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KRISZTINA SZÖKE, JAN E. BRINCHMANN

Key Words. Angiogenesis • Adipose tissue • Endothelial cells • Cellular therapy • Mesenchymal stem cells

ABSTRACT

Inadequate blood supply to tissues is a leading cause of morbidity and mortality today. Ischemic symptoms caused by obstruction of arterioles and capillaries are currently not treatable by vessel replacement or dilatation procedures. Therapeutic angiogenesis, the treatment of tissue ischemia by promoting the proliferation of new blood vessels, has recently emerged as one of the most promising therapies. Neovascularization is most often attempted by introduction of angiogenic cells from different sources. Emerging evidence suggests that adipose tissue (AT) is an excellent reservoir of autologous cells with angiogenic potential. AT yields two cell populations of importance for neovascularization: AT-derived mesenchymal stromal cells, which likely act predominantly as pericytes, and AT-derived endothelial cells (ECs). In this concise review we discuss different physiological aspects of neovascularization, briefly present cells isolated from the blood and bone marrow with EC properties, and then discuss isolation and cell culture strategies, phenotype, functional capabilities, and possible therapeutic applications of angiogenic cells obtained from AT.

INTRODUCTION

Inadequate blood supply to tissues is a leading cause of morbidity and mortality today. Narrowing of the vessels of the arterial tree may be caused by a range of diseases and environmental factors, with slightly different sets of etiological factors affecting large and medium-sized arteries and arterioles [1]. Obstructions of large and medium-sized arteries are frequently amenable to surgical or endovascular repair procedures. However, some of these procedures require the replacement of obstructed vessels with patent vessels obtained from other parts of the patient. The availability of redundant vessels is obviously limited. This has opened up a very active research field where the aim is to create arterial vessels by tissue engineering using biomaterials and autologous cells [2]. Further down the arterial tree, ischemic symptoms caused by obstruction of arterioles and capillaries are currently not treatable by replacement or dilatation procedures [3]. These symptoms most commonly occur in the limbs, where the disorder is called peripheral vascular disease (PVD), and in the heart, where arteriolar obstruction is one of the causes of refractory angina pectoris. Attempts to treat PVD using angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), or hepatocyte growth factor (HGF) have been disappointing [4]. Over the past decade, researchers have turned to the use of cells in attempts to produce neovascularization of ischemic tissues [5, 6]. In this concise review we briefly describe the mechanisms involved in de novo blood vessel formation, summarize the results of clinical trials using cells to treat microvascular tissue ischemia, and then focus on adipose tissue as a source of cells with therapeutic angiogenic potential.

NEOVASCULARIZATION—DE NOVO BLOOD VESSEL FORMATION

Formation of new blood vessels is a complex and integrated process that is not yet completely understood. It is important during embryological organogenesis, in the course of organ growth after birth, in the course of restoration of blood supply to ischemic tissues, and in the establishment of blood supply to tumors [5]. Neovascularization is the term used for the physiological processes of angiogenesis, vasculogenesis, and arteriogenesis, which represent different aspects of this complex process (Fig. 1). In angiogenesis, new microvessels are generated from pre-existing vasculature by the proliferation and migration of endothelial cells (ECs). These vessels play an important part in the repair mechanism of damaged tissues [5]. Hypoxia is an important stimulus for the expansion of the vascular bed, particularly through the effects of hypoxia-inducible factors (HIFs) [7]. HIFs upregulate angiogenic factors such as VEGF, which
stimulate both physiological and pathological angiogenesis. Vasculogenesis, on the other hand, refers to the formation of blood vessels by the recruitment and differentiation of undifferentiated endothelial progenitor cells at the site of new vessel growth [5]. This process is regulated by growth factors such as VEGF, FGF, transforming growth factor, and angiopoietin-1 and by their receptors, including VEGF receptor 1 (VEGFR1/FLT1), VEGFR2 (KDR/FLK1), and Tie-2 [8]. Until recently, the term vasculogenesis was used only to describe blood vessel formation in the embryo. However, this process has now also been shown to contribute to adult blood vessel formation [9, 10]. Arteriogenesis involves the expansive growth of collateral arteries by sprouting of pre-existing vessels to form collateral bridges between arterial networks via the migration and proliferation of ECs and SMCs. Growth factors and cytokines released endogenously in response to tissue ischemia act to promote neovascularization. Abbreviations: Ang 1, angiopoietin-1; EC, endothelial cell; EPC, endothelial progenitor cell; FGF, fibroblast growth factor; HIF, hypoxia-inducible factor; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor.

Figure 1. The processes of angiogenesis, vasculogenesis, and arteriogenesis. New microvessels are generated from pre-existing vasculature by the proliferation and migration of mature ECs in the classic process of new vessel growth, angiogenesis. Vasculogenesis involves participation of undifferentiated EPCs, which circulate to sites of new vessel growth, where they differentiate into mature ECs. Arteriogenesis involves the expansive growth of collateral arteries by sprouting of pre-existing vessels to form collateral bridges between arterial networks via the migration and proliferation of ECs and SMCs. Growth factors and cytokines released endogenously in response to tissue ischemia act to promote neovascularization. Abbreviations: Ang 1, angiopoietin-1; EC, endothelial cell; EPC, endothelial progenitor cell; FGF, fibroblast growth factor; HIF, hypoxia-inducible factor; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor.

ENDOTHELIAL CELLS IN BONE MARROW AND PERIPHERAL BLOOD

During embryogenesis, endothelial and hematopoietic lineages have common lineage precursors [11]. These common precursors, sometimes called hemangioblasts, are located in embryonic vessel walls at least through part of embryonic development.
Adipose tissue is a source of cells with angiogenic potential

Adult adipose tissue (AT) is one of the largest and most plastic tissues in the body. AT is the source of a range of hormones and cytokines, is a main reservoir of energy, and frequently goes through periods of expansion and shrinkage. Not surprisingly, AT is one of the most highly vascularized tissues in the body. A very close anatomical and physiological relationship has been demonstrated in AT between blood vessels, perivascular cells, and adipocyte precursor cells [21]. Mesenchymal stromal cells (MSCs) may differentiate to adipocytes and may be the earliest adipocyte precursor cells in AT. However, MSCs also have a role as perivascular cells, thus stabilizing new blood vessels [22, 23]. At the same time, the vasculature may have a causal role in the physiological functions of AT by controlling the number of microvessels and by remodeling existing vessels. Indeed, angiogenesis has been shown to be of great importance for the modulation of adipogenesis and obesity [24]. Thus, AT is an easily available, sometimes greatly superfluous tissue where new blood vessels are constantly being made in adult life.

The availability of ample amounts of tissue has generated a search for interesting and useful cell populations within AT. For this, variable amounts of liposuction material can be collected under local anesthesia by minimally invasive interference. After removal of blood from the liposuction material, the connective tissue keeping the remaining tissue together is digested using collagenase. Adipocytes, which make up the majority of the bulk of this tissue, are separated from other cells by gentle centrifugation. The pellet recovered from this centrifugation step is called the stromal vascular fraction (SVF) of AT. Analysis of SVF revealed that AT is a source of cells with multilineage differentiation potential [25, 26]. However, it soon became clear that SVF is, in fact, a heterogeneous population of cells. Several markers can be used to distinguish the populations contained within SVF, but the most useful may be CD31 and human leukocyte antigen (HLA) DR, which are molecules normally expressed on ECs. Upon phenotypic characterization of SVF, these molecules separate SVF cells into two populations: those coexpressing CD31 and HLA DR, approximately 20%–40% of the SVF, and those expressing neither of these [27]. After some weeks of in vitro culture, the plastic-adherent CD31⁺ HLA DR⁺ population expressed surface markers typical of MSCs [28]. These cells are frequently called adipose tissue-derived stem cells (ADSCs or ASCs), although we prefer to call them adipose tissue-derived mesenchymal stromal cells (AT-MSCs) to mark their ontogenetic relationship to bone marrow (BM) MSCs and at the same time distinguish them from other stem cells that may be found within the SVF. Transcription profiling analysis shows that the CD31⁺ HLA-DR⁺ cells within SVF overexpress transcripts associated with both arterial and venous endothelium and mostly resemble microvascular cells [27]. Under the culture conditions used in this study, which were optimized for MSC culture with Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium and no gelatin coat on the plastic surface, the CD31⁺ SVF cells did not proliferate in vitro. Later we successfully isolated and in vitro expanded CD31⁺ cells from AT using other cell culture conditions and showed that these were indeed bona fide ECs [29].
uncultured AT-MSCs to allow phenotypic and molecular charac-
terization. Comparisons of uncultured AT-MSCs with their cul-
ture-expanded offspring showed that plastic-adherent cell cul-
ture induced considerable differences in gene expression and
surface molecules [27]. Most notable, perhaps, was the expres-
sion of CD34 by practically all the uncultured AT-MSCs. This mol-
ecule, which is also expressed at low levels by most ECs, was lost
over the first few passages of plastic-adherent cell culture. Thus,
culture-expanded adipose-derived stem cells appear as a rela-
tively homogeneous population. They adhere to the definition of
MSCs provided by the International Society for Cell Therapy
based on their phenotype (CD73−, CD90+, CD105−, CD11b/
CD14+, CD19/CD73b, CD34+, CD45−, HLA DR−); their plastic-
adherent properties; and their multipotent differentiation poten-
tial to adipogenic, chondrogenic, and osteogenic lineages [28].

The CD31− population of SVF expresses very much lower levels
of mRNAs encoding EC molecules such as CD144, CD31,
vWF, VEGFR2, and VEGFR1 than does the uncultured CD31+ sub-
set of SVF [27]. At the same time, the CD31+ cells secrete a range
of soluble factors. Some, such as VEGF and HGF, are known to
promote neovascularization [32]. Using the mouse ischemic hind
limb model to determine the neovascularization potential, the
stromal cell fraction of mouse and human SVF was found to im-
prove angiogenesis mainly by the secretion of angiogenic growth
factors [33]. Similar mechanisms were shown to act when rat
AT-MSCs protected skin flaps against ischemia-reperfusion in-
jury [34]. However, other investigators showed that injected AT-
MSCs improved the ischemic score also by differentiation to
CD31+ ECs within ischemic tissues [35–37]. Yet other studies failed
to demonstrate the differentiation of adipose-derived cells toward
the endothelial lineage [38], possibly because of differences in pas-
sage number and culture conditions. Then, in 2008, a number of
studies appeared that suggested that MSC populations derive from
blood vessel walls and that they may be identical to the pericytes
[22, 39, 40]. A landmark paper by Crisan et al. described the in situ
and in vitro links between MSCs and pericytes, identifying a popu-
lation of CD146+CD34+CD45−CD56+ cells as pericytes in several
tissues [22]. These cells also expressed the classic MSC markers
CD44, CD73, CD90, and CD105 in vivo but did not express endo-
thelial markers CD31, CD144, vWF, or UEA-1. Crisan et al. con-
cluded that cultured perivascular cells from a variety of tissues
exhibit a phenotype that is very similar to that of BM-MSCs [22].
Because of their role as pericytes in most tissues, MSCs were now
suggested to have an important role in vasculogenesis by stabilizing
the vasculature [22, 23, 40–42]. Through interaction with ECs [23],
the MSCs are thus able to stimulate angiogenesis [35, 43]. In conclu-
sion, most evidence today suggests that the main role of AT-MSCs in
blood vessel biology may be as pericytes to secrete angiogenic fac-
tors and stabilize the interactions between ECs.

**ENDOTHELIAL CELLS IN ADIPOSE TISSUE**

Based on cell surface expression of CD31 and HLA DR, intracellu-
lar expression of vWF, and very high expression of mRNAs typical
of ECs, the CD31+ subset of SVF cells was considered to consist of
ECs, most likely microvascular ECs [29]. Previously, several at-
ttempts had been made to isolate ECs from SVF using plastic at-
chament techniques and positive selection strategies [44–48].

We recently used a combination of negative and positive immu-
nomagnetic isolation to derive a pure population of CD31+ cells
from AT [29]. Depending on the amount of liposuction starting
material, several tens of millions of uncultured ECs could be ob-
tained. These cells were readily expandable on a gelatin coat
with an endothelial culture medium supplemented with fetal
bovine serum (FBS). Later, we replaced the FBS with human
plasma supplemented with human platelet lysate (PLP). This has
enabled us to culture the ECs directly on plastic surfaces, which
means that the culture system is entirely humanized. These AT-
ECs proliferate rapidly through at least 15–20 population dou-
bings. Since the starting number of cells is already high, several
hundred millions of ECs may be obtained after a relatively brief
period of in vitro expansion. The AT-ECs form functional blood
vessels in Matrigel following subcutaneous injection into immu-
nodeficient mice. Interestingly, the vessel formation was more
dense and robust when AT-ECs were combined with the AT-
MSCs, suggesting that the MSCs adopt a supportive role similar
to that of pericytes under these conditions [29].

Nevertheless, the identity of the AT-ECs still remains contro-
versial. Since they express CD144 (vascular endothelial cadherin)
and vWF but do not express CD133, CD45, or CD14, they are not
likely to represent a population of early EPCs [49, 50]. Expression
of genetic markers typical of both the arterial and the venous
side of capillaries suggests that they may be microvascular endo-
thelial cells (MVECs). However, it has been shown that MVECs
strongly express CD141 [51], which was only weakly expressed or
absent on AT-ECs. Based on their phenotype (CD34+CD133−
vWF+CD144+VEGFR2+endothelial nitric oxide synthase−
CD31+), AT-ECs most resemble late outgrowth EPCs or ECFCs
[49, 50]. A population of ECFCs isolated from peripheral blood
has recently been described [20]. The isolation procedure was
different from that used to isolate EPCs [13], and the authors sug-
gested that these cells most closely resemble microvascular cells.
In collaboration with this group, we are now performing studies com-
paring the blood-derived ECFCs and the AT-ECs in terms of gene
expression, phenotype, and vessel-forming functionality. This study
should also help to clarify the somewhat confusing terminology
used for human ECs.

**THERAPEUTIC POTENTIAL OF ADIPOSE TISSUE-DERIVED
ANGIOGENIC CELLS**

AT, then, contains two populations of cells with different func-
tionalities that may contribute to neovascularization: the bona
fide ECs and AT-MSCs. These two nonoverlapping populations
make up more than 60% of the SVF and may be isolated in large
numbers from a relatively small amount of liposuction material.
Some clinical studies are based on uncultured SVF [52]. The adva-
tag is that isolation of SVF from liposuction material is a relative-
ly rapid procedure, which in fact may be performed au-
tomatically in the operating room [53–55]. The disadvantages
are the lower numbers of cells, a relatively uncontrolled mixture
of cell populations, and the fact that the functionality of uncul-
tured AT-ECs may be different from culture-expanded AT-ECs
[29]. For isolation of pure populations of AT-MSCs and AT-ECs,
negative immunomagnetic isolation procedures may be used for
both [27, 29]. This leaves no immunomagnetic beads in the re-
sulting cell population, a fact that should make the procedure
acceptable for cells to be used for treatment of patients. Both
cell populations are readily expandable during in vitro culture, and both may be cultured using human PLP, which makes the entire ex vivo expansion procedure free of xenogeneic proteins. Thus, the ex vivo isolation and culture procedure is likely to be acceptable to national regulatory authorities.

The availability of autologous ECs is likely to be crucially important both for the tissue engineering of arteries and for cell therapy for microvascular disease. Most ECs express HLA class II antigens [56]. Uncultured AT-ECs express HLA II molecules but lose these quickly from the surface upon cell culture [29]. Human AT-MSCs cultured in FBS express HLA II at the mRNA level but not on the surface [27]. However, according to our recent observation, when human PLP is used as a supplement, some of the AT-MSCs express HLA class II antigens. Both of these cell populations are likely to upregulate HLA class II molecules in an inflammatory environment. Thus, allogeneic angiogenic cells are likely to be rejected by an allospecific immune response directed toward their HLA class II molecules. The same may well be the fate for autologous angiogenic cells cultured in FBS, where xenogeneic antigens presented by autologous HLA class II molecules may induce an immune response. Autologous cells expanded in human medium supplements, however, are likely to be well tolerated in a transplantation situation.

However, there are still issues that need to be solved. One such issue is whether there are important phenotypic and functional differences in fat obtained from different sites [57]. The immunomodulatory property of AT-MSCs is also an important issue. It has been shown that AT-MSCs promote engraftment and prevent or treat severe graft-versus-host disease in allogeneic stem cell transplantation [58, 59]. Treatment with immunosuppressive cells might conceivably activate dormant infections or tumors, although results in this area are contradictory [60–63]. Also, in vitro culture of cells could activate transformation pathways and lead to tumor formation. MSCs are known to occasionally form tumors in mice [64], but neither tumors nor ectopic tissue formation following injections of MSC in humans has been reported after more than 10 years of follow-up [64–66]. Clinical application of AT-MSCs may therefore be considered to be safe. The preclinical and clinical experience with cultured ECs is still limited [67]. Thus, additional studies are needed to fully elucidate the safety and reproducibility of the in vitro expanded AT-ECs.

Based on the ready availability of large numbers of autologous cells, AT-ECs are likely to be attractive EC candidates for scientists involved in tissue engineering of arterial vessels. However, very few clinical trials using cell-based approaches to tissue engineer blood vessels have so far been performed [68]. In contrast, a huge number of clinical trials of stem cell therapy have been performed in attempts to moderate the outcome of another arterial disease, acute myocardial infarction (AMI) [69, 70]. Most of these have used uncultured populations of autologous cells derived from the bone marrow. The results of these trials are so far that “stem/progenitor cell treatment was not associated with statistically significant changes in the incidence of mortality [relative risk] 0.70, 95% CI 0.40–1.21] or morbidity (the latter measured by reinfarction, hospital readmission, restenosis and target vessel revascularization)” [69]. It is possible that the treatment outcome could have been improved by injection of autologous cells with angiogenic potential. However, AT-ECs and AT-MSCs need to be cultured in vitro to obtain the number of cells likely to induce neovascularization. This takes several weeks, by which time the acute phase of AMI has passed, and a therapeutic opportunity may have been lost. Cell culture-expanded autologene angiogenic cells from AT could be provided to patients in the acute phase of AMI, but as described above, these cells are likely to be rejected by alloimmune responses. Finally, relatively large numbers of uncultured autologous SVF cells may be procured within hours in an acute AMI situation. Human clinical trials using these cells are known to be under way, but no results have yet been published [52].

Ischemic symptoms caused by obstruction of arterioles and capillaries are not accessible to replacement or dilatation procedures and are currently treatable only by cell-based strategies for neovascularization. In the heart, this illness is called refractory angina pectoris. A number of clinical trials have been performed in groups of patients with refractory angina (Table 1). Most of these trials have used mononuclear cells from bone marrow (BM-MNCs). These are uncultured cells in which the fraction of hematopoietic stem cells is less than 1%, and the fraction of EPSCs is less than that. However, some recent studies have used cell culture-expanded MSCs (Table 1). All of these studies have reported beneficial effects, some even after long observation periods. The mechanism of the beneficial effect is uncertain. In fact, cells injected into the heart usually do not remain there very long; they migrate to the lung, spleen, and other organs [71]. Those cells that remain in the heart usually die or do not function. There may be several explanations for this. To survive, cells need the appropriate signals from their environment. This is particularly important for cells expanded in vitro adherent to molecules on plastic surfaces. These environmental signals may not be available in the myocardium. Also, cells injected into ischemic myocardium may find the microenvironment too hostile to promote survival. Recently we injected several different populations of human MSCs into the border zones of 1-week-old myocardial infarctions in immunodeficient rats [72]. They all induced surprisingly good functional improvement. At 4 weeks, only a small fraction of the injected cells could be recovered in the murine myocardium. This study and all other studies reporting beneficial effects of cells injected into ischemic hearts suggest that the benefit is mediated by paracrine factors [73]. A priori, a combination of autologous AT-ECs and AT-MSCs injected intramyocardially in patients with refractory angina should do better than any of the cells injected in studies published to date (Table 1), because the potential for direct contribution to neovascularization is considerably greater for these cells. To achieve this, however, the problem of the survival of cultured cells injected into myocardium needs to be solved.

Also for critical limb ischemia that has not been amenable to dilatation procedures, a number of cell-based trials to establish neovascularization have been performed (Table 2). Again, the cells most commonly used have been BM-MNCs, and clinical improvement has also been recorded in these groups of patients. Naturally, direct proof of involvement of the injected cells in the establishment of new blood vessels is not available, but in several of these studies evidence of improved blood supply to the ischemic regions could be demonstrated. In a mouse model of hind limb ischemia, evidence supporting the survival and direct contribution to new blood vessel formation by injected human AT-MSCs has been published [35–37]. This would suggest that the likelihood of survival of injected angiogenic cells in limb tissues is better than in the myocardium. If so, injection of combinations of autologous AT-ECs and AT-MSCs is likely to give an
even better clinical outcome than those reported in the studies cited in Table 2.

**CONCLUSION**

Traditional risk factors such as smoking, diabetes, hypercholesterolemia, hypertension, and age itself can lead to endothelial injury requiring repair of the vasculature. Surgical and catheter-based procedures are constantly improving the treatment options for many patients with tissue ischemia, but diseases mainly affecting arterioles and capillaries are likely to never be amenable to surgical or dilatation procedures. For these, cell-based therapeutic strategies will remain the best treatment options.

Two populations of cells with different functionalities exist in the SVF of AT that may contribute to neovascularization. Both may be used in the uncultured state, when the cell numbers will be lower but the cells are quickly available, and after in vitro expansion. Combinations of in vitro expanded AT-ECs and AT-MSCs yield robust vasculogenesis in Matrigel plugs injected into immunodeficient rodents, suggesting that these cells might also provide relief from ischemia in human clinical situations. However, several issues have to be addressed in order to get full therapeutic benefit from these cells. Most importantly, the cells must be seen to survive and directly contribute to new blood vessel formation. Another important issue is the mode of administration: should the cells be injected into the arterial tree supplying the ischemic area, or into the tissue actually exposed to ischemia? In addition, the role of supportive angiogenic cytokines and growth factors such as VEGF, FGF, HGF, and angiopoietin-1 remains unresolved. If one or more of these should be found to be beneficial, a decision has to be made whether the cytokines should be provided by supplemental injection or by genetically manipulating the injected cells.

The success of cell-based therapies depends on whether the engrafted cells differentiate into functional vascular cells and whether those cells can produce paracrine signals that encourage survival of the cells in the ischemic environment. Animal studies will be required to understand induced vasculogenesis in

Table 1. Clinical trials of cell-based therapy in therapeutic angiogenesis for refractory angina

<table>
<thead>
<tr>
<th>Clinical study</th>
<th>Type and no. of cells</th>
<th>Delivery</th>
<th>No. of patients/follow-up</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuchs et al. [74]</td>
<td>Unfractionated BM cells (32.6 ± 27.5 × 10^6/ml nucleated cells containing 2.6 ± 1.6% CD34&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>IM</td>
<td>10 patients/3 months</td>
<td>Improved CCSAC, exercise duration, stress-induced ischemia score</td>
</tr>
<tr>
<td>Vicario et al. [75]</td>
<td>Unfractionated BM cells (0.089 ± 0.023 × 10&lt;sup&gt;6&lt;/sup&gt;/kg nucleated cells)</td>
<td>IC</td>
<td>15 patients/12 months</td>
<td>Improved quality of life, CCSAC, myocardial perfusion</td>
</tr>
<tr>
<td>Fuchs et al. [76]</td>
<td>Unfractionated BM cells (28 ± 27 × 10&lt;sup&gt;6&lt;/sup&gt;/ml nucleated cells containing 2.2 ± 1.4% CD34&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>IM</td>
<td>27 patients/3 and 12 months</td>
<td>Improved CCSAC, exercise duration, stress-induced ischemia score</td>
</tr>
<tr>
<td>Beeres et al. [77]</td>
<td>BM-MNCs (84 ± 29 × 10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>IM</td>
<td>25 patients/12 months</td>
<td>Improved CCSAC, quality of life, LVEF, regional wall motion; reduced ischemic area</td>
</tr>
<tr>
<td>Tse et al. [78]</td>
<td>BM-MNCs (10&lt;sup&gt;5&lt;/sup&gt;/injection)</td>
<td>IM</td>
<td>12 patients/44 ± 10 months</td>
<td>No change in LVEF; most of the patients developed major cardiovascular events</td>
</tr>
<tr>
<td>Briguori et al. [79]</td>
<td>BM-MNCs (10&lt;sup&gt;7&lt;/sup&gt;/injection)</td>
<td>IM</td>
<td>10 patients/12 months</td>
<td>Improved CCSAC, myocardial perfusion, quality of life</td>
</tr>
<tr>
<td>Boyle et al. [80]</td>
<td>Mobilized CD34&lt;sup&gt;+&lt;/sup&gt; (66.9 ± 17.6 × 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>IC-XRF</td>
<td>5 patients/12 months</td>
<td>Improved CCSAC, quality of life</td>
</tr>
<tr>
<td>Tse et al. [81]</td>
<td>BM-MNCs (1 × 10&lt;sup&gt;6&lt;/sup&gt;/2 × 10&lt;sup&gt;6&lt;/sup&gt; per 0.1 ml)</td>
<td>IM</td>
<td>28 patients/6 months</td>
<td>Improved exercise tolerance, CCSAC, NYHA class, LVEF</td>
</tr>
<tr>
<td>Losordo et al. [82]</td>
<td>Mobilized CD34&lt;sup&gt;+&lt;/sup&gt; (5 × 10&lt;sup&gt;5&lt;/sup&gt;, 1 × 10&lt;sup&gt;5&lt;/sup&gt;, or 5 × 10&lt;sup&gt;6&lt;/sup&gt; cells/kg)</td>
<td>IM</td>
<td>24 patients/12 months</td>
<td>Improved angina frequency, CCSAC, exercise tolerance</td>
</tr>
<tr>
<td>Pompilio et al. [83]</td>
<td>CD34&lt;sup&gt;+&lt;/sup&gt; BMSCs (4–12 × 10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>IM</td>
<td>5 patients/12 months</td>
<td>Improved CCSAC, myocardial perfusion; increase in collateral score</td>
</tr>
<tr>
<td>van Ramshorst et al. [84]</td>
<td>BM-MNCs (100 × 10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>IM</td>
<td>50 patients/6 months</td>
<td>Improved CCSAC, LVEF, quality of life, summed stress score</td>
</tr>
<tr>
<td>Hosse et al. [85]</td>
<td>BM-MNCs (2 × 10&lt;sup&gt;6&lt;/sup&gt;/injection)</td>
<td>IM</td>
<td>8 patients/18 months</td>
<td>Improved CCSAC; reduced ischemic area</td>
</tr>
<tr>
<td>Wang et al. [86]</td>
<td>BM-CD34&lt;sup&gt;+&lt;/sup&gt; (5.6 ± 2.3 × 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>IC</td>
<td>112 patients/6 months</td>
<td>Improved CCSAC, myocardial perfusion, exercise capacity; reduced angina frequency episodes</td>
</tr>
<tr>
<td>Lasala et al. [87]</td>
<td>BM-MSCs + BM-MNCs (7.5 × 10&lt;sup&gt;6&lt;/sup&gt;/population)</td>
<td>IC</td>
<td>10 patients/6 months</td>
<td>Improved LVEF, quality of life</td>
</tr>
<tr>
<td>Friis et al. [88]</td>
<td>BM-MSCs derived EPCs (21.5 × 10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>IM</td>
<td>31 patients/6 months</td>
<td>Improved LVEF, exercise capacity, clinical symptoms</td>
</tr>
<tr>
<td>Losordo et al. [89]</td>
<td>Mobilized CD34&lt;sup&gt;+&lt;/sup&gt; (1 × 10&lt;sup&gt;5&lt;/sup&gt; or 5 × 10&lt;sup&gt;5&lt;/sup&gt; cells/kg)</td>
<td>IM</td>
<td>167 patients/12 months</td>
<td>Improved angina frequency, exercise tolerance</td>
</tr>
<tr>
<td>Tuma et al. [90]</td>
<td>BM-MNCs (8.19 ± 4.3 × 10&lt;sup&gt;6&lt;/sup&gt; + CD34&lt;sup&gt;+&lt;/sup&gt; (1.65 ± 1.42 × 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>PRCSP</td>
<td>14 patients/24 months</td>
<td>Improved CCSAC, LVEF; reduced ischemic area</td>
</tr>
<tr>
<td>Haack-Sørensen et al. [91]</td>
<td>BM-MSCs (21.5 × 10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>IM</td>
<td>31 patients/12 months</td>
<td>Improved CCSAC, nitroglycerine consumption, physical limitation, angina frequency, quality of life</td>
</tr>
</tbody>
</table>

Abbreviations: BM, bone marrow; BM-MNC, bone marrow mononuclear cell; BM-MSC, bone marrow mesenchymal stem cell; CCSAC, Canadian Cardiovascular Society Angina Classification; EPC, endothelial progenitor cell; IC, intracoronary infusion; IC-XRF, intracoronary-x-ray fluoroscopy; IM, intramyocardial injection; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association; PRCSP, percutaneous retrograde coronary sinus perfusion.
Table 2. Clinical trials of cell-based therapy in therapeutic angiogenesis for critical limb/hand ischemia

<table>
<thead>
<tr>
<th>Clinical study</th>
<th>Type and no. of cells</th>
<th>Delivery</th>
<th>No. of patients/follow-up</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lenk et al. [92]</td>
<td>CPCs (CD34&lt;sup&gt;+&lt;/sup&gt; CD144&lt;sup&gt;+&lt;/sup&gt;) (39 ± 24 x 10⁶)</td>
<td>IA</td>
<td>7 patients/12 weeks</td>
<td>Improved pain-free walking distance, ABI, tissue blood perfusion</td>
</tr>
<tr>
<td>Huang et al. [93]</td>
<td>Mobilized PB-MNCs (3 x 10⁹)</td>
<td>IM</td>
<td>28 patients/3 months</td>
<td>Less pain; smaller ulcer size; improved tissue blood perfusion and ABI; increased limb salvage</td>
</tr>
<tr>
<td>Koshikawa et al. [94]</td>
<td>BM-MNCs (3.67 ± 0.53 x 10⁹), CD34&lt;sup&gt;+&lt;/sup&gt; (4.94 ± 2.45 x 10⁹); CD34&lt;sup&gt;+&lt;/sup&gt; CD133&lt;sup&gt;+&lt;/sup&gt; (2.52 ± 1.57 x 10⁹)</td>
<td>IM</td>
<td>7 patients/6 months</td>
<td>Improved perfusion and ulcer size; less pain</td>
</tr>
<tr>
<td>Bartsch et al. [95]</td>
<td>BM-MNCs (83 ± 34 x 10⁹)</td>
<td>IM and IA</td>
<td>13 patients/13 months</td>
<td>Less pain; disappearance of gangrene; neovascularization</td>
</tr>
<tr>
<td>Wester et al. [100]</td>
<td>BM-MNCs (1.3 ± 6.67 x 10²)</td>
<td>IM</td>
<td>12 patients/24 weeks</td>
<td>Improved ABI, rest pain, and pain-free walking time</td>
</tr>
<tr>
<td>Chochola et al. [101]</td>
<td>BM-MNCs (3.49 ± 10⁶)</td>
<td>IA</td>
<td>24 patients/1 year</td>
<td>Improved limb salvage; improved ulcer healing and collateral vessel development</td>
</tr>
<tr>
<td>Cobellis et al. [102]</td>
<td>BMCs (10⁹)</td>
<td>IA</td>
<td>10 patients/12 months</td>
<td>Improved tissue blood perfusion, ABI, capillary densities, and pain-free walking distance</td>
</tr>
<tr>
<td>Napoli et al. [103]</td>
<td>BMcs + oral antioxidants, L-arginine therapy (5 x 10⁴/ml)</td>
<td>IA</td>
<td>36 patients/18 months</td>
<td>Improved pain-free walking distance, ABI, and ulcer healing</td>
</tr>
<tr>
<td>Kawamoto et al. [104]</td>
<td>Mobilized CD34&lt;sup&gt;+&lt;/sup&gt; (10⁵ or 5 x 10⁵) or 10⁶ cells/kg</td>
<td>IM</td>
<td>17 patients/12 weeks</td>
<td>Improved ulcer size, exercise capacity, and transcutaneous partial oxygen pressure; less pain</td>
</tr>
<tr>
<td>Amann et al. [105]</td>
<td>BM-MNCs (1.1 ± 1.1 x 10⁹)/BM total nucleated cells (3 ± 1.7 x 10⁶)</td>
<td>IM</td>
<td>51 patients/6 months</td>
<td>Increased limb salvage; improved ABI, pain-free walking distance, and tissue blood perfusion</td>
</tr>
<tr>
<td>Burt et al. [106]</td>
<td>CD133&lt;sup&gt;+&lt;/sup&gt; (2.5–5 x 10⁶/injection)</td>
<td>IM</td>
<td>9 patients/12 months</td>
<td>Improved amputation-free survival, quality of life, exercise capacity, perfusion, and collateral formation; less pain</td>
</tr>
<tr>
<td>Lasala et al. [107]</td>
<td>BM-MNCs (30 ± 10³) with BM-MSCs (30 ± 10⁶)</td>
<td>IA</td>
<td>10 patients/10 ± 2 months</td>
<td>Improved quality of life, exercise capacity, perfusion, and collateral formation</td>
</tr>
<tr>
<td>Kolvenbach et al. [108]</td>
<td>BMcs (17.2 ± 10⁶ CD34&lt;sup&gt;+&lt;/sup&gt;; 7.8 ± 10⁶ CD133&lt;sup&gt;+&lt;/sup&gt;; 0.5–5.7 x 10⁶ VEGFR2&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>IM</td>
<td>8 patients/9.2 months</td>
<td>Improved ABI</td>
</tr>
<tr>
<td>Lara-Hernandez et al. [109]</td>
<td>Mobilized EPCs (CD34&lt;sup&gt;+&lt;/sup&gt; CD133&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>IM</td>
<td>28 patients/14 months</td>
<td>Improved ABI; less pain; increased limb salvage</td>
</tr>
<tr>
<td>Sprengers et al. [110]</td>
<td>EPCs/BM-MNCs</td>
<td>IA</td>
<td>110–160 patients</td>
<td>Ongoing</td>
</tr>
<tr>
<td>Procházková et al. [111]</td>
<td>BM-MSCs</td>
<td>IM</td>
<td>96 patients/4 months</td>
<td>Increased limb salvage</td>
</tr>
<tr>
<td>Walter et al. [112]</td>
<td>BM-MNCs (87 ± 29 x 10⁹ or 178 ± 113 x 10⁹)</td>
<td>IA</td>
<td>40 patients/30.2 months</td>
<td>Improved ulcer healing; reduced rest pain; negative limb perfusion</td>
</tr>
<tr>
<td>Powell et al. [113]</td>
<td>BM-MNCs (136 ± 41 x 10⁹)</td>
<td>IM</td>
<td>46 patients/6 months</td>
<td>Improvement in amputation-free survival and ulcer healing</td>
</tr>
<tr>
<td>Idei et al. [114]</td>
<td>BM-MNCs (1.8 ± 0.5 x 10⁹), CD34&lt;sup&gt;+&lt;/sup&gt; (3.5 ± 1.4 x 10⁹)</td>
<td>IM</td>
<td>97 patients/4.8 years</td>
<td>Improved pain-free walking time, ulcer healing, tissue blood perfusion, and ABI</td>
</tr>
<tr>
<td>Lu et al. [115]</td>
<td>BM-MSCs/BM-MNCs</td>
<td>IM</td>
<td>41 patients/24 weeks</td>
<td>Improved ABI, amputation-free survival, perfusion index, ulcer healing, and quality of life</td>
</tr>
<tr>
<td>Murphy et al. [116]</td>
<td>BM-MNCs (1.7 ± 0.7 x 10⁹)</td>
<td>IM</td>
<td>29 patients/1 year</td>
<td>Improved ABI, amputation-free survival, perfusion index, ulcer healing, and quality of life</td>
</tr>
<tr>
<td>Perin et al. [117]</td>
<td>BM-MNCs vs. aldehyde dehydrogenase bright BM-MNCs (1.3 ± 1 x 10⁹ vs. 1.36 ± 0.59 x 10⁹)</td>
<td>IM</td>
<td>21 patients/12 weeks</td>
<td>Improved ABI; unchanged ulcer grade</td>
</tr>
<tr>
<td>Gabr et al. [118]</td>
<td>BM-MNCs (1.11 ± 10⁹)</td>
<td>IM</td>
<td>20 patients/3 months</td>
<td>Improved pain-free walking distance, resting pain, skin conditions, and ABI</td>
</tr>
</tbody>
</table>

Abbreviations: ABI, ankle brachial index; BMC, bone marrow cell; BM-MNC, bone marrow mononuclear cell; BM-MSC, bone marrow mesenchymal stem cell; CLI, critical limb ischemia; CPC, circulating blood-derived progenitor cell; EPC, endothelial progenitor cell; IA, intra-arterial injection; IM, intramuscular injection; PB-MNC, peripheral-blood mononuclear cell; VEGFR, vascular endothelial growth factor receptor.
the suboptimal ischemic vascular environment and to ensure that treatment with angiogenic cells is safe. Issues such as nurturing the local environment and appropriate delivery methods are key issues that need to be resolved before successful regenerative therapies will be effective in patients.

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AUTHOR CONTRIBUTIONS
K.S.: conception and design, collection and/or assembly of data, manuscript writing; J.E.B.: conception and design, manuscript writing, financial support, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST
The authors indicate no potential conflicts of interest.
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