication) have normal or near normal resting lower extremity blood flow, they exhibit skeletal muscle abnormalities compared with healthy controls (6). Paradoxically, despite having a reduced aerobic capacity, the skeletal muscle capillary density appears to be increased in patients with PAOD, and the relative increase in vascular density appears to be proportional to the extent of skeletal muscle hypoxia (6, 24). This could be viewed as a potentially beneficial adaptation and as an attempt to compensate for the reductions in blood flow.

Capillary density remains the most commonly assessed histomorphological measure of perfusion in preclinical models. Endothelial cells are quantified with the use of immunohistochemical techniques, and the capillary densities of ischemic tissue and contralateral normal tissue are compared (10). Because muscle atrophy is a common phenomenon in the hindlimb ischemia models, determination of the number of “muscle fiber capillary contacts” rather than the number of “capillaries per millimeter squared” may be more accurate, as the former is less influenced by changes in muscle fiber size. Regardless of the assessment used, a context for the interpretation of the findings is unclear. Additional tissue assessments that may prove valuable include changes in muscle fiber type, fibrosis, and evidence of the extent of cell death and proliferation (14).

Measurements of Lower Extremity Blood Flow

Calf blood pressure provides a simple and rudimentary method of assessing blood flow deficits. Calf blood pressure is usually measured with a Doppler flowmeter. The pulse in the posterior tibial artery in the lower calf is detected by the Doppler probe with contact gel, and the systolic blood pressure in the calf is measured with a small blood pressure cuff. The calf blood pressure ratio is defined as the ratio of the pressure in the ischemic hindlimb to that in the contralateral nonischemic hindlimb (ischemic-to-normal ratio). Therefore, this model can only be applied to situations of unilateral limb ischemia, and care must be taken to account for different levels of anesthesia and the effects on blood pressure.

Another measurement of lower extremity blood flow can be achieved with LDPI. LDPI uses a beam from a 2-mW helium-neon laser that sequentially scans a 12 × 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. Although Doppler flow measurements are useful for identifying flow deficits relative to
a nonoccluded contralateral limb, they may be less useful in identifying subtle changes in flow.

Finally, flow probes may be implanted over major inflow vessels (e.g., common iliac artery, internal iliac artery) to the ischemic limb, and flow changes in response to vasodilator administration (e.g., adenosine or papaverine) can be readily determined (3, 7). 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 hindlimb. 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. This establishes a potential to mistakenly attribute changes in flow to possible adaptations in the collateral circuit introduced by an arteriogenic treatment.

**Direct Measures of Perfusion to Ischemic Tissue**

The best means of assessing perfusion to the ischemic limb is to measure the capacity for collateral-dependent blood flow. This can be achieved 1) when blood flow that is delivered to the collateral-dependent tissue is measured and 2) when the resistance of the collateral circuit is the dominant resistance determining blood flow. The first condition is most easily achieved by measuring blood flow to the distal hindlimb (e.g., calf muscles) with the use of microspheres. Regional blood flow is proportional to the number of microspheres trapped in the area of interest. One can determine tissue blood flow with the following equation: sample flow (ml/min) ÷ radioactivity in reference sample = tissue flow ÷ radioactivity in organ. Achieving the latter condition is more challenging. Simple vasodilator administration can be problematic because isolated administration of vasodilator therapy to the collateral-dependent tissue is difficult, and systemic effects of a reduced blood pressure can confound an experiment. In a similar manner, anesthesia, if associated with excessive hypotension, can alter these measures of perfusion. It is possible to circumvent this problem by using muscle contractions during exercise in vivo (63) or in situ (57, 61). Muscle contractions impart the most powerful stimulus for vasodilation. As a result, vascular resistance of the collateral circuit is dominant, and blood flow to the calf muscles is collateral dependent (62). Because of the rigorous nature of the measurement conditions, this assessment of collateral blood flow has been thus far relegated to experimental studies.

**Anatomic Measures of Blood Flow**

Although contrast angiography is sometimes used to visualize collateral vessels, the complexity of the collaterals, the 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. Angiographic data, when present, are valuable mainly as a correlate to other measures such as calf blood flow, even when detailed analysis systems are used. Magnetic resonance imaging (MRI) is a rapidly advancing approach for regional blood flow assessments, as well as assessments of blood vessel function and integrity. MRI can provide a number of data points to help researchers understand and quantitatively measure lower extremity perfusion. Buschmann et al. (7) used MRI to visualize the femoral artery in multiple cross-sectional views; when viewing perpendicular to the direction of flow, these researchers were able to obtain assessments of collateral-dependent flow in a manner similar to the microsphere method described above. Contrast-enhanced magnetic resonance, with first-pass gadolinium-based contrast agent, can be used to visualize arteries and to obtain regional and muscle-specific perfusion measures.

**CONSIDERATIONS FOR PRECLINICAL TO CLINICAL APPLICATION IN TRIALS OF ANGIOGENESIS**

Preclinical hindlimb ischemia models have been used extensively to investigate potential therapeutic angiogenic agents. Angiogenesis is the growth and proliferation of blood vessels from existing vascular structures, whereas therapeutic angiogenesis seeks to employ this phenomenon as a method to increase perfusion to ischemic tissue (29). Over the past two decades, a large number of cytokine growth factors that stimulate endothelial cell proliferation in vitro and, either directly or indirectly, induce angiogenesis ex vivo or modulate angiogenesis in vivo have been identified (17). The vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) families have been the most frequently studied, and these factors may be the most potent. VEGF itself is not one gene but consists of a family of five different genes (VEGF-A, -B, -C, -D, and -E); in addition, in the most commonly referred-to form, the VEGF-A gene, four or more isoforms can be produced by alternate splicing. These VEGF isoforms differ in their extracellular matrix binding properties, with the 121 isoform being the weakest heparin binding and the 206 isoform being the strongest. VEGF-A also exists in vivo as dimers, either homodimers (i.e., 121:121) or heterodimers (i.e., 121:165), and each of the single isoform polypeptide chains can bind to other VEGF genes. The VEGF ligands bind to one of three known VEGF receptors (58). The complexity of the VEGF system is minimal compared with the complexity of the FGF family. The FGF family contains at least 20 members, with the most significant factors being acidic FGF (FGF-1) and basic FGF (FGF-2). Other important angiogenic growth factors include platelet-derived growth factor, placental growth factor, hepatocyte growth factor, and the angiopoietins. The majority of these angiogenic growth factors have been tested in various preclinical hindlimb ischemia models, and advantageous outcomes have been reported (1, 3). Human studies have since begun, and placebo-controlled trials using FGF-2 and VEGF121 have been completed.

In our consideration of human trials of therapeutic angiogenesis, only placebo-controlled trials will be reviewed. The first human studies with FGF were performed at the National Institutes of Health, where Lazerous et al. (33) administered intravascular FGF-2 to 11 patients with intermittent claudication in a phase I, double-blind, placebo-controlled, dose escalation trial (33). FGF-2 was shown to be well tolerated, and a small study showed plethysmographic evidence of improved lower extremity blood flow in the FGF-2-treated group. The Therapeutic Angiogenesis With Recombinant Fibroblast Growth Factor-2 for Intermittent Claudication (TRAFFIC) trial was a phase II, double-blind, placebo-controlled study that compared intra-arterial infusions of recombinant FGF-2 with
placebo (35). In total, 190 patients with moderate-severe intermittent claudication and infrainguinal obstructive atherosclerosis were randomly assigned to receive bilateral intraarterial infusions of placebo (one dose) or FGF-2 (2 doses) at 30-day intervals. The study showed a significant increase in the primary end point (change in peak walking time at 90 days), and this change was most evident in the single FGF-2 bolus group (1.77 min for single bolus vs. 0.6 min for placebo, \( P = 0.026 \)). The second FGF-2 bolus did not provide any additional benefit over the single bolus. TRAFFIC remains the only phase II trial of therapeutic angiogenesis to show benefit in its primary efficacy measure.

The Regional Angiogenesis With Vascular Endothelial Growth Factor in Peripheral Arterial Disease (RAVE) trial was a phase II, double-blind, placebo-controlled trial comparing intramuscular injections of VEGF121 adenovirus (AdVEGF121) with placebo (47). In total, 105 patients with chronic, stable, predominately unilateral intermittent claudication and resting ankle-brachial systolic blood pressure index of <0.8 were randomly assigned to receive intramuscular injections of low-dose AdVEGF121, high-dose AdVEGF121, or placebo. All groups, including the placebo group, demonstrated similar increases in the primary end point (change in peak walking time at 12 wk). There was no difference in any secondary measures of efficacy. Edema was reported more often in the groups that received AdVEGF121 compared with placebo; in addition, the fraction of patients with edema was higher in those that received the highest dose of AdVEGF121.

LIMITATIONS OF USING ANIMAL STUDIES TO PREDICT OUTCOMES IN HUMANS

Both basic FGF (bFGF) (1, 61) and VEGF121 (19, 37) had positive efficacy outcomes in preclinical studies, and VEGF administration has been linked to edema formation. The outcomes in human trials, certainly for VEGF121 and to a lesser extent for bFGF, have been much less impressive, highlighting the limitations inherent in animal studies. Surgically induced hindlimb ischemia is not equivalent to human PAOD. Ligation and excision in animals are typically sudden, involve only one arterial segment, and occur in the presence of healthy surrounding vasculature. Human PAOD is much more complex. Vascular obstructions, except in rare cases, develop gradually, frequently involve multiple vascular sites, and are more prevalent at points of turbulent flow and high transmural pressure. Inflow vessels (aortic and iliac arteries), conduit vessels (femoropopliteal arteries), and run-off vessels (tibial and pedal arteries) are all often involved. Additionally, the surrounding vasculature, even in the absence of obvious vascular obstructions, often contains atherosclerotic disease and may not be capable of responding to cytokine growth factors in the same way that “healthy” tissue does (5a). Similarly, other comorbid illnesses frequently associated with human PAOD (diabetes, hypertension, hypercholesterolemia) are usually absent in animal models, also perhaps altering the angiogenic response. Clinical presentations appear to differ as well. Human clinical syndromes often do not correlate with the location of the obstruction or the extent of atherosclerotic disease, whereas the degree of vascular injury often correlates with outcome in animal models. Clearly, the complexities in the pathophysiology and clinical presentation of PAOD make the development of model systems that represent a broad cross section of patients with the disease a challenge. The infrequent use of techniques to assess collateral blood flow capacity is another limitation of preclinical models. This is perhaps the best way to assess changes in perfusion to the ischemic limb but has seldom been employed because of the complexities involved. Instead, total perfusion to the ischemic limb is often used, which may lead to false estimates of efficacy.

FUTURE PRECLINICAL AND CLINICAL TRIALS

Despite their limitations, preclinical studies have the potential to provide a great deal of data to aid clinical development and may provide information that is useful beyond the simple answer of “did the agent show efficacy or no efficacy.” For example, groups have been able to optimize dosing regimens (e.g., site, nature of delivery, duration), which could be critical to the development of a tissue environment more conducive to arteriogenesis (2, 34). Preclinical models also offer the unique opportunity to explore the feasibility of combination therapy, which is often difficult to employ in clinical trials, such as the effects of administering multiple angiogenic growth factors or combining angiogenic therapy with exercise (38, 64). Furthermore, the limitations of preclinical models, once identified, can sometimes be overcome. For example, transgenic animals, such as apoE−/− and diabetic mice, may have endothelial-mediated responses to ischemia that more closely resemble human PAOD and therefore might be better suited to predict efficacy.

Selecting the appropriate preclinical model and choosing the proper end point to assess efficacy are critical for designing future preclinical trials. Importantly, some models are more useful for studying intermittent claudication, whereas others are more suited for studying critical limb ischemia. Likewise, some methods for assessing changes in perfusion are more accurate than others, and techniques that assess the capacity for collateral-dependent flow (such as postexercise microsphere administration or postexercise MRI) are favorable. Therapeutic agents that show efficacy in this “new generation” of preclinical trials will merit further assessment in clinical trials. Focusing on changes in perfusion at early stages, as opposed to clinical surrogates such as peak walking time and ankle-brachial index, would improve human clinical trials. Ultimately, a successful human trial would also be able to demonstrate an increase in collateral-dependent blood flow. Although this measurement is even more challenging in humans, it has been achieved in the past with plethysmography (33, 54). MRI may eventually provide better and more reproducible measures of perfusion.

In summary, PAOD covers a spectrum of disorders that are the result of impaired perfusion, usually to the lower extremity. PAOD is a disease in need of new therapeutic treatment modalities. Therapeutic angiogenesis is an investigational approach designed to improve tissue perfusion, and presently the field of therapeutic angiogenesis remains in its infancy, with successes being reported in some instances and disappointments in others. If the field is to advance, scientifically rigorous translational studies carried out in appropriate animal models will be necessary to develop and assess potential proangiogenic therapies before their use in humans. Consequently, we hope the information contained in this study will help investigators...
make informed decisions regarding the planning of future studies such that these therapies may one day find their way into everyday clinical practice.

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