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Stem Cell Therapy in PAD☆

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Abstract Critical limb ischemia (CLI) continues to form a substantial burden on Western health care. Despite recent advances in surgical and radiological vascular techniques, a large number of patients is not eligible for these revascularisation procedures and faces amputation as their ultimate treatment option. Growth factor therapy and stem cell therapy — both therapies focussing on augmenting postnatal neovascularisation — have raised much interest in the past decade. Based on initial pre-clinical and clinical results, both therapies appear to be promising strategies to augment neovascularisation and to reduce symptoms and possibly prevent amputation in CLI patients. However, the underlying mechanisms of postnatal neovascularisation are still incompletely understood. Both fundamental research as well as large randomised trials are needed for further optimisation of these treatment options, and will hopefully lead to needed advances in the treatment of no-option CLI patients in the near future.

Peripheral arterial disease (PAD) is a growing health-care problem. A prevalence ranging from 3% to 10% has been reported for the general population,[1] increasing up to 29% in primary health-care populations.[2] These figures are likely to increase in the coming years, due to improving life expectancy of the Westernised population,[3] and increasing prevalence of risk factors such as diabetes and obesity. Patients with PAD experience a high risk of cardiovascular events (i.e., myocardial infarction and stroke),[4,5] which increases with increasing severity of limb symptoms.[1] Critical limb ischaemia (CLI), defined as chronic rest pain or tissue necrosis caused by progressive PAD, is thus associated with the highest cardiovascular risk.

Despite its relatively low incidence at 0.05–0.1% per year,[1,6] CLI imposes a disproportionately large medical and economical burden on Western health care. It is associated with surgery, hospitalisation and poor quality of life (QoL).[7,8] In Europe and North America, there are 413 000 hospital discharges of patients with chronic PAD annually[1]; in the Netherlands, CLI has a reported prevalence between 0.04% and 0.1%, and leads to approximately 1700 hospital admissions per year, with an average stay of 25 days. Amputation is the most common reason for admission (57% of the admissions).[9] QoL scores of CLI patients have been reported

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to be worse than scores obtained from patients with cancer and chronic heart disease.[8,10]

The prognosis for CLI patients is poor, with 5-years survival rates of 50% or less.[11,12] The presence of cardiovascular risk factors and co-morbidity importantly contributes to reduced survival. Treatment of CLI patients should therefore not only aim at relieving symptoms of CLI but also at strict cardiovascular risk-factor management to prevent progression of systemic atherosclerosis. Although, for some patient groups, primary amputation is considered the best choice of treatment,[13] full mobility after amputation is only achieved in 25–50% of patients, whereas perioperative mortality is estimated to be 5–20%, and a second amputation is required in approximately 30% of patients.[14] For most CLI patients, limb salvage therefore remains the primary goal of contemporary treatment.[13] Despite the rapidly developing radiological and surgical intervention techniques, the therapeutic options in CLI patients are limited: approximately 40% of the patients are not eligible for surgical or radiological revascularisation, either due to the extent or location of atherosclerotic lesions or due to extensive co-morbidity.[15,16] No effective pharmacological therapy is currently available.[17] New limb-salving treatment options for CLI are therefore being explored. Therapies focussing on (the stimulation of) postnatal neovascularisation have raised much interest in the past decade. Here, we discuss the current standing of these therapies and appraise the future perspectives of cell therapy.

Postnatal Neovascularisation Therapies

Postnatal neovascularisation refers to the process of new vessel formation in the adult. In the past decade, new insights into this process have arisen, and therapies stimulating postnatal neovascularisation, that is, growth-factor therapy and stem cell therapy, have been studied both in preclinical and clinical settings.

Growth-factor therapy augments postnatal neovascularisation by supplying proangiogenic growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (bFGF) or hepatocyte growth factor (HGF), to the ischaemic tissue either in the form of a recombinant protein or by means of gene therapy to introduce genes encoding for such a pro-angiogenic factor. In total, eight patients' series and four randomised controlled trials have reported on the use of growth-factor therapy in CLI patients. Initial uncontrolled studies investigating growth-factor therapy in patients with CLI showed promising results. However, larger randomised trials in patients with CLI and intermittent claudication were mainly disappointing and reported only limited success[18,19] (see Ref. 20 for further reading on these trials). Several factors have been suggested to have negatively influenced the results of the randomised trials, such as the administered dose, the achieved duration of gene expression, heterogeneity between patients included in the trials and poor end point selection.[21] Furthermore, the clinical studies conducted thus far have only focussed on the induction of a single proangiogenic factor, while the induction of a single growth factor may be insufficient to drive the postnatal neovascularisation process. Interest within the field of growth-factor therapy now appears to be shifting to the induction of more than one single proangiogenic factor, and randomised placebo-controlled trials are currently focussing on the effects of gene transfer of key transcription factors, such as hypoxia-inducible factor (HIF)-1α.[22]

After the discovery of bone marrow-derived endothelial progenitor cells (EPCs) in peripheral blood in the 1990s, many publications reported on the role of EPCs in postnatal neovascularisation. The mechanisms by which EPC augment postnatal neovascularisation are thought to be, on the one hand, by homing and direct incorporation into existing blood vessels, facilitating the sprouting of new capillaries, and, on the other hand, by paracrine effects, stimulating resident endothelial cells in the vascular wall to proliferate.[23–31] Initial clinical studies on the effects of stem or progenitor cell therapy in patients with PAD or CLI showed encouraging results. Thus far, over 30 clinical studies reported on the use of bone marrow-derived or peripheral blood-derived stem cells in patients with PAD and CLI, most of them reporting improvements in clinical parameters, such as rest pain, pain-free walking distance, ankle–brachial pressure index and/or transcutaneous oxygen pressure (see Ref 32 for an in-depth review of these studies).

Stem Cell Therapy for PAD: Where Do We Stand?

Majority of studies on cell therapy in PAD have been small studies in CLI patients, lacking double-blind controls. Large, randomised, placebo-controlled trials are needed to confirm the results of the initial case reports and patient series. Currently, eight randomised, placebo-controlled clinical trials on the effects of stem cell therapy in patients with CLI are being conducted worldwide; of which four are European, investigator-driven trials (see Table 1).

Of note, most of the reported studies investigating the effects of stem cell therapy in CLI patients have been conducted in relatively young Asian patients with thromboangiitis obliterans.[33] In Caucasian patients however, the main cause of CLI is atherosclerotic PAD, which is associated with a high presence of cardiovascular risk factors. These cardiovascular risk factors have been reported to impair the functional capacities of stem and progenitor cells[34] and may negatively influence the effects of stem cell therapy. It is therefore important to evaluate the effects of stem cell therapy also in Westernised populations. The ongoing randomised clinical trials mentioned in Table 1 will provide initial insights into the efficacy of stem cell therapy in atherosclerotic PAD patients.

Further fundamental as well as clinical research is necessary to answer the many unanswered questions regarding stem cell therapy – the optimal cell population to be administered, the optimal route of administration, the optimal dose, patient selection criteria[32] – and to develop optimised, well-tailored stem cell therapy for PAD patients. Several different cell populations have been used in clinical studies and were reported beneficial; however, no direct comparison between cell types has been performed in a clinical study so far. In general, it is currently believed that different cell populations work together in vascular repair and postnatal neovascularisation, and that these cells act via

Table 1 Currently ongoing randomised, placebo controlled trials investigating the effects of stem cell therapy in CLI patients.

Year started	Name (NCT)	Number of patients	Country	Intervention
2006	JUVENTAS Trial (00371371)	110—160	The Netherlands	Intra-arterial BM-MNC
2007	BONMOT Trial (00434616)	90	Germany	Intramuscular BM-MNC
2007	RESTORE-CLI Trial (00468000)	150	United States of America	Intramuscular ''Aastrom TRC'' BM cells
2007	*Harvest Technologies* (00498069)	48	United States of America	Intramuscular BM aspirate concentrate
2007	ABC Trial (00539266)	108	The Netherlands	Intramuscular BM-MNC
2007	ACT34-CLI Trial (00616980)	75	United States of America	Intramuscular CD34+ cells
2008	MESENDO Trial (00721006)	30	United States of America	Intramuscular ''stem cell mixture''
2009	BALI Trial (00904501)	110	France	''Implantation'' of BM-MNC

Overview consists of all relevant trials registered in the US NIH trial register (clinictrials.gov).
NCT Number: Study identification number in the clinical trial register of the U.S. National Institutes of Health (clinicaltrials.gov).

direct incorporation into the endothelial layer and endothelial differentiation, by supplying angiogenic factors via a paracrine mechanism to resident cells or by a combination of both. The synergy of different cell types remains to be clarified, which may lead to the identification of the most potent (combination of) cell types to be used for stem cell therapy.

Both intramuscular injection of cells, intra-arterial injection, or the combination of both have been reported as effective ways to administer stem cell therapy. With intramuscular injection, local depots of stem cells are created in the ischaemic muscle, which could augment neovascularisation by facilitating cell-to-cell contact, cell transdifferentiation and paracrine mechanisms; but survival of the stem cell may be reduced since the depots are laid down in an ischaemic environment. With intra-arterial administration, stem cells travel to the border zone of ischaemia in the nutrient- and oxygen-rich circulation, thus providing a favourable environment for survival and engraftment, but cell uptake from the circulation to the ischaemic tissue may be limited. Clinical studies comparing different routes of administration have not yet been reported but will help to identify the most effective way of delivering stem cell therapy to the ischaemic tissue.

Remarkable differences in the number of stem cells isolated from the bone marrow and in the numbers of administered cells have been reported between studies, with a range of approximately 125-fold for the number of isolated cells. However, no relation between this varying cell dose and the obtained effects has been observed between these studies. A dose-escalating study investigating the effects of increasing numbers on injected cells will allow further optimisation of stem cell therapy.

It is still largely unknown what determines the efficacy of stem cell therapy in a patient. Preclinical and clinical results hint at the influence of the patient's age, the severity of the disease, the presence of risk factors such as diabetes, hypertension and smoking behaviour and the degree of stem cell dysfunction. In fact, a direct relation between the number of cardiovascular risk factors and the number of EPCs and their function has been demonstrated in patients with cardiovascular disease.[34—39] Further research on the influence of patient characteristics on the effects of stem cell therapy will help to determine patient suitability for stem cell therapy.

Future Perspectives

Both growth factor and cell therapy appear to be promising strategies to augment neovascularisation in CLI patients. The therapies are still being developed and optimised, hopefully leading to better tailored treatment for PAD patients in general, and for patients with CLI specifically.

A logical direction for future research may be to combine growth factor and cell-based therapy. Intramuscular gene therapy could be administered in the calf muscles as pre-treatment of the target tissue to augment homing of intra-arterially administered progenitor cells. Furthermore, gene therapy of progenitor cells *ex vivo* could be applied before injecting them into the patient to enhance the homing, migration, incorporation and/or (trans)differentiation capacities. In addition, progenitor cell dysfunction may be an interesting therapeutic target. Despite the promising effects reported for autologous cell therapy, the fact that functional capacities of progenitor cell from patients with cardiovascular disease are hampered is becoming more and more apparent, as are the negative effects of cardiovascular risk factors on the function of these cells. Hampered functional capacities, such as impaired homing to ischaemia, and reduced vascular outgrowth might limit the effects of cell therapy. It has been demonstrated that these EPC dysfunctions can be (partially) reversed by systemic pharmacological interventions with statins,[40] peroxisome proliferator-activated receptor (PPAR) gamma agonists,[41] erythropoietin[42] or angiotensin II receptor antagonists.[43] Besides the already known benefits of risk-factor management, this underscores the importance of strict treatment of cardiovascular risk factors in PAD patients. Furthermore, *ex vivo*-pre-treatment of progenitor cells with drugs targeting cardiovascular risk factors, such as statins and certain anti-hypertensive drugs, has been shown to improve cellular

function *in vitro* and increase neovascularisation capacity *in vivo*.[40,44–50] Thus, not only the treatment of cardiovascular risk factors supplemental to stem cell therapy, but also administration of pre-treated, functionally improved autologous stem cells might further augment the efficacy of stem cell therapy.

In vitro selection of cells may be another option to further optimise stem cell therapy. The exact (combination of) cell types responsible for the effects of cell therapy is not yet known, but selection of proangiogenic cell types and filtering out unwanted or undesirable (possibly pro-atherosclerotic) cells may improve therapeutic efficacy. In culture conditions, different methods can lead to outgrowth of cells with an endothelial phenotype. Early outgrowth EPC can be cultured in 4–7 days by plating mononuclear cells in a specific medium that facilitates endothelial outgrowth.[23] These cells are mostly monocyte-derived and seem to act mainly by the secretion of proangiogenic factors.[51] Preclinical studies showed beneficial effects of these EPCs in models of hindlimb ischaemia.[52] Late outgrowth EPC emerge in culture after 3–4 weeks as colonies of confluent, cobblestone-shaped cells with robust proliferative capacity.[53] They most likely derive from a CD34+ cell population and act by incorporating in newly formed vasculature. While *ex-vivo* cultured early outgrowth EPCs have been applied clinically, late outgrowth EPCs have not been used in the clinic yet, but seem promising because of their profound proliferative capacity and the already obtained preclinical effects. *In-vitro* differentiation towards an endothelial progenitor cell type may thus further augment the effects of cell therapy.

Although the need for new revascularisation options in 'no-option' CLI patients is high and current evidence for the efficacy of therapeutic neovascularisation is appealing, the possibility of side effects should not be disregarded. Thus far, the number and extent of reported side effects is minimal, but no sufficiently long-term follow-up of patients treated with such cell therapy is currently available. Continued monitoring and long-term follow-up of all patients treated with stem cell therapy is warranted to provide solid ground for long-term safety conclusions.

Until now, the effects of postnatal neovascularisation therapy have predominantly been studied in no-option CLI patients, which is a patient group in urgent need of such new treatment options. Furthermore, no-option CLI patients form, by definition, a patient group in which neovascularisation therapy will not interfere with conventional treatment options, and hence will not delay a possible limb salvaging therapy. By contrast, the hampered functional capacities of stem cells of CLI patients might render no-option CLI patients less suited as the initial patient group in which to study the effects of neovascularisation therapy. However, it should be noted that not all no-option patients are incurable, since no-option patients also include patients who are inoperable due extensive co-morbidity, but in which an amputation is not inevitable *per se*. Furthermore, the results obtained in CLI patients thus far have also led to the initiation of cell therapy studies in patients with less severe stages of PAD, such as intermittent claudication. Intermittent claudication patients differ substantially from CLI patients, with regard to the severity of PAD (non-end-stage disease), the amount of vascular damage and the possibly different extent of stem cell dysfunction. These studies will provide insights in the effects of such patient and disease characteristics on the effectiveness of stem cell therapy.

In summary, postnatal neovascularisation by means of growth factor therapy, cell-based therapy or a combination of both, appears to be a viable alternative in the treatment of CLI patients without other treatment options. Ongoing randomised clinical trials and new (pre)clinical studies further elucidating underlying mechanisms of postnatal neovascularisation will hopefully lead to crucial advances in the treatment of PAD in the near future.

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