Angiogenic therapy, which involves the use of an exogenous stimulus to promote blood vessel growth, is an attractive approach for the treatment of ischemic diseases. It has been shown in animal models that the stimulation of blood vessel growth leads to the growth of the whole vascular tree, improvement of ischemic tissue perfusion and improved muscle aerobic energy metabolism. However, very few positive results have been gained from Phase 2 and 3 clinical angiogenesis trials. Many reasons have been given for the failures of clinical trials, including poor transgene expression (in gene-therapy trials) and instability of the vessels induced by therapy. In this Review, we discuss the selection of preclinical models as one of the main reasons why clinical translation has been unsuccessful thus far. This issue has received little attention, but could have had dramatic implications on the expectations of clinical trials. We highlight crucial differences between human patients and animal models with regards to blood flow and pressure, as well as issues concerning the chronic nature of ischemic diseases in humans. We use these as examples to demonstrate why the results from preclinical trials might have overestimated the efficacy of angiogenic therapies developed to date. We also suggest ways in which currently available animal models of ischemic disease could be improved to better mimic human disease conditions, and offer advice on how to work with existing models to avoid overestimating the efficacy of new angiogenic therapies.

**Introduction**

**Ischemic diseases: the clinical picture**

Ischemic diseases make up a group of cardiovascular diseases that result from inadequate oxygenation of tissues such as the heart (causing coronary artery disease), brain (cerebrovascular disease) and peripheral muscles (peripheral arterial disease). The principal pathophysiological process causing ischemic diseases is atherosclerosis. Atherosclerosis is a progressive disease that usually affects large arteries, in which the accumulation of lipids, inflammatory cells and fibrous material in the inner arterial wall culminates in the formation of stenotic and occlusive lesions (Lusis, 2000). Humans with significant stenosis of the arterial lumen experience reversible ischemic pain during exercise, which manifests as stable angina pectoris or intermittent claudication. Progression of the stenosis is associated with ischemic pain at rest, which manifests as unstable angina pectoris or critical chronic limb ischemia. Acute and life-threatening cardiovascular events, such as myocardial infarction and stroke, can occur upon rupture of an atherosclerotic lesion and subsequent atherothrombotic events.

**Conventional treatment strategies**

Treatment of ischemic diseases is strongly based on prevention of disease progression. Primary prevention involves addressing lifestyle-related issues such as smoking, a sedentary lifestyle, poor diet and being overweight. Primary and secondary prevention involves pharmacological management of cardiovascular risk factors such as hyperglycemia, hyperlipidemia and hypertension (Norgren et al., 2007). Although statins have been reported to decrease atherosclerotic plaque size and improve the function of the vascular endothelium (Davignon, 2004; Ylä-Herttuala et al., 2011), no pharmacological treatment exists to treat the ischemic tissue. In patients with intermittent ischemic symptoms, anticoagulants, vasodilators and exercise training can be used to relieve symptoms. Revascularization procedures, such as percutaneous transluminal angioplasty, intravascular catheter-mediated thrombolysis, thrombendarterectomy or bypass surgery are performed in patients with critical symptoms to improve blood circulation (Gibbons et al., 2003; Antman et al., 2004; Norgren et al., 2007). However, many patients cannot be treated with conventional revascularization strategies because of a poor overall health status or underlying comorbidities. Moreover, a substantial portion of patients undergoing revascularization procedures does not benefit from the treatments or experiences restenosis, resulting in poor prognosis and diminished quality of life. These challenges have encouraged the search for novel therapeutic alternatives to treat ischemic diseases.
Concept of angiogenic therapy
The essential role of angiogenesis in the growth and recovery of tissues gave birth to the concept of angiogenic therapy – i.e. promoting blood vessel growth as a potential therapeutic approach for the treatment of ischemic diseases (Folkman, 1971). The aim of angiogenic therapy in ischemic diseases is to stimulate blood vessel growth in areas of poor vascularization in an attempt to increase blood supply, and to support tissue function and recovery. Blood vessel growth can be induced by three means: angiogenesis, arteriogenesis and vasculogenesis (Fig. 1). Angiogenesis is defined as the process of capillary vessel growth, and can occur by: (1) proliferation of pre-existing vascular endothelial cells into new capillary sprouts (sprouting angiogenesis); (2) enlargement of pre-existing capillaries; or (3) by intussusception or bridging of pre-existing vessels into smaller daughter vessels (splitting angiogenesis) (Fig. 1) (Risau, 1997; Rissanen et al., 2005; Makanya et al., 2009; Carmeliet and Jain, 2011). Arteriogenesis is defined as the process of growth of collateral arteries, whereby pre-existing arterioles enlarge and remodel into large vessels that bypass the arterial occlusion (Simons, 2005; Schaper, 2009). Arteriogenesis is initiated by blood-pressure- and shear-stress-mediated mechanisms, whereas angiogenesis is triggered by hypoxia, resulting in the production of vascular growth factors in the ischemic tissue. In ischemic diseases, both angiogenesis and arteriogenesis take place endogenously. Similarly, during angiogenic therapies, stimulation of both angiogenesis and arteriogenesis might be beneficial (Rissanen et al., 2005). Whereas angiogenesis and arteriogenesis are well known to take place in adult tissues, by definition vasculogenesis (i.e. the de novo formation of the vascular plexus from endothelial precursor cells) is usually considered to take place only during early development. However, the incorporation of vascular stem or progenitor cells into vessel structures (i.e. postnatal vasculogenesis) has gained attention in some studies of angiogenic cell therapies. Thus, the relevance of vasculogenesis in adult tissues remains unclear (Asahara et al., 1997; Rafii and Lyden, 2003; Balsam et al., 2004; Ziegelhoeffer et al., 2004; Fang and Salven, 2011).

Methods for inducing blood vessel growth
Stimulation of vascular growth can be achieved by exogenous administration of pro-angiogenic agents such as members of the vascular endothelial growth factor family (notably VEGF-A_{165} and the mature form of VEGF-D) (Ferrara, 2004; Ylä-Herttuala et al., 2007), members of the fibroblast growth factor family (notably FGF-2 and FGF-4) (Ylä-Herttuala and Alitalo, 2003; Murakami and Simons, 2008), members of the platelet-derived growth factor family (notably PDGF-BB) (Fredriksson et al., 2004), angiopoietins (notably ANG-1) (Davis et al., 1996), hepatocyte growth factor (HGF) (Aoki et al., 2000; Rissanen and Ylä-Herttuala, 2007), insulin-like growth factors (IGFs) (Delafontaine et al., 2004), members of the hypoxia-inducible factor family (notably HIF-1α) (Pajusola et al., 2005; Patel et al., 2005) and nitric oxide synthase (NOS) (Brevetti et al., 2003b; Cooney et al., 2006), among others (Fig. 1).

To deliver the angiogenic agents, three main approaches have been tested in preclinical settings: protein, gene and cell therapies. In protein therapy, recombinant proteins are used directly to induce therapeutic effects (Ruel and Sellke, 2003). However, a major

**Fig. 1. Post-ischemic vascular repair mechanisms and the growth factors involved.** The top half of the diagram shows angiogenic vascular repair processes that can take place after an ischemic insult (arterial occlusion), which is displayed in the bottom half of the diagram. Upon an arterial occlusion, arteriogenesis (i.e. collateral growth) is induced by the redirection of blood flow, causing increased shear stress and subsequent cytokine production in the vascular endothelium. Factors such as VEGF and NO are responsible for the enlargement and growth of the collaterals, whereas factors such as PDGF and FGF mediate the stabilization of the vessels by recruiting pericytes. The hypoxic tissue distal to the occlusion (bottom half of the diagram) expresses transcription factors such as HIF, which enables the production of angiogenic proteins such as VEGF and ANG, which are involved in the modulation of the distal vasculature to make connections to the opening collaterals (angiogenesis). Angiogenesis can include sprouting, intussusception or capillary enlargement (see text for details). Postnatal vasculogenesis might also contribute to post-ischemic vascular repair via the incorporation of circulating endothelial progenitor cells into the forming vascular structures.
limitation of this approach is the very short half-life of exogenous proteins in target tissues, resulting in only transient therapeutic effects (Annex and Simons, 2005). In contrast, gene therapy uses non-viral or viral vectors to carry a gene construct encoding a therapeutic protein into target tissues, where it is abundantly expressed by the target cells (Ylä-Herttuala and Alitalo, 2003). The idea of cell therapy in its present form is that transfected cells function as protein factories with the capability of producing multiple endogenous growth factors, meaning that the transplanted cells will induce vascular growth mainly in a paracrine manner, rather than directly replacing damaged cells (Menasché, 2010). However, at least at the preclinical level, the efficacy of cell therapies has not yet reached that of gene therapy to promote vascular growth.

**Therapeutic potential of angiogenic factors**

The potential of angiogenic therapies to revascularize tissues has been extensively studied in animal models of myocardial and peripheral ischemia (summarized in Table 1). Using these models, preclinical studies using several individual angiogenic factors, including VEGFs, FGFs, HIF-1α and HGF, have showed significant improvements in clinically relevant end points such as increased regional perfusion, improved exercise tolerance and tissue energy metabolism, improved myocardial function, and protection against ischemic damage. Although most of these factors have also been tested in clinical trials, the promising preclinical potential has thus far not been translated into clinical success.

**Testing candidate therapies in models of myocardial ischemia**

Animal models have been used to demonstrate the potential of angiogenic therapies for myocardial ischemia. In experimental settings, myocardial ischemia is most commonly induced by ligation of the anterior descending branch of the left coronary artery. The occlusion can be achieved by introducing a catheter-mediated embolization coil or a surgical ligature to block the coronary flow, or by surgically placing ameroid constrictors around the coronary vessels to resemble more slowly progressing occlusion (Watanabe et al., 1998; Hughes et al., 2003; Madeddu et al., 2006; Abarbanell et al., 2010; Gao et al., 2010) (Table 1). In a porcine model of myocardial ischemia, intramyocardial injection with a plasmid encoding VEGF caused neoangiogenesis followed by improved regional myocardial perfusion and function (Choi et al., 2006). In another study, plasmid- or adenovirus-mediated VEGF-A gene transfer induced significant post-ischemia neovascularization and improved left ventricular function in a rat model of myocardial infarction (Hao et al., 2007). More recently, adenovirus-mediated overexpression of VEGF-B in a porcine model of myocardial ischemia was shown to induce myocardium-specific vascular growth, and to improve myocardial perfusion and ejection fraction (Lähteenvuo et al., 2009). Adenoviral gene transfer of FGF-2 was shown to enhance arteriogenesis and echocardiographic parameters of left ventricular function in a porcine model of chronic ischemia (Horvath et al., 2002). In addition, intracoronary injection of adenoviral FGF-4 resulted in improved myocardial perfusion and increased regional function in a porcine model of stress-induced myocardial infarction (Gao et al., 2004).

**Testing candidate therapies in models of peripheral ischemia**

Angiogenic factors have also shown promise in experimental models of peripheral ischemia. Experimental limb ischemia is typically induced by ligation and/or excision of the common

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**Table 1. Most commonly used in vivo models of myocardial and hindlimb ischemia**

| Species | Model | References |
|---------|-------|------------|
| **Myocardial ischemia** | | |
| Mouse | LAD ligation | Salto-Tellez et al., 2004; Gao et al., 2010 |
| Rat | LAD ligation | Selye et al., 1960; Pfeffer et al., 1979 |
| Rabbit | LAD ligation | Morales et al., 2002 |
| | LAD ligation | Katsanos et al., 2012 |
| | LCX ligation | Edwards et al., 2002 |
| Swine | LAD ligation | Iwanaga et al., 2004 |
| | LCX ligation | Watanabe et al., 1998; Lähteenvuo et al., 2009 |
| **Hindlimb ischemia** | | |
| Mouse | CFA (with or without vein) ligation and/or excision | Masaki et al., 2002; van Weel et al., 2004; Limbourg et al., 2009 |
| | SFA (with or without vein) ligation and/or excision | Couffinhal et al., 1998; Masaki et al., 2002; Limbourg et al., 2009 |
| | CFA and SFA ameroid constriction | Yang et al., 2008 |
| Rat | CIA ligation | Paek et al., 2002; Tang et al., 2005 |
| | SFA ligation and/or excision | Yang et al., 1995; Takeshita et al., 1997; Herzog et al., 2002 |
| | CIA and CFA ameroid constriction | Tang et al., 2005 |
| Rabbit | CFA excision | Pu et al., 1994; Takeshita et al., 1994 |
| | SFA excision | Rissanen et al., 2003a |
| | PFA ligation | Rissanen et al., 2005 |
| | CFA ameroid constriction | Baffour et al., 2000 |

CIA, common iliac artery; CFA, common femoral artery; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; PFA, profound femoral artery; SFA, superficial femoral artery.
Challenges associated with ischemic animal models

Femoral artery or common iliac artery, with or without accompanying ligation of potential collateral sources (Pu et al., 1994; Couffinhal et al., 1998; Waters et al., 2004; Madeddu et al., 2006; Limbourg et al., 2009) (Table 1). In ischemic rabbit hind limbs, plasmid or adenoviral gene transfer of VEGF-A demonstrated neoangiogenesis, resulting in increased skeletal muscle perfusion (Takeshita et al., 1996; Korpiisto et al., 2008a). Additionally, adenoviral VEGF-A was shown to induce the growth of the whole vascular tree, including the growth of collateral arteries (Rissanen et al., 2005). Furthermore, adenoviral gene transfer of either placental growth factor (PIGF; a member of the VEGF family) or VEGF-A improved local perfusion, aerobic energy metabolism and exercise tolerance in ischemic rabbit hind limbs (Gowdak et al., 2000; Korpiisto et al., 2008b). Repeated (but not single) intramuscular injections of plasmid encoding VEGF-A were found to increase microvasculature, resulting in effective protection against ischemic muscle damage (Olea et al., 2009). Furthermore, adenovirus-mediated overexpression of FGF-4 was reported to induce therapeutic angiogenesis and arteriogenesis, increasing local muscle perfusion (Rissanen et al., 2003a). Finally, proteins such as the transcription factor HIF-1α and HGF, which can simultaneously stimulate the expression of multiple growth factors involved in post-ischemic vascular response and tissue recovery, have been reported to achieve more physiological angiogenic responses than individual growth factors alone (Taniyama et al., 2001; Patel et al., 2005; Pyun et al., 2010; Li et al., 2011). Notably, tissue edema has been acknowledged as a side effect of angiogenic treatments (Weis and Cheresh, 2005); growth factors such as the mature form of VEGF-D, capable of stimulating the growth of lymphatic vessels, might help to relieve this side effect (Rissanen et al., 2003b).

Results from clinical trials

Despite these promising results from preclinical studies, clinical trials testing angiogenic therapies have thus far failed to demonstrate clear-cut evidence of therapeutic efficacy: improvements in primary outcome measures such as improved exercise performance, decreased rates of amputation, or decreased mortality have not been observed (Tongers et al., 2008; Belch et al., 2011; Hedman et al., 2011; Mitsos et al., 2012). In addition, evidence of biological activity of the drugs (such as increased angiogenic factor levels in plasma or improved regional perfusion at injection sites) has rarely been reported (Baumgartner et al., 1998; Hedman et al., 2003; Gupta et al., 2009; Muona et al., 2012). However, an acceptable safety profile and improvement in secondary or supportive end points such as symptomatic improvement has been achieved (Baumgartner et al., 1998; Hedman et al., 2003; Gupta et al., 2009; Muona et al., 2012).

Several explanations have been provided to account for the lack of efficacy in clinical trials. Concerning the angiogenic drug, problems including inadequate therapeutic doses, insufficient duration of exposure, compromised delivery and poor vector transduction efficiency have been discussed (Rissanen and Ylä-Herttuala, 2007; Gupta et al., 2009; Hedman et al., 2011; Zachary and Morgan, 2011; Mitsos et al., 2012). In addition, potential patient-related issues proposed to underlie inefficacy include defects in the response to angiogenic stimuli due to existing comorbidities, the use of other medications, circulating angiogenic inhibitors, lack of target receptor expression in target tissues, lack of viable muscle tissue required for a therapeutic response, and growth factor resistance in a chronically ischemic environment (Simons and Ware, 2003; Ylä-Herttuala and Altitalo, 2003; Rissanen and Ylä-Herttuala, 2007; Gupta et al., 2009). However, one of the major shortcomings in this area – and one that has not yet been rigorously acknowledged – is the lack of animal models that accurately recapitulate key features of the human disease. This deficiency has probably contributed to the inadequate and over-optimistic conclusions drawn from preclinical data thus far, and to the poor outcome of drug development. Improved preclinical models, and improved preclinical study design, will be essential prerequisites to move forward in developing treatments for ischemic diseases. We address these challenges in the remainder of this article.

Challenges in modeling ischemic diseases

The ideal animal model of cardiovascular ischemic disease does not currently exist. At one extreme, it can be claimed that a truly accurate model is not possible, given that the clinical disease is so heterogeneous and each patient is unique. In addition to arterial occlusions, patients with ischemic diseases have various comorbidities (Table 2) that can affect the development and severity of atherosclerotic plaques, and the responsiveness of muscle tissue to the subsequent ischemic injury. Thus far, none of the existing animal models of ischemic disease can fully reproduce the comorbidities of chronic ischemia that occur in humans, or are too complicated to be used in preclinical development. Importantly, using simple models to test a new therapy can lead to overly optimistic results of that therapy’s potential to treat a complex chronic condition. In the following sections, we discuss the main problems with currently used ischemia models and suggest how models could be improved to better resemble human ischemic diseases.

Acute ligation models

The occlusion pathophysiology and tissue recovery that occur after an acute arterial ligation are very different in animal models than in human chronic ischemic diseases. An experimental acute vessel occlusion (Fig. 2A) results in an immediate vascular response that generates a powerful, endogenous stimulus for collateral growth (i.e. arteriogenesis) in otherwise healthy animals. This type of acute ischemia induced in young, healthy animals mainly reflects the situation in a limited subgroup of patients (such as young patients with traumatic injuries or arterial emboli), who require immediate medical interventions and are not typically enrolled in angiogenic therapy clinical trials. Under physiological conditions, an acute arterial occlusion causes pre-existing collaterals to function as possible anastomoses to bypass the occlusion (both in animal models and in humans). The occlusion generates a pressure gradient between the proximal and distal ends of the occluded vessel, resulting in a redirection of blood flow towards the collaterals (Fig. 2A). Subsequently, the increased flow generates a dramatic rise in collateral artery wall shear stress, which again alters endothelial gene expression (Pipp et al., 2004). As a primary response, nitric oxide (NO)-induced vessel vasodilatation and flow-mediated arterial remodeling take place, decreasing vascular resistance by increasing the collateral vessel diameter (Heil et al.,
Indeed, multiple lines of evidence in the literature illustrate the remarkably fast recovery of perfusion after sudden occlusion of a major artery in animal models of experimental ischemia, suggesting the existence of a strong natural compensatory collateral response (Hershey et al., 2001; Scholz et al., 2002; Buschmann et al., 2003; Tang et al., 2005; Yang et al., 2008; Ziegler et al., 2010).

Table 2. Animal models of ischemic diseases

| Co-morbidities | Method of induction | Species | References |
|---------------|---------------------|---------|------------|
| Hyperlipidemia resulting in atherosclerosis | Atherogenic diet (WD, HFD, HCD, Paigen diet) | M, Rb, Sw, NHP | Daugherty, 2002; Getz and Reardon, 2012 |
| | Genetic knockout (LDLR, ApoE, ApoBEC1) and/or transgenesis (ApoB<sup>100/100</sup>, ApoE2, ApoE3, ApoC3) | M, Rb | Getz and Reardon, 2012 |
| | Spontaneous mutation enrichment by selective breeding (LDLR, ApoB<sup>100/100</sup>) | Rb, Sw | Zaragoza et al., 2011 |
| Plaque rupture | Spontaneous (advanced age) on a genetic background | M, Rb, Sw | |
| | Vessel injury (mechanical injury, balloon injury, photochemical injury) with/without HCD diet | M, Rb | |
| Diabetes | Spontaneous mutation enrichment with/without obesity phenotype | M, Rb, Sw, NHP | Rees and Alcolado, 2005; Chatzigeorgiou et al., 2009 |
| | Diet/nutrition (diabetogenic diet, atherogenic diet) | M, R, Sw | Chatzigeorgiou et al., 2009 |
| | Chemical (alloxan, streptozotocin, goldthioglucose) with/without obesity phenotype | M, Rb, Sw | Rees and Alcolado, 2005; Chatzigeorgiou et al., 2009 |
| | Surgical (partial pancreatectomy) | R, Rb, Sw, D, NHP | Rees and Alcolado, 2005; Chatzigeorgiou et al., 2009 |
| | Genetic knockout/transgenesis (IRS-1,2,3; GLUT-2,4; PPARs, GK, β3R, UCP-1, hIAPP, Ins<sup>2<sub>24ig</sub></sup>) | Mainly M | Plum et al., 2005; Rees and Alcolado, 2005; Chatzigeorgiou et al., 2009 |
| Diabetes-accelerated atherosclerosis | Chemically induced diabetes on atherosclerotic background | M, Rb, Sw | Gerrity et al., 2001; Goldberg and Dansky, 2006; Wu and Huan, 2007 |
| | Diet-induced diabetes on atherosclerotic background | M, Sw | Xi et al., 2004; Goldberg and Dansky, 2006; Wu and Huan, 2007 |
| | Genetically induced diabetes on atherosclerotic background | M | Goldberg and Dansky, 2006; Wu and Huan, 2007 |
| Hypertension | Spontaneous mutation enrichment with/without diet (high-salt diet) | M, R | Pinto et al., 1998; Lerman et al., 2005 |
| | Genetic knockout, transgenesis (mRen2) | M, R | |
| | Surgical (renal artery constriction) | M, R | |
| | Chemical (angiotensin II infusion, DOCA) with/without surgical renal mass reduction | M, R | |
| Oxidative stress | Genetic knockout/transgenesis (SOD-1,2,3; GPx1, OKD48) | M | Melov, 2002; Oikawa et al., 2012 |
| | Chemicals generating free radicals | M | Melov, 2002 |
| Heart failure | Myocardial ischemia (coronary artery occlusion) | M, R, Sw, D, Sh | Halapas et al., 2008; Dixon and Spinale, 2009; Patten and Hall-Porter, 2009 |
| | Pressure overload (aortic constriction/banding, valvular stenosis, renal artery constriction, angiotensin II infusion) | M, R, Sw, D, Sh | Halapas et al., 2008; Dixon and Spinale, 2009 |
| | Volume overload (mitral regurgitation) | R, Rb, D | Halapas et al., 2008; Dixon and Spinale, 2009 |
| | Genetically induced dilated cardiomyopathy (TNF-α overexpression, muscle lim protein knockout) | M | Patten and Hall-Porter, 2009 |
| | Tachycardia (ventricular pacing) | Rb, Sw, D, Sh | Halapas et al., 2008 |

β3R, β3 receptor; D, dog; DOCA, deoxycorticosterone acetate; GK, glucokinase; GLUT, glucose transporter; GPx, glutathione peroxidase; HCD, high-cholesterol diet; HFD, high-fat diet; hIAPP, human islet amyloid polypeptide; Ins, insulin (gene); IRS, insulin receptor substrate; LDLR, low-density lipoprotein receptor; M, mouse; mRen, mouse renin(gene); NHP, non-human primate; OKD, Keap-1-dependent oxidative stress detector; PPAR, peroxisome proliferator-activated receptor; R, rat; Rb, rabbit; Sh, sheep; SOD, superoxide dismutase; Sw, swine; TNF-α, tumor necrosis factor alpha; UCP, uncoupling protein; WD, western diet.
Challenges associated with ischemic animal models

**A** Acute ligation model

- Arteria iliaca communis
- Hypoxia
- HIF
- VEGF
- Ischemic tissue damage (necrosis)
- Acute inflammation

Angiogenesis

Pressure gradient

Redirection of flow

Collateral growth

Tissue regeneration

**B** Gradual ligation model

- Arteria iliaca interna
- A. femoralis communis
- A. femoralis superficialis
- A. femoralis profunda

Flow gradually directing towards collaterals

Decreasing pressure gradient

Increasing collateral growth

No need for tissue regeneration

**C** Ischemic diseases

- Atherosclerotic plaque

Insufficient collateral growth

No tissue regeneration

Chronic hypoxia

HIF

Replacement of muscle by fibrous tissue

Vegetation

Chronic inflammation

Endothelial dysfunction

Angiogenesis

Fig. 2. Differences in collateral growth and tissue recovery in animal models versus humans with arterial occlusive disease. (A) After an acute arterial ligation (shown here in a mouse), there is a strong pressure gradient between the proximal and distal sides of the occlusion (orange line). This redirects the blood flow into adjacent arterioles and causes a strong shear-stress-mediated opening of collateral channels, which restore blood flow into the hypoxic areas. In the hypoxic tissues, ischemic tissue damage (necrosis) occur if the blood flow is not restored within the first hours after the occlusion. Necrosis induces acute inflammation, and recruited inflammatory cells produce angiogenic cytokines such as VEGF. The hypoxia itself activates factors such as HIF that stimulate the production of VEGF among other factors, and angiogenesis. Distal angiogenesis, along with the growth of collaterals, contributes to the tissue recovery by the formation of connections between the collaterals and the distal capillaries. (B) Upon a gradual occlusion, for example by using an ameroid constrictor, the slow development of the occlusion produces an accommodation window for the collaterals to open. The pressure gradient is never as high as after acute ligation because the blood flow is gradually redirected to the adjacent arterioles, which have time to open to balance the pressure change caused by the gradual occlusion with the constrictor. There is no acute tissue damage, because the collaterals start to compensate for the lack of blood flow before it completely stops. Therefore, there is also less inflammation. The amount of hypoxia and tissue damage varies according to the availability of collaterals and causes comparable distal angiogenic responses. If collaterals are effectively recruited, there is very little tissue hypoxia and therefore little endogenous growth factor upregulation, but also no tissue damage requiring recovery. (C) In human ischemic diseases, atherosclerotic plaques develop all around the arterial tree, causing blood pressure to decrease gradually following each plaque. Thus, a single occlusion in the periphery causes a very small pressure gradient between the proximal and distal sides of the occlusion, as the blood pressure is low already above the occlusion. Accordingly, there is very little blood flow to redirect towards the collaterals and minimal shear stress to open the collaterals, which results in insufficient collateralization. Distal tissue suffers from gradually developing chronic hypoxia that manifests by the replacement of muscle fibers with less-energy-consuming fibrotic tissue and chronic inflammation. The tissue might be less prone to respond to angiogenic stimuli owing to endothelial dysfunction and other factors. The insufficient collaterals and angiogenic signaling limit tissue recovery, eventually leading to total necrosis and loss of tissue function if not effectively treated. Artery names are shown in panel A.
The strong collateralization in animal models provides inflow to ischemic muscles in a way that does not occur in human patients; this could have a major impact on the results of angiogenic studies. Furthermore, an acute arterial occlusion leading to distal tissue hypoxia causes the activation of an acute inflammatory-angiogenic-myogenic response against ischemic tissue injury (Fig. 2A) (Scholz et al., 2003; Silvestre et al., 2008). The outcome of this response involves: the recruitment and activation of inflammatory cells; VEGF-induced activation, proliferation and migration of endothelial cells; and activation of satellite cells in the periphery of the ischemia-injured myofibers. These events all support the formation of new blood vessels – i.e. angiogenesis and regeneration of the ischemic muscle. Thus, endogenous arteriogenesis and angiogenesis have a profound contribution to the recovery of blood flow and muscle function in animal models with acutely induced ischemic injuries. In contrast, these endogenous recovery mechanisms do not function sufficiently in patients with chronic ischemic injuries. In contrast, these endogenous recovery mechanisms do not function sufficiently in patients with chronic ischemic symptoms and co-morbidities (Fig. 2C). Thus, acute ligation models should not be used to model chronic ischemia. If they must be used, the results should be interpreted with extreme caution because both vascular and muscular responses to the ischemic insult are very different in these models compared with humans with ischemic diseases. These differences could dramatically affect the effects of angiogenic therapies.

Gradually developing occlusions
Gradually tightening constrictors such as ameroids are often used to model chronic ischemia. Such systems are thought to better recapitulate human ischemic disease pathophysiology and atherosclerotic plaque progression than acute ligations (Tio et al., 1999; Baffour et al., 2000; Tang et al., 2005; Yang et al., 2008). However, a gradually developing occlusion does not cause chronic ischemia in otherwise healthy animals. Instead, the gradual development of the occlusion results in an accommodation window during which the muscle has time to adapt to hypoxia (i.e. to grow collaterals (Fig. 2B). Hence, a smaller amount of necrosis, acute inflammatory responses and endogenous angiogenic stimuli take place than with acute arterial occlusion (Fig. 2B). Thus, ischemia (defined by ischemic tissue injury) might not in fact take place. Instead, gradually developing occlusions might just involve transient tissue hypoxia (Fig. 2B) and thus model a milder form of ischemia. This might be more suitable than an acute ischemia model in certain settings, e.g. for studying hypoxia-sensitive tissues such as the myocardium or brain. In humans with chronic ischemia, the lack of vast necrosis, acute inflammation and endogenous angiogenic stimuli are similar to what is observed in gradual occlusion models. However, the crucial difference between the experimental models and patients is that the patients, owing to their co-morbidities, do not have sufficient growth of collaterals to counteract the evolution of the plaques, despite this accommodation window (Fig. 2C). Thus, the patients have chronic tissue damage whereby necrotic tissue is replaced by fibrotic tissue, and acute inflammation is replaced by chronic inflammation. Furthermore, these patients not only have decreased endogenous angiogenic stimuli, but also diminished angiogenic signaling because there is very little viable tissue left to mediate the effects (Fig. 2C).

Another reason for the insufficient collateralization in patients with ischemic diseases might be stenotic lesions throughout the major feeding arteries: in the legs, the carotid arteries, the aorta and the coronary vessels, among others (Fig. 2C). This results in a gradually decreasing pressure gradient along the arterial tree away from the heart [demonstrated as a decreased ankle-brachial blood pressure index (ABI) in peripheral arterial disease], which might hinder the opening of collateral vessels owing to the lack of shear stress and decreased flow when an arterial occlusion takes place (Fig. 2C). Thus, to better mimic the human ischemic disease, it might be essential to control collateralization in animal models, instead of controlling the speed of occlusion.

Atherosclerotic models
A plain ischemia model is never an ischemic disease model. Unfortunately, most animals do not naturally develop atherosclerotic plaques nor an atherosclerotic ischemic disease. There are several genetic and/or diet-induced models of atherosclerosis and related co-morbidities [involving rodents, rabbits, pigs and even non-human primates (Table 2)], but they are often not suitable for modeling ischemia. For example, the Watanabe heritable hyperlipidemic (WHHL) rabbit, which is genetically prone to hyperlipidemia, develops sporadic, spontaneous atherosclerotic plaques; however, plaque development is slow, and the sporadic nature of the plaques makes it difficult to use this animal as an ischemia model (Watanabe, 1980; Shiomi and Ito, 2009). IGF-II/LDLR−/−ApoB100 mice are hyperlipidemic with type 2 diabetes and exhibit early-onset severe atherosclerotic plaques (Heinonen et al., 2007). However, even with 80-100% stenosis in coronary vessels, the cardiac outcome is normal in these mice owing to adaptive remodeling and myocardial hibernation (Heinonen et al., 2011). Thus, it seems that genetic modifications of two important ischemic risk factors (hyperlipidemia and diabetes) is not enough to mimic human ischemic disease because of compensatory mechanisms that occur in young, otherwise healthy animals. Combining more co-morbidities in a single model might reduce the compensatory responses and create a more human-like disease progression. However, even then, the potential of animals to spontaneously recover from ischemia needs to be well validated, while keeping in mind that patients do not recover in the same way.

Models for endothelial dysfunction and oxidative stress
Impairment in the functional properties of the endothelium (i.e. endothelial dysfunction) has been proposed as another potential cause for the lack of natural regenerative responses in humans suffering from ischemic disease (Kinnaird et al., 2008; Boodhwani and Sellke, 2009; Sun et al., 2009). Endothelial dysfunction seems to be related especially to oxidative stress and the reduction of endothelial NO production and signaling (McQuaid and Keenan, 1997; Boger, 2004; Sun et al., 2009; Vita and Hamburg, 2010). Endothelial dysfunction is also intensified in the presence of different co-morbidities such as diabetes, hyperlipidemia, chronic inflammation or the lack of the nutrifying blood flow (Brevetti et al., 2003a; Münzel et al., 2008; Vita and Hamburg, 2010). Although endothelial dysfunction might be present in many of the current animal models for ischemic diseases, the specific role of oxidative stress on therapeutic outcomes has rarely been explored owing to the confounding effects of different co-morbidities. For example, hyperglycemia in diabetes causes oxidative stress, but might also
have direct effects on endothelial dysfunction. Specific knockout models for oxidative stress have been developed (Table 2) and might offer a useful tool for the future study of the specific effects of oxidative stress on angiogenic therapies.

**The impact of aging on ischemic disease**

Most animal studies have failed to acknowledge the impact of aging on endogenous repair mechanisms and exogenous therapeutic outcomes. Aging is associated with diminished post-ischemic recovery both in humans and animals (Faber et al., 2011; Lähteenvuo and Rosenzweig, 2012). The muscle composition in elderly patients is affected not only by age-related changes, but also by diet, physical activity and hormonal control. This can result in replacement of the muscle tissue with fat and connective tissue, age-related decline of muscle fibers, reduced muscle performance or progressive deficits in metabolic capacity (Rissanen et al., 2002). Although physical activity has been proven to positively influence almost all aspects of cardiovascular diseases, elderly patients are not always able to exercise owing to disease-related pain, tiredness or weakness. In contrast, animals, by evolution, have been programmed to move to ensure survival. Using aged animals would help to better mimic the human disease. However, in animals, the detection of significant atherosclerotic stenoses, before they become life threatening but still cause ischemic symptoms, will require substantial numbers of animals, careful monitoring and further developments in non-invasive imaging techniques.

**Predicting clinical potential using currently available animal models**

Until the ideal preclinical model is developed, we need to work with our less-than-perfect animal models. Because it will be difficult to create a single animal model that can completely mimic the human ischemic disease, we need to acknowledge that results from a single model will probably not predict how a treatment will work in the clinic. To better predict the potential of new angiogenic therapies in clinical trials, we might thus need to use several different disease models to evaluate the effects of different co-morbidities on the therapeutic efficacy in a step-by-step manner, using clinically relevant end points.

**Experimental setups**

There are some obvious differences between animals and humans with ischemic disease, including physiology, body size and the distance over which collateral arteries need to grow. Currently, these are features that must be controlled by the requirements of clinical trial applications, at least to some extent, by using larger animal models to support data from small rodents before a therapy can be tested in clinics. However, in terms of cardiovascular ischemic diseases, there is no requirement to use disease models – only ischemia models – when applying for permission to test a novel therapeutic agent in clinical trials. In light of the problems with the currently available ischemia models that we have discussed here, there is a clear need for updating the requirements, because the models do not accurately predict how the treatment will work in patients. A step-wise analysis of the effects of different co-morbidities, such as diabetes, hypercholesterolemia, oxidative stress and aging, on the efficacy of angiogenic therapies needs to be examined. Proven efficacy in heterogeneous animal models (instead of only in a very specific homogenous animal population) will increase the probability that a therapy will work in the clinic.

Heterogeneity of preclinical models will probably increase variation in the results, but this should not be seen as a disadvantage. Rather, investigators should study the reasons underlying variation in response to angiogenic therapy (e.g. if the duration or the level of hyperglycemia affects angiogenic responses in diabetic animals). Identifying a limiting factor or co-morbidity that results in a poor treatment response can also help in the selection of patients for clinical trials. Importantly, publishing not only positive findings, but also neutral and especially negative findings, might offer crucial information that could be used to determine why the clinical trials carried out thus far have mostly failed.

**Preclinical end points**

There are currently also major differences between the end points used in preclinical animal studies and clinical trials. In animal models, demonstrating histological evidence of angiogenesis and healing of perfusion after an acute arterial occlusion is common practice. In contrast, in clinical studies the end points in addition to evidence of improved perfusion include healing of chronic ischemic ulcers, decreased amputation rates, decreased mortality, improved quality of life and improved walking distance. Of course, the lack of true chronic ischemic disease models means that end points such as decreased amputation rate or decreased mortality cannot be studied, because results obtained with non-diseased animals would probably lead to overly optimistic predictions. However, the functionality of the newly formed angiogenic vessels and the benefit of the angiogenic therapy on ischemic muscle survival should be regularly studied in animal models. Besides histological evidence of vascular growth, there should be requirements to demonstrate improved blood flow in the immediate area of the angiogenic therapy as well as to demonstrate therapeutic benefits on muscle function or energy metabolism.

**Conclusions**

In this Review, we have discussed the selection of preclinical models as one of the main reasons for the failures in clinical angiogenic trials. It is an obvious issue, that has received little attention. However, this has probably had, and will continue to have, a great impact on expectations from the clinical trials, if not acknowledged properly. As we have discussed, a simple ischemia model is never a disease model. For example, the induction of ischemia using acute ligations or gradually occluding constrictors in animal models might have produced an overly optimistic view of the potential of angiogenic therapies in humans, in whom chronic arterial disease blood-pressure- and shear-stress-related recovery mechanisms are quite different than in the animal models. In addition, disease-related co-morbidities that are present in humans can strongly affect the results of angiogenic therapies in the clinics. These co-morbidities (Table 2) should be more carefully taken into account in preclinical testing of angiogenic therapies so as to better predict the clinical effects of the therapy. To ensure the future success of angiogenic therapies in the clinic, it is essential that more attention is focused on developing animal models that better mimic human ischemic disease.
Challenges associated with ischemic animal models

Reviewer: Disease Models & Mechanisms (DMM)
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