Vascular disease and stem cell therapies

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Introduction and background: Peripheral vascular disease is the leading cause of limb ischemia (LI). LI is manifested by claudication, ischemic rest pain, ulcers or gangrene. It is the result of peripheral arterial disease due to atherosclerosis. Over the last decade, several centers around the world have initiated clinical trials utilizing stem cells as a treatment for this disease.

Sources of data: Published medical literature, clinical trials announced in clinical trials.gov and TCA cellular therapy experience.

Areas of agreement: There is general agreement that stem cells are useful for LI.

Areas of controversy: These arise from the type of cells, dose, route of administration and methods to evaluate efficacy.

Growing points: Growing evidence suggests that bone marrow derived-mesenchymal stem cells are as good as or superior to mononuclear cells, and a combination of both cell types may be even better.

Areas timely for developing research: Based on current trials and publications, several promising biological products could become part of the therapeutic arsenal for LI. This may include combinations of more than one type of adult/induced pluripotent stem cells/embryonic stem cells, use of stem cells with growth factors or extracellular matrix molecules.

Keywords: limb ischemia/cell therapy/therapeutic angiogenesis/clinical trials

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Introduction

Limb ischemia (LI) is the term used for patients with claudication, chronic ischemic rest pain, ulcers or gangrene attributed to inadequate blood flow or arterial occlusive disease. Chronic LI (CLI) is the end result of peripheral arterial disease due to atherosclerosis. Other aggravating conditions include hypercholesterolemia, hypertension, cigarette smoking and diabetes. Less frequent causes of CLI include Buerger's disease, or thromboangiitis obliterans, and some other forms of arteritis. A large percentage of patients with CLI have coexisting diseases, such as
cardiovascular and renal disorders. In a recent issue of The American College of Cardiology, 30% of patients with severe LI will suffer a major amputation and 25% will die within a year. The conventional treatment options of surgical or percutaneous re-vascularization are limited to ~70% of patients. The remainder of this population will face amputations, gangrene, infectious complications and even death.

The rationale for the use of cell therapies in the treatment of LI

Over the last 10 years, several centers have addressed the use of cell-based therapies to restore blood flow in patients with critical LI. Angiogenesis has been considered the only means of adult neovascularization, which consists of new blood vessel development from preexisting vasculature. Vasculogenesis on the other hand refers to blood vessel formation from endothelial progenitors that differentiate in situ and was thought to be limited to embryologic development. However, the existence of bone marrow-resident and/or circulating endothelial progenitor cells (EPCs) has provided evidence that post-natal vasculogenesis also occurs in adults. Although, a definition of complete immunophenotype for EPCs is still lacking, it has been accepted that these cells co-express at least ‘typical’ stem cell markers, such as CD34, CD133 and ALDH. New blood vessel formation is a complex process, involving degradation of the basement membrane by proteases, proliferation and migration of EPCs, lumen formation, basement membrane assembly, recruitment of pericytes or vascular smooth muscle cells (VSMCs), vascular maturation and finally blood flow. Several growth factors play pivotal roles in different aspects of this process. Vascular endothelial growth factor and fibroblast growth factor regulate many functions of EPCs, including proliferation, migration, extracellular proteolysis and tube formation activity. Platelet-derived growth factor (PDGF), induces recruitment and migration of pericytes/VSMCs to the newly formed vessels. These events take place in a very coordinated fashion, in order to generate mature stable vessels. The lack of any of these components produces immature and permeable vessels.

Accordingly, the concept of infusing EPCs or a biological source of EPCs was visualized by many researchers as a potent therapeutic modality to decrease tissue ischemia by development of new blood vessels.
Sources of EPCs or other cell products utilized in cell therapy for CLI

In order to reduce ischemia by improving blood flow, several clinical trials have been designed to assess whether the intramuscular, intra-arterial or intravenous infusion of a cell product, supposedly enriched in EPCs was a safe and technically feasible modality.

In most cases, the source of EPCs has been obtained via bone marrow aspirate or preparation of peripheral blood stem cells (PBSCs), after mobilization with hematopoietic growth factors.

Unsorted bone marrow mononuclear cells

Following the aspiration of a relatively small volume (20–60 ml) of bone marrow and its processing through density gradient centrifugation, a fraction of light-density mononuclear cells (MNCs) is obtained. The resulting fraction is composed mainly (95%) of myeloid cells. Stem/progenitor cells at different stages of commitment along the hematopoietic, endothelial and/or mesenchymal lineages, represent not >2–4% of MNCs. Since procedures for BM aspiration are not standardized, the effectiveness of preparations of unsorted MNCs is limited; in most cases authors perform a truncated immunophenotype by assessing the presence of representative stem cells, such as CD34$^+$ and CD133$^+$, which account for not >2–5% of MNCs.

Unsorted PBSCs

MNCs can be obtained by mobilization of bone marrow hematopoietic stem cells with growth factors (usually granulocyte-colony-stimulating factor). MNCs, enriched in several subsets of hematopoietic stem cells (CD34$^+$, CD133$^+$; range 2–6%), are obtained from the bloodstream by aphaeresis, a common and expeditious laboratory procedure.

Purified bone marrow- or peripheral blood-derived stem cells

Stem/progenitor cell populations can be purified from the MNC fraction by several methods. A common approach is through magnetic activated cell sorting, which permits a simple and fast isolation of several types of stem cells (CD34$^+$, CD133$^+$, ALDH$^+$).
Mesenchymal stem cells

Mesenchymal stem cells (MSCs) also known as stromal cells reside in the bone marrow and in lower concentration in other tissues such as placenta, muscle and fat. These cells can be ex vivo expanded and induced, either in vitro or in vivo, to terminally differentiate into several phenotypes. The isolation, culture, ex vivo expansion and the multipotential attributes of these cells, have made MSCs an attractive candidate for cell therapies, including the treatment of CLI.

Use of MSCs as a single cell product

The option to utilize MSCs to treat CLI patients has been based on the translation of animal studies to the clinical practice. In these studies, it has been shown that the peri-infarct transplant of MSCs in a swine model enhances angiogenesis after myocardial infarction.

Use of MSCs in combination with a source of EPCs

Based on the complexity of vasculogenesis, it is reasonable to envision a therapeutic product that may provide EPCs and the other cell phenotypes, growth factors and factors involved in the ‘production’ of a mature and functional new blood vessel.

MSCs have most of the desired attributes to complement the role of EPCs. Accordingly, TCA cellular therapy, LLC has developed a combination stem cell product that contains both cell phenotypes. To support the role of MSCs, it is worthwhile to mention that de novo formation of VSMC/pericytes progenitors (VSMC/PC) occurs after the differentiation of perivascular mesenchymal cells in a PDGF-B-dependent process.

Additional evidence for the relationship between these two cell types in the development of a mature vascular system is provided by the finding that MSCs express typical pericyte transcripts, such as NG2, CD146, CD271 and CD140B. The contribution of MSCs to the development of angiogenesis also includes their production of an extended cytokine and growth factor profile, a vast array of extracellular matrix molecules as well as the expression of several counter receptors associated with matrix- and cell-to-cell adhesive interactions.

Therefore, MSCs can be an outstanding co-partner cell type for EPCs in the design of a comprehensive treatment for LI.

MSCs have also the advantages of easy ex vivo expansion, cryopreservation and characterization. In addition, their lack of HLA class II surface antigens permits their use as an allogeneic (no-self) product.
Completed clinical trials based on the use of stem cells for the treatment of LI

As seen in Table 1 results of several non-randomized clinical studies, including the pioneer work of Tateishi-Yuyama et al., have shown that transferring a source of EPCs into ischemic limbs is safe and feasible. In turn, this form of therapy produced clinical benefits noticeable by a modest decrease in ischemic symptoms and an increase in ankle-brachial index (ABI), transcutaneous oxygen pressure and exercise tolerance. It has been hypothesized that the observed clinical effect(s) is associated with an improvement of angiogenesis, formation of new collaterals and/or augmentation of endothelium-dependent vasodilatation. However, in most cases, the lack of appropriate angiographic and/or perfusion scintigraphy follow-up makes this assumption dubious. The use of nuclear perfusion imaging to measure blood flow

Table 1 Selected published data showing the use of cellular products to treat patients with critically limb ischemia

<table>
<thead>
<tr>
<th>Study</th>
<th>Patient enrolment</th>
<th>Cell productb</th>
<th>Efficacy as changes in hemodynamic or clinical parameters/follow-upb</th>
<th>Clinical effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tateishi-Yuyama et al.</td>
<td>22 MNCs</td>
<td>ABI, TcPO2, WT/24 weeks</td>
<td>TA</td>
<td></td>
</tr>
<tr>
<td>Saigawa et al.</td>
<td>8 MNCs (CD34⁺)</td>
<td>ABI, TcPO2,4 weeks</td>
<td>TA</td>
<td></td>
</tr>
<tr>
<td>Miyamoto et al.</td>
<td>12 MNCs + Plat</td>
<td>Rest pain, WT, PS/4 weeks</td>
<td>TA</td>
<td></td>
</tr>
<tr>
<td>Nizankowski et al.</td>
<td>10 MNCs (CD34⁺, AC133⁺)</td>
<td>TcPO2, ABI, angio, PS/12 months</td>
<td>Not discussed</td>
<td></td>
</tr>
<tr>
<td>Kawamura et al.</td>
<td>30 PBSCs</td>
<td>ABI, WT, ulcer healing/2.4 mo</td>
<td>TA</td>
<td></td>
</tr>
<tr>
<td>Bartsch et al.</td>
<td>13 MNCs</td>
<td>ABI, TcPO2, VOP/2–13 months</td>
<td>TA</td>
<td></td>
</tr>
<tr>
<td>Cobellis et al.</td>
<td>10 MNCs (two infusions)</td>
<td>ABI, WT/12 months</td>
<td>Blood flow and capillary density</td>
<td></td>
</tr>
<tr>
<td>Matoba et al.</td>
<td>74 MNCs</td>
<td>QoL, WT, ABI, TcPO2/25.3 months</td>
<td>TA</td>
<td></td>
</tr>
<tr>
<td>Burt et al.</td>
<td>9 PBSCs (CD133⁺)</td>
<td>QoL/3–6 months</td>
<td>Leg perfusion</td>
<td></td>
</tr>
<tr>
<td>Mizuno et al.</td>
<td>8 MNCs + Plat + EPC into a ‘cultured dermal substitute’</td>
<td>ABI, angio, tomography, PS/6 months</td>
<td>TA</td>
<td></td>
</tr>
<tr>
<td>Lasala et al.</td>
<td>10 MNCs + MSCs</td>
<td>QoL, WT, ABI, TcPO2, angio; TA PS/12–18 months</td>
<td>TA</td>
<td></td>
</tr>
<tr>
<td>G. P. Lasala, submitted for publication</td>
<td>26 MNCs + MSCs</td>
<td>QoL, WT, ABI, PS/16.8 ± 5.9 TA months</td>
<td>TA</td>
<td></td>
</tr>
</tbody>
</table>

The studies depicted in this table correspond to non-randomized, single-group assignment, single-center Phase I clinical trials.

bPlat, Platelets; ABI, ankle-brachial pressure index; angio, angiography; QoL, quality of life; TcPO2, transcutaneous oxygen pressure; PS, 99 mTc-TF perfusion scintigraphy; VOP, venous occlusion plethysmography; WT, pain-free walking time; follow-up time: TA, therapeutic angiogenesis.
Clinical trials in progress utilizing cell therapy for the treatment of CLI

The National Institute of Health publishes clinical trials that have been approved by the Food and Drug Administration, European medicines agency and other national regulatory bodies under an identification number and activation status in clinicaltrials.gov as shown as of January 2011 in Table 2.

Areas of agreement

There is a general global consensus that stem cells are safe and could become a promising alternative for patients with severe LI who are not candidates for conventional therapies.

Areas of controversy

Cell types

As seen in Tables 1 and 2, there is a variety of cell products utilizing stem cells alone or in combination with other cells, multiple growth factors and/or biological products. In general terms, preliminary results from phase I and II trials suggest safety and improvement of CLI with various cellular products, including cell combinations (MSCs–MNCs) (G. P. Lasala, submitted for publication). Phase III clinical trials will help elucidate the degree of efficacy of these new and diverse cellular therapies.

Nevertheless, it is important to emphasize that the foundation on which all the clinical trials used so far is supported by conflicting information on the identification and characterization of EPCs, the ‘repair’ cell. This paradoxical topic connotes the lack of a unique EPC marker, the paucity of EPCs in circulation and the known phenotypic and functional overlapping between EPCs, hematopoietic cells and mature endothelial cells.
<table>
<thead>
<tr>
<th>Identifier</th>
<th>Location</th>
<th>Study condition</th>
<th>Phase</th>
<th>Cell product/patient enrollment</th>
<th>Main study design</th>
<th>Secondary outcome/time frame</th>
</tr>
</thead>
<tbody>
<tr>
<td>01257776</td>
<td>Spain</td>
<td>Recruiting</td>
<td>I/II</td>
<td>MSC/36</td>
<td>R; SE; OL; PA</td>
<td>ABI/1–12 months</td>
</tr>
<tr>
<td>00883870</td>
<td>India</td>
<td>Ongoing</td>
<td>I/II</td>
<td>MSC/20</td>
<td>R; PL; SE; PA</td>
<td>TcPO₂, ABI/6 months</td>
</tr>
<tr>
<td>00922389</td>
<td>India</td>
<td>Ongoing</td>
<td>II</td>
<td>PBSC + G-CSF/36</td>
<td>R; SE; OL; PA</td>
<td>TcPO₂/12 months</td>
</tr>
<tr>
<td>00371371</td>
<td>Nether-land</td>
<td>Recruiting</td>
<td>I/II</td>
<td>MNC/110</td>
<td>R; SE; OL; PA</td>
<td>Rest pain, TcPO₂, ABI/6 months</td>
</tr>
<tr>
<td>010019681</td>
<td>USA</td>
<td>Recruiting</td>
<td>I</td>
<td>Cord blood MSC/25</td>
<td>NR; UNCO; OL; SE</td>
<td>WT, QoL/24 months</td>
</tr>
<tr>
<td>01232673</td>
<td>Czech R</td>
<td>Ongoing</td>
<td>II</td>
<td>MNC/40</td>
<td>R; SE; PA; OL</td>
<td>ABI, ABI/4 months</td>
</tr>
<tr>
<td>01206865</td>
<td>China</td>
<td>Not yet open</td>
<td>I/II</td>
<td>Cord blood MSC/50</td>
<td>R; SC; SE; PA; OL</td>
<td>ABI, WT, ulcer healing/3 months</td>
</tr>
<tr>
<td>009193900</td>
<td>USA</td>
<td>Recruiting</td>
<td>I</td>
<td>CD133⁺/24</td>
<td>R; PA; DB; SE</td>
<td>Vascular hemodynamic function/6 months</td>
</tr>
<tr>
<td>00442143</td>
<td>Denmark</td>
<td>Recruiting</td>
<td>I</td>
<td>MNC/10</td>
<td>NR; UNCO; SE; SGA, 0L</td>
<td>ABI, TcPO₂, ulcer healing/4 months</td>
</tr>
<tr>
<td>00434616</td>
<td>Germany</td>
<td>Recruiting</td>
<td>II/III</td>
<td>MNC/90</td>
<td>R; PLCO; SE; PA; DB</td>
<td>ABI, TcPO₂, WT, angiography/3 months</td>
</tr>
<tr>
<td>00488020</td>
<td>MNC/10</td>
<td>Recruiting</td>
<td>I</td>
<td>UNCO; SE; PA; OL</td>
<td>QoL/6 months</td>
<td></td>
</tr>
<tr>
<td>00677404</td>
<td>Iran</td>
<td>Recruiting</td>
<td>I/II</td>
<td>MNC + G-CSF/50</td>
<td>R; UNCO; SE; PA; OL</td>
<td>ABI, WT, Rest pain/6 months</td>
</tr>
<tr>
<td>01245335</td>
<td>USA</td>
<td>Not yet open</td>
<td>III</td>
<td>MNC/210</td>
<td>R, SE; PA; DB</td>
<td>Change in Li classification, VAS, ulcer size/6 months</td>
</tr>
<tr>
<td>00282646</td>
<td>Germany</td>
<td>Recruiting</td>
<td>I/II</td>
<td>MNC/40</td>
<td>R; PLACO; SE; PA</td>
<td>ABI, TcPO₂, WT, ulcer size/6 months</td>
</tr>
<tr>
<td>00595257</td>
<td>India</td>
<td>Ongoing</td>
<td>I/II</td>
<td>MNC/60</td>
<td>R; PA; OL</td>
<td>Hemodynamic response/60 days</td>
</tr>
<tr>
<td>01269580</td>
<td>Italy</td>
<td>Recruiting</td>
<td>I</td>
<td>Vascular progen. cells/109</td>
<td>Observational; prospective</td>
<td>Not indicated/12 months</td>
</tr>
<tr>
<td>00919516</td>
<td>USA</td>
<td>Recruiting</td>
<td>I</td>
<td>MNC/36</td>
<td>UNCO; SE; SGA; OL</td>
<td>ABI, VAS, Ulcer healing/3 months</td>
</tr>
<tr>
<td>00306085</td>
<td>Italy</td>
<td>Recruiting</td>
<td>I</td>
<td>MNC/20</td>
<td>NR; PLACO; SE; SGA, DB</td>
<td>ABI, Ulcer healing, angiography/6 months</td>
</tr>
</tbody>
</table>

*Identifier, ClinicalTrials.gov NCT; CD34⁺, subset stem cells prepared from MNC; CD133⁺, subset stem cells prepared from MNC; G-CSF, granulocyte-colony-stimulating factor; DB, double blind; OL, open label; PA, parallel assignment; PLACO, placebo control; R, randomized; SE, safety/efficacy; SGA, single-group assignment; UNCO, uncontrolled; TcPO₂, transcutaneous oxygen pressure; VAS, pain assessment using a visual analogue scale.*
**Dose and route of administration**

Most of the trials have been performed with single doses, so that no conclusion can be drawn at this time. The intra-arterial and intramuscular (IM) routes have been tested. The intravascular route, which is more invasive and therefore has more complications, requires the use of IV contrast, which is contraindicated in patients with renal insufficiency. The IM route, used in more studies, is simple to perform and has shown safety and efficacy. Based on these simple facts, it is the opinion of the authors that the IM route should be the preferred method of delivery.

**Methods to evaluate efficacy**

The endpoints need to be measurable and comparable for efficacy. The primary endpoint in most studies has been amputation, death and amputation-free survival. The area of most controversy deals with assessment of efficacy as defined by improvements in clinical and hemodynamic parameters, as follows:

(i) ABI has been widely used, but it is unreliable in patients with calcified arteries who have normal or high ABIs. Patients with diabetes (DM) tend to have more calcified vessels and 41% of patients with CLI have DM. It is operator dependent when used with a blood pressure cuff and requires very well-trained personnel. This technique is now more reliable with the new commercially available oscillometry devices.

(ii) Transcutaneous oxygen pressure has been used in several trials, but it is influenced by skin thickness,36 blood pressure and temperature. It is operator dependent since it has to be taken in the exact same site as previous measurements.

(iii) Walking time has not been standardized for speed, distance and inclination. It is somewhat reliable in unilateral disease, but it losses accuracy in bilateral disease since it is unclear which of the extremities is responsible for the claudication.

(iv) Angiography is operator dependent since it relies on the amount of contrast and rate of infusion. It is difficult to quantify the interpreter’s findings, which usually requires more than two observers. In addition, it is contraindicated in renal insufficiency, a common co-morbidity in patients with CLI.

(v) Nuclear perfusion studies used exclusively in the extremities is unreliable, as the amount of radioactive isotopes injected at different times varies. It is the opinion of the authors that scintigraphy utilizing instead a comparison of ratio with other tissues (like, the brain muscle/brain ratio)4,11 appears to be the most reliable and reproducible method available at this time (G. P. Lasala, submitted for publication).
Growing points

New cellular products continue to emerge in addition to the therapeutic biological arsenal that has evolved over the last decade. The safety and efficacy of these new therapies is challenged by the ability of the scientific community to digest this rapidly evolving field.

Areas timely for developing research

Emerging therapeutic options, not yet fully developed, include the use of MSC stimulated with epidermal growth factor; EPCs in combination with allogeneic dermal substitutes, and induced pluripotent stem cells and/or embryonic stem cell.

Conclusions

The examination of the clinical trials for CLI performed over the last decade leave no doubt that biomedicine is facing a paradigm shift from traditional revascularization techniques to a completely new field of therapeutic opportunities, which includes CLI patients who have no re-vascularization options. They have proven safety with different degrees of efficacy. As clinical trials continue to develop and new discoveries arise, it is imperative to stay updated with these new therapies for untreated conditions.

Conflict of interest

Shareholders of TCA Cellular Therapy, LLC.

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TCA Cellular Therapy is a private research company owned by health care professionals who strongly believe in stem cell therapies.
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