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1. Introduction

As knowledge about the cellular and molecular mechanisms that control vessel growth grew during the last two decades, therapeutic manipulation of angiogenesis was increasingly regarded as one of the most promising areas of translational research. Based on its potential to target key steps in the pathogenesis of disease groups with great impact on public health, therapeutic blockage and stimulation of angiogenesis emerged years ago, as the holy grail of research on new strategies to treat cancer and arterial occlusive diseases respectively. However, the result of these two related development processes were quite different. Anti-angiogenic therapy is today a reality in the treatment of cancer and diseases associated with pathological vessel growth such as in the retina. In contrast, no strategy based on the concept of stimulating angiogenesis (usually referred as “therapeutic angiogenesis”) has so far reached widespread clinical use.

1.1 The clinical problem: critical limb ischemia

Arterial occlusive disease (AOD) is the leading cause of morbidity and mortality in industrialized countries, and represents a problem of growing dimensions for developing countries (Beaglehole et al., 2003). Clinically, AOD includes acute myocardial infarction, stroke and peripheral arterial disease (PAD). PAD is defined as the obstruction of arterial blood flow, in areas other than the brain and heart (Garcia, 2006). When it affects the lower limbs, the clinical picture is silent in its early stages, progressing to intermittent claudication when the obstruction of blood flow reaches 50% of normal. Out of patients with intermittent claudication, between 15 and 20% are estimated to progress to critical limb ischemia (CLI) (Dormandy et al., 1989; Second European Consensus Document, 1991). The term critical limb ischemia (CLI) refers to the final stages of PAD, when chronic lack of blood supply sets off a cascade of pathophysiologic events that lead to rest pain, trophic lesions of the leg or both (Varu et al., 2010). The international consensus definition of CLI is the following: any patient with chronic ischemic rest pain, ulcers, or gangrene attributable to objectively proven arterial occlusive disease (Norgren et al., 2007). CLI patients represent approximately 1% of patients with PAD. The prognosis of these patients can be compared to some other malignancies, showing overall survival rates below 50% in 5 years (DormandyThomas, 1988).
The diagnosis of established CLI is straightforward, and based on the physical examination, the ankle:brachial index (ABI), and on several imaging methods. Despite progresses in medical care, the grim significance of CLI diagnosis does not seem to have changed significantly during the last decade, remaining a predictor of poor survival and poor outcomes (Varu et al., 2010). Observational studies of patients with CLI roughly agree on a 50% amputation-free rate after 1 year (with part of these patients still presenting symptoms), 25% of patients undergoing a major amputation and 25% will have died. In areas with poorer medical care, these numbers maybe even worse.

Current treatment of CLI is complex, involving both surgical and non-surgical approaches. The goal of treatment is to restore adequate blood supply. Ultimately, success should include relief of ischemic pain, healing of ischemic ulcers, prevention of limb loss, improvement of patient function and quality of life, while also prolonging survival. Revascularization, either surgical or endovascular, is the most straightforward and intuitive strategy to reach these goal. Unfortunately, revascularization is not always feasible for several reasons. First, CLI is not only a disease of large vessels, and widespread microvascular and/or surrounding tissues involvement at the time of diagnosis (resulting in poor outflow vessels in the limb) may hamper its success. Second, medical comorbidities, invariably present in CLI patients, may also limit surgery as a viable option. Last, even in patients referred to surgical revascularization, an extremely high immediate post-operative mortality rate that reaches 11.6% according to a recent meta-analysis (Albers et al., 2006) demonstrates the need for a thorough assessment of risks and benefits, underscoring the challenge of treating CLI. For patients for whom revascularization is not feasible for one of these reasons, limb loss rates after 6 months are near 50% (Brass et al., 2006), and amputation remains as the only option to treat CLI.

In conclusion, CLI represents a condition characterized by considerable morbidity and mortality, a high impact on quality of life, and suboptimal treatment options. These characteristics highlight the importance of devising new strategies to treat CLI.

1.2 Understanding the problem: pathophysiology of CLI

From a pathophysiological stand-point, CLI affects both macrovascular and microvascular systems, and is characterized by chronic deprivation of blood supply to limb tissues. With time, several changes in the structure and function of the vascular tree occur, including diminished sensitivity to vasodilatory stimuli, decreased wall to lumen ratio, lower nitric oxide production, and capillary microthrombi formation due to impaired anticoagulant function of the endothelium (Varu et al., 2010).

The natural response to tissue ischemia is the activation of several molecular and cellular pathways that result in the formation of new blood vessels, involving processes such as angiogenesis and arteriogenesis. However, in CLI patients, as in other forms of AOD, this response is not sufficient to provide the adequate amount of blood to ischemic tissues.

2. Therapeutic angiogenesis

Thirty years ago, Folkman observed that the development and maintenance of an adequate microvascular supply is as an essential condition for growth of neoplastic tissue (Folkman, 1971), laying the ground for the development of anti-angiogenic therapies and also for therapeutic angiogenesis. Later, the idea of boosting the formation of collateral vessels as a way to treat CLI, a strategy known as "therapeutic angiogenesis", was stimulated by
angiographic and clinical observation that spontaneous development of collateral vessels was sufficient to preserve ventricular function in patients with ischemic heart disease (Habib et al., 1991). Therefore, it was not very long after the identification of the first angiogenic growth factors that the first experiments using Vascular endothelial growth factor (VEGF-A) were initiated, aiming to improve blood flow to ischemic tissues (Takeshita et al., 1994).

2.1 The rationale for therapeutic angiogenesis

For therapeutic angiogenesis to be justified from a theoretical standpoint, at least one of the following two conditions must be true: (1) the presence of decreased levels of angiogenic growth factors in ischemic tissues, or (2) the possibility of optimizing the endogenous response to ischemia through the use of supra-physiological doses of these factors. Regarding the first hypothesis, Schultz and colleagues demonstrated a positive correlation between the expression of VEGF-A stimulated by hypoxia in monocytes and the number of collaterals in the myocardium of patients with chronic coronary ischemia (Schultz et al., 1999). Furthermore, senile rabbits and mice exhibit subnormal angiogenic response, attributed to decreased levels of VEGF-A in ischemic tissues, and supplementation with exogenous VEGF-A reversed this subnormal angiogenic response (Rivard et al., 1999). A single study that evaluated the concentrations of these factors in muscle and skin of patients with PAD found normal levels of VEGF-A, and high levels of other angiogenic growth factor FGF-2, suggesting a relative deficiency of VEGF-A in these tissues (Palmer-Kazen et al., 2004). Regarding the second hypothesis, several studies have shown that supraphysiological doses of VEGF optimize the angiogenic response (Post et al., 2001), and this was indeed the rationale for most of the initial protocols for therapeutic angiogenesis. It should be noted however, that supraphysiological doses of VEGF-A and FGF-2 have also been shown to induce the formation of aberrant vessels (Sola et al., 1997; Ozawa et al., 2004), suggesting that the therapeutic window for this strategy may be narrow. For this reason, a detailed comprehension of the molecular and cellular events that govern the formation of new blood vessels is of paramount importance for the development of a successful therapeutic angiogenesis strategy.

2.2 The “how-to” of new blood vessels formation

In the human embryo, development of the vascular system begins with the formation of the blood islands, where hematopoietic and endothelial cell (EC) precursors (angioblasts) coexist (Choi et al., 1998). The differentiation of angioblasts in EC is called vasculogenesis, and is the process by which primitive tubes of EC are formed. Several additional steps are needed until a functional and mature vascular system is formed. During these processes, vascular sprouts emerge from pre-existing tubes, a process known as angiogenesis, and mural cells (pericytes and smooth muscle cells - SMC) are incorporated, a process termed arteriogenesis (Conway et al., 2001). The regulation of these steps is made through a complex network of vascular growth factors. To mention some, VEGF plays a critical role from early to later stages, which was demonstrated in knockout mice for the VEGF gene, which do not develop a vascular system (Carmeliet et al., 1997). Moreover, in an unusual observation, heterozygosity for the VEGF gene (+/-) determined the mortality of all animals, indicating the need for optimal concentrations of VEGF for vascular formation. The expression of VEGF occurs, among other stimuli, in response to hypoxia (Shweiki et al., 1992). It is now recognized that hypoxia induces expression of HIF-1α, which binds to a
promoter region of VEGF gene, driving its expression in ischemic areas (Kimura et al., 2000). FGF is another growth factor that promotes the proliferation and migration of EC. PDGF is essential for the recruitment of components of the vascular wall such as SMC, that stabilize EC by preventing their unregulated proliferation (Benjamin et al., 1998). Angiopoietin 1 is involved in the maintenance of vascular integrity (Thurston et al., 1999) and recruitment of smooth muscle cells (Suri et al., 1996). In conclusion, the formation of a mature arterial system starts with the proliferation of EC (vasculogenesis), followed by the sprouting of new capillaries (angiogenesis), and the maturation of the newly formed vessel by pericytes, smooth muscle cells and extracellular matrix (arteriogenesis).

Angiogenesis also occurs in post-embryonic life, in physiological situations such as the ovulatory cycle, wound healing, and in response to tissue ischemia, as in CLI. In addition, several pathological conditions are associated with increased angiogenesis, such as inflammation, proliferative retinopathy and growth and spread of tumors (Carmeliet, 2003). In general terms, it is acknowledged that post-natal angiogenesis recapitulates the mechanisms described in the embryo.

2.3 Gene therapy as the preferred platform for therapeutic angiogenesis

One of the most attractive aspects of therapeutic angiogenesis is that by increasing the formation of collateral vessels, one would be only intensifying what happens spontaneously in ischemic tissue. The simplicity of this idea, associated with the huge unmet needs of patients with CLI, resulted in a relatively rapid transition from preclinical to phase I and II trials, which were initiated with a level of expectation that was probably too unrealistic considering the amount of basic and translational research on post-natal angiogenesis available at that time.

Gene transfer was rapidly considered the preferred method for therapeutic angiogenesis. Using gene therapy (GT), genes of vascular growth factors could be directly injected into ischemic muscle, which would function both as the major therapeutic target, as well as a production site for these proteins. Several reasons make CLI an attractive target for GT. These include not only the limitations of available treatments for CLI, but also a series of very specific characteristics of CLI treatment that will be discussed next.

2.3.1 Requirement of lower levels of expression

A major obstacle to the success of GT in some hereditary diseases is the need to obtain high and sustained expression of the therapeutic gene. Initial observations suggested that therapeutic angiogenesis required minimal and only transient expression of angiogenic growth factors (Dor et al., 2002). This was interpreted as a possibility to obtain therapeutic effects with less and more simple vectors, resulting in higher safety, and lower costs.

2.3.2 Availability of animal models

The existence of animal models that reproduce the clinical outcome of CLI allowed the completion of preclinical studies and accelerated the development of clinical trials. In this model, hindlimb ischemia is generated by excision of the femoral artery of the animal (usually rabbit or rat) leg (Pu et al., 1993).

2.3.3 Expression of naked DNA by skeletal muscle

Perhaps the most important factor to boosted studies in GT-based therapeutic angiogenesis from the 90’s on was an intriguing observation in 1990 by Wolff and colleagues, that pure
DNA or RNA could be absorbed and expressed by skeletal muscle cells of mice without the participation of any particular system of gene transfer (Wolff et al., 1990). Although the mechanism of DNA capture by the target cell remains unknown (Wolff et al., 2005), the confirmation of this observation opened the way for the use of non-viral vector (usually in the form of pure plasmid DNA) in CLI studies.

2.3.4 Lower complexity of strategies based on non-viral vectors
The major advantage of non-viral vectors is their safety profile. These vectors do not integrate into the host genome (no risk of insertional mutagenesis), do not trigger severe inflammatory reactions (absence of innate immune response to the vector), which would be a huge limitation in an already inflamed tissue, and their effectiveness is not limited by previous immunity of the host. In addition, non-viral vectors are simple to produce, at lower costs when compared to viral vectors.

3. Preclinical data
Using variations of the model of hind limb ischemia (Pu et al., 1993), the effectiveness of therapeutic angiogenesis in small animal models was demonstrated by intra-arterial, intravenous and intramuscular administration of recombinant proteins or by gene transfer of several vascular growth factors (Takeshita et al., 1994; Takeshita et al., 1994; Bauters et al., 1995; Tsurumi et al., 1996; Garcia-Martinez et al., 1999; Shimpo et al., 2002). However, as discussed in the next section, these studies should be regarded more a proof of concept tool than as an indicator of efficacy in clinical studies.

In order to have a more critical view of these studies, it is important to briefly review general characteristics of the hindlimb ischemia model and of tools to evaluate efficacy in these studies.

3.1 The hindlimb ischemia model
In the classic hindlimb ischemia model, ligation and excision of the iliac or femoral artery reduces blood flow to levels between 30%, inducing a series of events that trigger angiogenesis and arteriogenesis over 3-4 weeks, after which resting blood flow is restored to about 60-70% of baseline (Scholz et al., 2002). In order to more accurately reproduce the progressive clinical course of CLI, a minority of studies used stepwise ligation of vessels, as well as constricting devices (Tang et al., 2005). Angiographic study of these animals show that collaterals emerging from the side branches of internal iliac artery and reaching the distal portions of the foot, as well as an extensive network of capillaries throughout the ischemic muscle are responsible for the partial restoration of blood flow (Takeshita et al., 1997). Because revascularization in this model has been shown to involve the basic mechanisms of angiogenesis and arteriogenesis (Asahara et al., 1997; Scholz et al., 2002), the model has been widely used in preclinical studies of GT-based therapeutic angiogenesis. However, one should acknowledge that the model does not exactly mimic the slow progression of atherosclerosis observed in patients with PAD. In addition, revascularization that occurs in this model is associated with intense capillary proliferation, whose real significance for perfusion has been challenged.

As in other areas of medical research, the translation of results from small animal models to humans is problematic. This is even more relevant for studies using gene transfer by direct
injection of non-viral vectors. Uptake and expression of non-viral vectors by skeletal muscle occurs mostly in the vicinity of the needle (Wolff et al., 1990). Therefore, the exposure of muscle fibers to vector is much higher in rats and rabbits, than in humans, even if multiple injections are used in humans. In addition, animals used in these studies are usually healthy and young, as opposed to patients with CLI. This is even more relevant because it has been shown that age impairs the angiogenic response (Zhuo et al., 2010).

3.2 Methods used to evaluate efficacy
Although the preclinical studies have unequivocally demonstrated that GT-based therapeutic angiogenesis increase capillary proliferation in ischemic tissues, the real significance of this increase to the effective perfusion remains uncertain. Thus, the use of functional methods to assess the clinical relevance of newly formed vessels is of utmost importance in preclinical studies. Usually, efficacy is measured by a combination of anatomic (usually counting of newly formed vessels in histological sections or angiographic studies) and functional methods. Perfusion studies with microspheres that are trapped in capillaries are considered the gold-standard, but are time-consuming. Therefore, doppler tissue imaging, which measures the skin blood flow in the ischemic limb, is one of the most frequently used functional method. The method is based on the assumption that skin perfusion reflects whole limb perfusion. Alternative methods such as measurements of oxygen tension, sestamibi scintigraphy and heat detecting cameras have also been used, along with clinical scores that evaluate the presence of ulcer healing, necrosis, etc. Whichever the method used, one should bear in mind potential pros and cons to avoid over-interpretation of preclinical data.

4. Data from clinical trials
In humans, GT-based therapeutic angiogenesis was first used in 1994 in a patient with CLI, who received an intra-arterial (distal to the obstruction) injection of plasmid DNA containing the cDNA of VEGF-A (Isner et al., 1996). The authors reported angiographic improvement after 12 weeks, associated with the development of angiomas and unilateral edema, two characteristics that enforced the biological effect of the treatment. The first phase I study also used plasmid DNA of the VEGF-A cDNA, delivered by multiple intramuscular injections in 9 CLI patients for whom revascularization was not feasible. Treatment was considered safe, and transient edema was the only adverse event. In addition, the authors reported increased levels of VEGF-A in serum, and improvements in ABI and in skin lesions, which could avoid amputation in 3 patients (Baumgartner et al., 1998). Two years later similar results were reported by the same group in 50 patients (Baumgartner et al., 2000). Subsequently, additional phase I studies confirmed the safety of these strategies (Comerota et al., 2002), paving the way for phase II studies.

4.1 Phase II studies
The first randomized double-blind study involved 54 patients with PAD amenable to angioplasty. In this study, VEGF-A gene transfer (n=35) or placebo (n = 19) were delivered intra-arterially by catheter after angioplasty. Gene transfer was performed using adenovirus (n=18) or non-viral vectors (liposome/plasmid-DNA) (n=17). Treatment proved to be safe. However, despite significant increase in the number of collaterals in the two treated groups compared to placebo, other efficacy parameters were not modified (Makinen et al.,
In the second randomized doubleblind study, 54 patients with CLI and diabetes were randomized to intramuscular injections of placebo or plasmid DNA with the VEGF-A cDNA. The primary efficacy endpoint (amputation at 100th day) was not significantly modified by treatment, but authors reported significant improvements in ABI, and in ulcer healing in treated patients compared to placebo (Kusumanto et al., 2006). Though not using non-viral vectors, the RAVE (Regional Angiogenesis with Vascular Endothelial Growth Factor) trial deserves to be discussed. In this trial, 105 patients with unilateral, exercise-limiting claudication were randomized to direct intramuscular injections of low or high doses of adenoviral VEGF121 (Kusumanto et al., 2006). Despite the observation of dose-dependent peripheral edema, which suggests that bioactive VEGF was indeed produced, the authors observed no significant change in primary or secondary endpoints. These results raised several important questions: First, it could be possible that the use of an isoform of VEGF that could have a shorter tissue half-life (as is the case of VEGF121 compared to VEGF165) could have limited treatment efficacy. Alternatively, the lack of validated endpoints, privileging physician-oriented as opposed to patient-oriented outcomes could also explain the negative results. Finally, failure could simply mean that use a VEGF isolated is not an effective strategy to obtain clinically-relevant therapeutic angiogenesis in CLI (Gupta et al., 2009).

After the first phase I trial with gene transfer of FGF-1 reported improvements in wound healing, pain and transcutaneous oxygen pressure after intramuscular injection of naked plasmid FGF-1 (Comerota et al., 2002), results from a phase II trial were reported. In this trial (TALISMAN 201 phase II trial in patients with CLI), patients were randomized to intramuscular injections of NV1FGF (n=59) or placebo (n=66) (Nikol et al., 2008). After 25 weeks, the proportion of patients that reached the primary endpoint (ulcer healing) did not differ significantly between treatment and control groups. However, amputation rate was significantly lower in NV1FGF-treated patients compared to placebo (37.3 vs 55.4%) and the strategy moved on to a phase III trial.

Because of its properties to regulate the expression of multiple downstream mediators of the angiogenesis cascade, including VEGF, thus acting as a “master-switch” agent, the transcription factor hypoxia-inducible factor (HIF) 1-α has been intensively studied as a candidate therapeutic gene for therapeutic angiogenesis (Gupta et al., 2009). The most extensively studied growth factor that is currently under clinical development for GT-based therapeutic angiogenesis is hepatocyte growth factor, a potent mitogen for several cell types (Bussolino et al., 1992). It has been shown that serum levels of HGF are elevated in patients with coronary artery disease, and that higher levels correlate with better prognosis (Lenihan et al., 2003). In the STAT phase II trial, 104 patients with CLI were randomized to treatment with placebo or HGF (Powell et al., 2008). No safety concerns were raised related to the therapeutic agent. On an intention-to-treat analysis, no significant differences in transcutaneous oxygen tension (TcPO2) were observed among patients treated with HGF (in increasing dose levels) or placebo. However, when patients that presented increases in TcPO2 before treatment were excluded, patients that received the highest dose level of HGF presented significant improvements in TcPO2 compared to the remaining groups. Again, these observations demonstrate the impact of endpoint selection in trial results and highlight the importance of identifying the most relevant endpoint to be used in therapeutic angiogenesis trials.

Following this study, the same group recently reported the results of the HGF-0205 trial. Patients received three sets of eight intramuscular injections of HGF plasmid every 2 weeks...
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(Powell et al., 2010). Injection sites were selected on an individual basis based on arteriographically defined vascular anatomy. In total, 21 patients were randomized to HGF treatment and 6 to placebo. No safety concerns were raised. HGF-treated patients presented significant improvements in toe-brachial index and rest pain compared to patients that received placebo. Complete ulcer healing at 12 months occurred in 31% of patients compared to 0% of placebo, though this difference was not statistically significant. Amputation rates (HGF 29 % vs. placebo 33%) and mortality were similar between groups. These results suggest that tailoring treatment to patient indivual characteristics might improve treatment outcomes. If confirmed, it is possible that the classical strategy to inject naked DNA into predefined sites of ischemic tissue with poor knowledge of the pharmacological and geographic distribution of the actual treatment agent could justify several negative results reported in previous human trials. One should bear in mind that when using a delivery strategy that is characterized mostly by local transfection, the proportion of ischemic tissue actually treated in a mouse or rat is very unlikely to be reached even by multiple injections in humans.

Additional studies have been recently reported using HGF plasmid-based therapeutic angiogenesis. In a multicenter, randomized, double-blind, placebo-controlled trial, 40 patients also received injections in sites selected by angiographic evaluation, on days 0 and 28 (Shigematsu et al., 2010). The overall primary improvement rate of the primary endpoint (improvement of rest pain or reduction of ulcer size) was significantly higher in HGF-treated patients (70.4%) compared to the placebo group (30.8%). An evaluation of quality of life also showed benefits of HGF treatment. Very recently, HGF plasmid intramuscular injections was also shown to improve several efficacy endpoints such as ABI, ulcer size, pain, in another phase I/IIa trial that treated 22 patients with CLI with of 2 or 4 mg of HGF plasmid, 2 times (Morishita et al., 2011).

Finally, a phase II trial was also conducted using gene transfer of an extracellular matrix protein that induces angiogenesis indirectly by interaction with integrins, Developmentally regulated endothelial locus (Del-1). In the DELTA trial, 105 patients with PAD were randomized to intramuscular injections of Del-1 plasmid in association with poloxamer 188, an agent that enhances transfection (Grossman et al., 2007). The control group received poloxamer 188 alone. No safety concerns were raised, but neither primary nor secondary endpoints were modified. Notably, improvement in these endpoints occurred in both treated and control groups, highlighting the importance of placebo effect in studies using new strategies such as gene therapy.

4.2 Safety concerns

To date, more than 1000 subjects have been treated for gene therapy for therapeutic angiogenesis in phase I and II trials, with adverse event rates that are similar to those in control groups (Varu et al., 2010). Still, there are important long-term safety concerns, stemming from theoretical but rather intuitive and important considerations, that still need to be addressed and weighted against the benefits of gene therapy-based therapeutic angiogenesis. These include the risk of accelerating pathologic angiogenesis in tumors, retina and atherosclerotic plaques. So far no evidence of these effects has been reported. Dose-dependent hypotension or proteinuria (> 1g/24h) have been reported in studies using VEGF-A (Henry, Rocha-Singh et al., 2001) and FGF-2 (Laham et al., 2000; Unger et al., 2000; Cooper et al., 2001) in the form of recombinant proteins, but in studies with gene therapy, where lower levels of proteins are expected to be expressed, these were not observed.
Studies with hepatocyte growth factor have not raised significant safety concerns. Of note, in one recent study, circulating levels of HGF were not detected, suggesting a restricted distribution of the therapeutic agent (Morishita et al., 2011).

5. Next steps

Preclinical and clinical trials have demonstrated the safety and potential efficacy of this strategy. Currently, the area is going through a phase in which new strategies and conceptual changes, developed after reevaluation of early clinical results and after a return from bedside to bench, are being tested in clinical trials. After almost 2 decades since the first human trial of GT for CLI, it is clear that several factors can be optimized in order to maximize the effectiveness of therapeutic angiogenesis. These include:

- **Animals x humans**: differences between animal models and the progression of CLI in humans, should be acknowledged, thus avoiding over-interpretation of results from animal studies.

- **Efficacy outcomes (animals)**: more relevant efficacy outcomes should be identified in preclinical models allowing more realistic translation of results from bench to bedside.

- **Efficacy outcomes (humans)**: CLI is diagnosed and monitored with the help of objective methods that include ABI determination, toe systolic pressures or transcutaneous partial pressure of oxygen (TcPO$_2$) (Varu et al., 2010). However, an important unanswered question that makes evaluation of treatment even more complex is the very definition of a salvageable versus a non-salvageable limb (Connelly et al., 2001; Rowe et al., 2009). As in other areas of medical research, treatment outcome measures are mostly physician- rather than patient oriented. In the context of CLI, outcomes such as ABI improvement, limb salvage and survival have always been considered the most significant outcomes that any new treatment should be able to improve. However, patient-oriented outcome research in the last years suggests that in selected populations these outcomes are not necessarily associated with improvements in quality of life (Varu et al., 2010). Whether classical outcomes such as ABI index, or more patient-oriented outcomes should be used as primary endpoint for efficacy assessment of therapeutic angiogenesis is an important and challenging question.

- **Basic science**: Continuing research efforts on the mechanisms of blood vessel formation are extremely important to allow more rational selection of therapeutic genes to treat CLI. These could include the use of “cocktails” of growth factor genes, or master-switch genes, that could induce an angiogenic response that is closer to that spontaneously observed during “physiologic” collateral formation.

- **Delivery strategies and vectors**: Better delivery strategies and/or more efficient non-viral vectors for GT-based therapeutic angiogenesis should be developed, allowing more widespread expression of the therapeutic gene in human skeletal muscle.

- **Towards a pharmacological approach to therapy**: Better understanding of variables that govern the pharmacokinetics of non-viral vector delivery to skeletal muscle, thus enabling a less empiric design of clinical trials are of paramount importance so that trial design with non-viral vectors delivered to skeletal muscle start to move towards a more pharmacological paradigm, that allow a more rational interpretation of data and planning of treatment. So far, actual “dosing” of the therapeutic proteins has not been possible. In fact, this could severely affect the possibility to to reproduce even very positive results in larger scale phase III trials.
6. Conclusions

Therapeutic manipulation of angiogenesis is increasingly regarded as a promising treatment alternative for patients with CLI, as results from a new generation of clinical trials are revealed. The rationale for the use of vascular growth factors to stimulate vessel growth in ischemic tissues is mainly the demonstration that ischemic tissues present a relative deficiency of these growth factors, and that their administration can boost vessel growth. In addition, several characteristics render CLI a very interesting target for gene therapy-based therapeutic angiogenesis. In this chapter we reviewed the molecular and cellular rationale of therapeutic angiogenesis, results from pre-clinical studies, and finally results from clinical trials that used gene therapy as a platform for therapeutic angiogenesis. In addition, we tried to provide a critical appraisal of limitations of small animal models (and of the translation of endpoints from these studies to clinical practice), as well as a discussion on the steps that still need to be taken in order to render gene therapy with non-viral vectors as a more predictable and controllable strategy.

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