Perivascular Delivery of Encapsulated Mesenchymal Stem Cells Improves Postischemic Angiogenesis Via Paracrine Activation of VEGF-A

Rajesh Katare,* Federal Riu,* Jonathan Rowlinson, Andrew Lewis, Rachel Holden, Marco Meloni, Carlotta Reni, Christine Wallrapp, Costanza Emanueli, Paolo Madeddu

Objective—To test the therapeutic activity of perivascular transplantation of encapsulated human mesenchymal stem cells (MSCs) in an immunocompetent mouse model of limb ischemia.

Approach and Results—CD1 mice underwent unilateral limb ischemia, followed by randomized treatment with vehicle, alginic microbeads (MBs), MB-encapsulated MSCs (MB-MSCs), or MB-MSCs engineered with glucagon-like peptide-1. Treatments were applied directly in the perivascular space around the femoral artery. Laser Doppler and fluorescent microsphere assessment of blood flow showed a marked improvement of perfusion in the MB-MSCs and MB-MSCs engineered with glucagon-like peptide-1 groups, which was associated with increased foot salvage particularly in MB-MSCs engineered with glucagon-like peptide-1–treated mice. Histological analysis revealed increased capillary and arteriole density in limb muscles of the 2 MSC groups. Furthermore, MB-MSCs engineered with glucagon-like peptide-1 and, to a lesser extent, MB-MSC treatment increased functional arterial collaterals alongside the femoral artery occlusion. Analysis of expression changes in ischemic muscles showed that MB-MSC transplantation activates a proangiogenic signaling pathway centered on vascular endothelial growth factor A. In contrast, intramuscular MB-MSCs caused inflammatory reaction, but no improvement of reparative vascularization. Importantly, nonencapsulated MSCs were ineffective either by intramuscular or perivascular route.

Conclusions—Perivascular delivery of encapsulated MSCs helps postischemic reperfusion. This novel biological bypass method might be useful in patients not amenable to conventional revascularization approaches.

(Key Words: collateral circulation • peripheral artery disease • stem cells)

Peripheral artery disease (PAD) affects up to 15% of people >55 years.1 Critical limb ischemia (CLI) is the end stage of lower extremity PAD in which severe obstruction of blood flow results in ischemic rest pain, ulcers, and high risk for limb loss. Surgical bypass surgery or percutaneous revascularization, the gold standard for the treatment of PAD, produces long-term benefit with a 5-year limb salvage rate of >80%.2 However, ~30% of patients with CLI cannot be revascularized because of multivascular disease or occlusions of small-caliber blood vessels, which are common in patients with diabetes mellitus and hypertension.3

Gene and stem cell therapies have been accredited to provide a possible alternative to interventional angioplasty.4,5 Clinical trials using bone marrow–derived mononuclear cells showed significant benefit, including improvement of ankle brachial index, transcutaneous partial pressure of oxygen, reduction of pain, and decreased need for amputation.6–9 Intramuscular, intra-arterial injection, or a combination of both represents the preferred route of cell therapy in clinical trials. Both the methods have limitations depending on the disease pattern. Intra-arterial infusion is not ideal for patients with occluded femoral artery because the stem cells will not reach the affected site by blood flow, whereas intramuscular delivery requires multiple injections to maximize the extension of therapeutic benefit. In BONE Marrow Outcomes Trial 1&2 (BONMOT-1&2), injections were placed instead along the occluded arteries with the intention to increase the formation of collaterals bypassing the occlusion.10 A common drawback of the above methods is that cell retention is generally low because of massive apoptosis in the
first few days after implantation. Microencapsulation strategies, surrounding the cells with a semipermeable polymeric membrane, have been proposed for enhancing cell viability. Here, the aim is to use the cells as local drug delivery factories, to secrete a cocktail of beneficial factors to elicit a therapeutic paracrine effect. Recently, we have developed a novel miniaturized encapsulation procedure where stem cell–containing core beads are surrounded with a permeable shell of biocompatible alginate, which provides a robust immunosuppressive barrier, whereas allowing for diffusion of inherent paracrine factors, such as vascular endothelial growth factor (VEGF). Furthermore, the optimized shell-to-core ratio ensures full cell viability. This new formulation (CellBeads) contains human mesenchymal stem cells (MSCs) genetically modified to express glucagon-like peptide-1 (GLP-1), an incretin hormone that has antiapoptotic, proangiogenic, and cardioprotective effects. Intracoronary injection of CellBeads passed feasibility and safety tests and showed therapeutic benefit in a porcine model of acute myocardial infarction. In addition, CellBeads have been evaluated in a clinical study to treat patients with posthemorrhagic stroke to limit the ensuing apoptotic damage. Based on these encouraging data, we propose that CellBeads might be used as an off-the-shelf cell product for wide-scale treatment of CLI.

Therefore, in view of clinical application, the present study investigates the feasibility of CellBead delivery into the perivascular space surrounding the occluded femoral artery and the efficiency of CellBead transplantation in improving reparative angiogenesis and collateralization in an immunocompetent mouse model of limb ischemia.

## Results

### Feasibility of Perivascular Cell Delivery

To assess the retention of microbeads (MB), cryosections from en bloc muscle samples were collected at day 7 after ischemia induction. Hematoxylin and eosin staining of these samples demonstrated the presence of MB-MSCs engineered with GLP-1 (MB-MSC-GLP) surrounding the femoral artery and vein, thus, confirming the feasibility of the procedure (Figure 1A). Furthermore, cell-transplanted muscles showed increased capillary (P<0.02 versus vehicle) and arteriole density (P<0.05 versus vehicle) at the site of implantation (Figure 1B–1D). Arterioles were oriented coaxially with the femoral artery.

### Improvement of Clinical Outcomes

Limb salvage (no necrotic toe) occurred in 33% and 42% of mice given vehicle or MB, respectively. This outcome was improved by MSC transplantation, with 66% salvage in MB-MSC and 75% in MB-MSC-GLP–treated groups. Global clinical outcome considering the number of mice

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**Figure 1.** A, Representative hematoxylin-eosin staining showing the persistence of microbeads encapsulated with mesenchymal stem cells transfected with glucagon-like peptide-1 (MB-MSC-GLP) along the femoral artery and vein at 7 days postischemia. B, Representative microphotographs from fluorescence microscopy showing MB-MSC-GLP, isocitric-B4 positive capillaries (box, blue arrowhead) and α-smooth muscle actin positive arterioles (box, pink arrowhead) around the femoral artery and vein. C, Bar graphs showing the quantitative analysis for the number of capillaries and arterioles around femoral artery and vein. D, Bar graph showing arteriole density. Scale bars, 100 μm. Data represented as mean±SE, n=4 per group. *P<0.05 and **P<0.01 vs Vehicle.
with ≥1 necrotic toes or foot necrosis/amputation is illustrated in Figure 2A. Analysis of contingency by the χ² test was not applicable because the data set comprised several values <1, and <20% of the values were >5. Therefore, an arbitrary score from 0 (no necrosis) to 6 (foot necrosis/amputation) was computed and used to compare the experimental groups. ANOVA detected a difference among groups pertaining to the severity score (P<0.01). MB-MSC-GLP–treated mice showed the lowest severity score (0.4±0.3; P<0.01 versus vehicle and P<0.05 versus MB), followed by MB-MSC (1.2±0.6), MB (2.4±0.7) and vehicle (3.0±0.7). Computation of Cohen d coefficient indicates a large biological effect of MB-MSC-GLP as compared with vehicle (1.3) and MB (1.1).

Improvement of Perfusion

Laser Doppler blood flow recovery was different among groups (2-way ANOVA, group effect: P<0.0001). Bonferroni multiple comparison analysis indicates an improvement of blood flow in MB-MSC-GLP– and MB-MSC–treated groups (P<0.001 versus vehicle or MB from day 7 to day 21; Figure 2B). Cohen d coefficient indicates that the biological effect of MB-MSC-GLP and MB-MSC treatments is large (2.8 and 2.4 versus vehicle and 2.0 and 1.9 versus MB, respectively). In contrast, no difference was observed between MB-MSC and MB-MSC-GLP groups.

Analysis of blood flow to the adductor muscle at 21 days postischemia using fluorescent microspheres indicates a significant difference among groups (ANOVA, group effect: P=0.02), with the MB-MSC-GLP group showing a large improvement compared with vehicle (P<0.01; Cohen d coefficient, 2.6) or MB (P<0.05; Cohen d coefficient, 1.9; Figure 2C). Moreover, the MB-MSC group showed higher blood flow levels compared with vehicle (P<0.05; Cohen d coefficient, 1.9). No difference was detected between MB-MSC and MB-MSC-GLP treatments. Moreover, no group difference was observed in perfusion of contralateral adductor.

Similarly, P O₂ levels in the ischemic adductor were markedly improved in the MB-MSC-GLP– or MB-MSC–treated groups compared with vehicle or MB (ANOVA, P<0.0001; Figure 2D). The effects in both treatment groups can be considered very large (Cohen d coefficient, 3.7 and 3.4, respectively).

Figure 2. A, Stacked bars showing % of necrotic toes at 2 days postischemia. B, Representative Doppler images at 21 days postischemia and line graph showing blood flow recovery. Green and yellow squares delimit the ischemic and contralateral foot, respectively; n=12 per group. C, Bar graph showing adductor muscle blood flow at 21 days, as assessed by fluorescent microspheres; n=5 per group. D, Bar graph showing the level of P O₂ at 21 days; n=9 to 12 mice per group. Data are mean±SE. *P<0.05, **P<0.01, and ***P<0.001 vs Vehicle; #P<0.05 and ###P<0.001 vs microbeads (MB). MB-MSCs indicate MB encapsulated with mesenchymal stem cells; and MB-MSC-GLP, MB encapsulated with mesenchymal stem cells transfected with glucagon-like peptide-1.
Promotion of Reparative Angiogenesis

Immunofluorescence microscopy showed a significant increase in the number of capillaries in ischemic adductor muscles of MB-MSC–transplanted mice (879±16 capillaries/mm²) compared with vehicle (673±13 capillaries/mm²; P<0.001; Figure 3A). Similarly increased were small arterioles (15.3±0.5 versus 9.1±0.3 arterioles/mm² in vehicle; P<0.001; Figure 3B). Analogous improvements were observed in the MB-MSC-GLP–treated group (891±20 capillaries/mm² and 14.6±0.8 arterioles/mm²; P<0.001 versus vehicle for both comparisons; Figure 3A and 3B). The effect of MB-MSC and MB-MSC-GLP treatments on neovascularization was very large (Cohen d coefficient, 5.5 and 5.0 for capillaries and 6.5 and 3.6 for arterioles, respectively). Use of MB alone did not produce any improvement (736±13 capillaries/mm² and 10±0.5 arterioles/mm²; P=NS versus vehicle; Figure 3A and 3B).

Collateral Formation Along the Occluded Artery

Cell therapy approaches mainly enhance the microvasculature downstream to the vascular occlusion. However, unless the artery blockage is removed or bypassed, it is unlikely that the increased microvascular bed will restore optimal perfusion. We verified whether the increased muscular blood flow and oxygenation observed in our study was associated with an increased collateralization along the femoral artery, and whether these collaterals were functionally operative. We found that MB-MSC-GLP– and MB-MSC–treated mice have an increased number of capillaries (460±12 and 389±23 versus 177±10 capillaries/mm² in vehicle; P<0.001; Figure 3C), small arterioles (10.3±0.3 and 9.0±0.4 versus 5.7±0.2 arterioles/mm² in vehicle; P<0.01; Figure 3D), and large arterioles (4.0±0.2 and 3.6±0.2 versus 2.2±0.2 arterioles/mm² in vehicle; P<0.01; Figure 3D) in the implant site around the arterial occlusion. The effects induced by MB-MSC-GLP and MB-MSC treatments were very large (Cohen d coefficient: 10.8 and 4.8 for capillaries, 7.6 and 4.7 for small arterioles, and 4.1 and 3.2 for large arterioles, respectively). Interestingly, pairwise comparison indicates that MB-MSC-GLP is superior to MB-MSC treatment in increasing capillary and small arteriole density (P<0.05 for both comparisons; Cohen d coefficient, 1.5 and 1.6 for capillaries and arterioles, respectively). Treatment with MB did not affect local vascularization (218±4 capillaries/mm², 5.3±0.6 small arterioles/mm² and 2.3±0.3 large arterioles/mm²; P=NS versus vehicle).

To verify the functional status of periocclusional vascularization, whole-mount preparations of the implantation site were analyzed after intravenous injection of the endothelial marker isocitron. Three-dimensional reconstruction of confocal microscopy images showed numerous functional capillaries and arterioles bridging the space between implanted MB-MSC-GLP and MB-MSC and the ischemic muscles (Figure 4; Movie I in the online-only Data Supplement).

Activation of Angiogenic Factors

To verify the mechanisms for improved angiogenesis, we measured gene and protein expression in limb muscles at 7 days after ischemia. Results of cytometric bead arrays show the expression of human angiogenic proteins in ischemic muscles of MB-MSC– and MB-MSC-GLP–treated mice (Figure 5), indicating persistance of paracrinally active human cells.

Figure 3. A and B, Representative microphotographs and bar graphs showing capillary (A) and arteriole (B) density in the ischemic adductor muscle at 21 days postischemia. Scale bars are 100 µm. C and D, Bar graphs showing capillary (C) and arteriole (D) density around femoral artery and vein (at the site of delivery of cells or vehicle). Data are means±SE, n≥5 in each group. ***P<0.001 vs Vehicle; ###P<0.001 vs microbeads (MB); ΦP<0.05 vs MB encapsulated with mesenchymal stem cells (MB-MSCs). MB-MSC-GLP indicates MB encapsulated with mesenchymal stem cells transfected with glucagon-like peptide-1.
Furthermore, RT-profiler polymerase chain reaction array showed a marked activation of murine genes associated with angiogenesis, such as endoglin, VEGF-A, sphingosine kinase 1, angiopoietin 4, interleukin-8, and heparanase, in ischemic muscles of MB-MSC– and MB-MSC-GLP–treated mice compared with vehicle (Table). Notably, the 2 treatments markedly reduced the expression of β2-microglobulin, which is a biomarker of peripheral vascular disease, 23 as well as the expression of several antiangiogenic genes, such as tissue inhibitor of metalloproteinase 2, tissue inhibitor of metalloproteinase 3, and thrombospondin 2, and proinflammatory genes associated with T lymphocytes induction of MSC apoptosis, including interferon-γ and tumor necrosis factor-α. Moreover, quantitative polymerase chain reaction confirmed the expression of the GLP transgene in limb muscles injected with MB-MSC-GLP (Figure III in the online-only Data Supplement).

The interaction of differentially regulated genes was then investigated using the STRING database (Figure 6). Results indicate that transplantation of MB-MSCs interferes with a molecular network centered on VEGF-A, interleukin-6, interferon-γ, and tumor necrosis factor-α, leading to changes in the balance between angiogenic mediators (the chemokines CXCL3 and CCL2/MCP1, the membrane glycoprotein endoglin, and the S1P activator sphingosine kinase 1), stabilizers of vascular growth (angiopoietin 4 and thrombospondin 1), and enzymes implicated in activation (heparanase) or inhibition (tissue inhibitor of metalloproteinases) of extracellular matrix degradation.

**Importance of Cell Delivery Route and Encapsulation**

Next, we evaluated the benefit of direct transplantation of encapsulated MSC in ischemic muscles. Intramuscular delivery of MB-MSCs was ineffective in improving the blood flow recovery or muscular capillary density in immunocompetent mice with limb ischemia (Figure IVA and IVB in the online-only Data Supplement). Furthermore, bead injection caused distortion of the muscle structure (Figure IVCi and IVCii in the online-only Data Supplement) and marked infiltration of inflammatory cells expressing isoelectin and the macrophage/microglial marker F4/80 (Figure IVCiii–IVCvi in the online-only Data Supplement). Therefore, perivascular delivery of the current CellBead formulation seems to be superior to intramuscular injection.

In separate experiments, we evaluated the therapeutic activity of perivascular or intramuscular transplantation of nonencapsulated MSCs. In both cases, allogeneic cell therapy was unable to produce improvement of blood flow recovery in immunocompetent mice with limb ischemia (Figure VA and VE in the online-only Data Supplement). Furthermore, no difference was observed in the capillary and arteriole density of muscles directly injected with nonencapsulated MSCs or vehicle (Figure VB–VD in the online-only Data Supplement). Perivascular delivery of nonencapsulated MSCs caused a mild increase in capillary density of the ischemic muscle (P<0.04 versus vehicle), but no change in arteriole density (Figure VF–VH in the online-only Data Supplement). To verify whether the lack of therapeutic activity is related to the rapid clearance of cells that are not protected by an immunoisolating shell, we next measured the expression of human angiogenic proteins in mice given nonencapsulated MSCs (either intramuscularly or perivascularly) or vehicle. Human proteins were undetectable in ischemic murine muscles from all studied groups. These data suggest functional inactivation of nonencapsulated MSCs at 7 days from transplantation in perivascular or interstitial muscular space.

**Discussion**

This study is the first to show the therapeutic activity of a cell product consisting of encapsulated, genetically modified MSCs, which were delivered perivascularly in a mouse model of limb ischemia. On stabilization at the implantation site, the cell product induces microvascular angiogenesis in the ischemic muscle as well as collateralization alongside the occluded femoral artery, leading to restoration of perfusion, oxygenation, and remarkable improvement of limb salvage. The therapeutic effect is mainly attributable to paracrine modulation of a molecular network in which VEGF-A represents the central hub.

The originality in the design of CellBeads consists of a miniaturized 2-step encapsulation, which is critical for the product
performance because it allows for improved bioavailability and tolerability (Figure I in the online-only Data Supplement). This technology will only work with nontumorigenic cell lines because proliferating cells would burst the specifically designed capsule and result in the loss of function. Importantly, independent investigation on toxicity and tumorigenicity on CellBeads is reassuring

Table. Modulation of Pro- and Antiangiogenic Genes in MB-MSC– and MB-MSC-GLP–Treated Groups Compared With the Vehicle as Measured by the RT-Profiler PCR Angiogenesis Array

<table>
<thead>
<tr>
<th>Genes</th>
<th>Description</th>
<th>Fold Changes in MB-MSCs vs Vehicle</th>
<th>Fold Changes in MB-MSC-GLP vs Vehicle</th>
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<tr>
<td>ANGPTL4</td>
<td>Angiopoietin 4</td>
<td>2.11</td>
<td>2.30</td>
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<tr>
<td>CCL2</td>
<td>Chemokine (C-C motif) ligand 2</td>
<td>1.80</td>
<td>2.14</td>
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<td>CXCL3</td>
<td>Chemokine(C-X-C motif) ligand 3</td>
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<td>ENG</td>
<td>Endoglin</td>
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<tr>
<td>HPSE</td>
<td>Heparanase</td>
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<td>2.89</td>
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<tr>
<td>IL8</td>
<td>Interleukin-8</td>
<td>2.75</td>
<td>3.01</td>
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<tr>
<td>SPHK1</td>
<td>Sphingosine kinase 1</td>
<td>4.22</td>
<td>2</td>
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<tr>
<td>THBS1</td>
<td>Thrombospondin 1</td>
<td>3.17</td>
<td>3.76</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Vascular endothelial growth factor A</td>
<td>4.12</td>
<td>2.49</td>
</tr>
<tr>
<td>B2M</td>
<td>β2-microglobulin</td>
<td>−2.85</td>
<td>−4.66</td>
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<td>TIMP2</td>
<td>Tissue inhibitor of metalloproteinase 2</td>
<td>−18.74</td>
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<tr>
<td>TIMP3</td>
<td>Tissue inhibitor of metalloproteinase 3</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>IFNG</td>
<td>Interferon-γ</td>
<td>−4.55</td>
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MB-MSCs indicates microbeads encapsulated with mesenchymal stem cells; MB-MSC-GLP, microbeads encapsulated with mesenchymal stem cells transfected with glucagon-like peptide-1; and PCR, polymerase chain reaction.
Furthermore, the novel approach described here combines several implementations of cell therapy in 1 single cell product. The CellBeads formulation enables long-lasting cell retention in the transplanted site and integrates the native paracrine activity of MSCs with a genetic modification conferring encapsulated cells with the ability to produce and secrete GLP-1, an incretin hormone that has both antiapoptotic and cytoprotective effects. Delivery of MB-MSC-GLP alongside the occluded femoral artery allows for authentic stimulation of collateralization together with potentiation of muscular vascularization.

Figure 6. Molecular network induced by microbeads encapsulated with mesenchymal stem cells (MB-MSCs) in murine ischemic muscle. Upregulated (A) and downregulated (B) murine genes are shown separately. In addition, the full network is shown (C) with indication of the nature of interaction. The size of each circle is proportional to the difference in expression as compared with vehicle. ANGPTL4 indicates angiopoietin 4; B2M, β2-microglobulin; CCL2, chemokine (C-C motif) ligand 2; CXCL3, chemokine(C-X-C motif) ligand 3; ENG, endoglin; HPSE, heparanase; IL6, interleukin-6; IL8, interleukin-8; IFNG, interferon-γ; PLG, plasminogen; SPHK1, sphingosine kinase 1; TIMP2, tissue inhibitor of metalloproteinase 2; THBS1, thrombospondin 1; THBS2, thrombospondin 2; TIMP3, tissue inhibitor of metalloproteinase 3; TNF, tumor necrosis factor; and VEGF-A, vascular endothelial growth factor A.
Individual elements of this approach proved to benefit post-ischemic angiogenesis. For instance, cell therapy with bone marrow–derived MSCs reportedly improves limb function, reduces the incidence of amputation, and attenuates muscle atrophy in a mouse model of limb ischemia. Moreover, MSCs promote lower limb perfusion and foot ulcer healing in patients with CLI, either given alone or in combination with endothelial progenitor cells. MSCs engineered with growth factors or antiapoptotic agents, including Akt, adrenomedullin, and angiopoietin, showed incremental enhancements of therapeutic activity in models of ischemia. Likewise, intracoronary delivery of GLP–1–overexpressing MSCs induces substantial cardiac recovery in an acute myocardial infarction model. Furthermore, the BONMOT-1&2 trials showed the feasibility and advantage of perivascular delivery of dispersed stem cells in CLI. Rapid inactivation of transplanted cells occurs, however, in an allogeneic setting, as documented by the present study. Encapsulation of human MSCs prevents this phenomenon, thus providing a potential means of treating patients with severely debilitating CLI with a single off-the-shelf cell product.

All the above concepts were integrated in the strategy of delivering immunoprotected MSCs to the vascular occlusion site. Our study shows for the first time that the procedure is feasible and therapeutically useful. Data highlight the additional benefit from 1 particular manifestation of the CellBead technology, whereby MSCs have been genetically modified to secrete a GLP-1 fusion protein. The GLP-1–enriched cell product showed superior foot-salvaging and collateral-forming capacities as compared with MB-MSCs. After arterial occlusion, preexisting vessels start enlarging through an arteriogenic process that is mainly triggered by increased shear stress, but also involves soluble factors, inflammatory cells, cell proliferation, and the remodeling of the extracellular matrix. We found that the arteriogenic process is enhanced by MB-MSC-GLP and, to a lesser extent, by MB-MSCs, most likely through secreted factors that are released into the surrounding environment in the immediate vicinity of the occluded artery. The method used in this study advantageously directs GLP-1 and other native growth factors to the anatomic region where collateralization is maximally desirable, resulting in a robust neovascularization of arterioles coaxially oriented with the femoral artery. This represents a significant improvement over conventional intramuscular cell therapy, which disperses cells and therapeutic mediators in an unpredictable manner.

In addition, perivascular delivery of MB-MSC-GLP enhanced the tributary microvascular bed in hindlimb muscles, as documented by increased counts of capillaries and small arterioles in the ischemic adductor. Measurement of human protein expression confirms that CellBeads are secreting paracrine factors at day 7 postimplantation, and this is accompanied by an upregulation of murine proangiogenic genes and corresponding downregulation of antiangiogenic genes. The functional improvements in the model are, therefore, likely to arise from a combination of direct effects of the beneficial secretion of proteins, such as VEGF-A from the CellBeads (which is highly conserved and, therefore, active in mouse), together with additional modulation of gene expression in the tissues local to the implant site by the secreted paracrine factors. These factors might reach the skeletal muscle by diffusion through the interstitium or via the expanded collaterals. Therefore, the method allows for additively advantageous implementation of proximal collateralization and distal microvascular angiogenesis.

The present CellBead formulation does not seem to be suitable for intramuscular delivery as benefits of cell therapy are overwhelmed by spatial restriction and excessive inflammatory reaction in the mouse muscle in this model. Studies in large animal models are necessary to compare feasibility and therapeutic activity of direct intramuscular injection versus delivery around the neurovascular femoral bundle.

In summary, the described methodology using microCellBead technology may offer potential as a treatment for peripheral vascular disease, whereby administration of multiple microCellBead depots within the vicinity of diseased vessels could promote revascularization and re-established blood flow to the ischemic limb. The approach is particularly attractive for use in patients in whom interventional revascularization is not amenable because of multiple or distal obstructions. Moreover, perivascular cell therapy might be used as an adjuvant treatment in conjunction with or preparation to operative revascularization.

Conclusions

After femoral arterial ligation in a mouse, application of CellBeads in the perivascular space enhances collateralization and neoangiogenesis through secretion of a variety of paracrine factors that may act directly as well as indirectly by inducing the upregulation of proangiogenic chemokines and downregulation of antiangiogenic genes. Functional neoangiogenesis made by capillaries and small-medium size arterioles is significantly enhanced around the site of administration. This leads to significantly improved blood flow, increased tissue oxygenation, and reduced toe necrosis. These results demonstrate the potential for CellBead technology in the treatment of peripheral vascular disease.

Disclosures

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**Significance**

Surgical bypass surgery or percutaneous revascularization is the gold standard for the treatment of peripheral vascular disease, producing long-term benefit, with a 5-year limb salvage rate of >80%. Gene or stem cell therapies have accredited to provide a possible alternative to surgical bypass surgery in patients with multivascular disease or occlusions of small-caliber blood vessels, which are commonly seen in those with diabetes mellitus or hypertension. However, the mode of cell delivery for optimal revascularization is highly debatable. The new methodology of administration of multiple microCellBead depots within the vicinity of the diseased vessels as described in this study could be attractive for use in these patients to promote revascularization and re-establish the blood flow to the ischemic limb. Moreover, perivascular cell therapy might be used as an adjuvant treatment in conjunction with or preparation to operative revascularization.
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<th>Encapsulation procedure</th>
<th>Advantage</th>
<th>Disadvantage</th>
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<td>A</td>
<td>Basic cell / alginate Bead</td>
<td>-</td>
<td>Cells on the surface have contact with patients immune system</td>
</tr>
<tr>
<td>B</td>
<td>One step encapsulation procedure for generation of a cell free outer shell</td>
<td>Immunoisolation is achieved</td>
<td>High ratio of outer shell volume to inner core volume results in large implant size</td>
</tr>
<tr>
<td>C</td>
<td>Two step encapsulation procedure with a spherical core bead</td>
<td>Shell/core ratio for reduced implant size</td>
<td>Diameters of more than 600 μm result in cell necrosis in the centre of the Bead</td>
</tr>
<tr>
<td>D</td>
<td>Miniaturized two step encapsulation procedure</td>
<td>Diameters of 400 μm result in full cell vitality</td>
<td>-</td>
</tr>
</tbody>
</table>

**Supplemental Figure I:** Advantages of the miniaturized two-step encapsulation procedure used to produce CellBeads (D) over other encapsulation procedures (A-C).

**Supplemental Figure II:** Representative cartoon image showing the femoral artery ligation and perivascular application site of microbeads.
Supplemental Figure III: Quantitative PCR analysis of GLP1 expression in mice injected respectively 18S was used as internal control and DDCt method has been used for analysis. n=3 per group.
Supplemental Figure IV: Effect of intra-muscularly delivered encapsulated MSC. A. Laser Doppler recovery in the 4 groups. B. Capillary density in ischemic muscles. C. Representative microscopy images of ischemic adductor muscles. Preparations are stained with haematoxylin and eosin (i&iii) or immuno-stained with isolectin B4 (green fluorescence) and F 4/80 antigen (pan-macrophage markers, red fluorescence) (ii,iv-vi). Nuclei are stained blue by DAPI.
**Supplemental Figure V:** Effect of intra-muscularly or peri-vascularly delivered non-encapsulated MSC.  

**A** & **E.** Laser Doppler recovery.  

**B** & **F.** Capillary density in ischemic muscles.  

**C** & **D.** Arteriole density (<50mm).  

**G** & **H.** Arteriole density (>50mm).  

*P<0.05 vs. vehicle.
**Supplemental Video I:** Three dimensional animation of the Z-stack images collected from the adductor muscle of mouse injected with MB-MSCs-GLP. Mouse were injected with isolectin-B4 before collecting the samples to identify the functional capillarries (green). Nuclei are stained by DAPI.
Materials and Methods
Ethics
Experiments using animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (the institute of Laboratory Animal Resources, 1996) and with approval of the British Home Office and the University of Bristol.

Preparation of microbead-encapsulated MSCs
Briefly, MSC were obtained from the BM of a single healthy male donor aged 33 years following informed consent. Primary cells were immortalised following stable transduction by a retroviral vector containing hTERT.1 A plasmid expression vector encoding GLP-1 was transfected into the parental cell line to produce cells that secrete a GLP-1 fusion protein which comprises of two GLP-1 molecules bound by an intervening peptide. Approximately 60-70 MSC were embedded into spherically shaped barium cross-linked alginate core beads with a diameter of ~120µm. To achieve immunoprotection of the encapsulated GLP-1 secreting cells, the corebeads were surrounded by a selectively permeable shell of pure alginate, which led to micro-CellBeads with a diameter of ~160µm (Online Supplemental Figure 1). The mean production rate of 100L GLP-1 CellBeads is about 3 pmol GLP-1 per hour. GLP-1 CellBeads were cryopreserved in 10% DMSO and stored at -80°C. Before use, the CellBeads were thawed and washed in Ringer’s solution to remove DMSO.

Controls of GLP-1 CellBeads (denoted as MB-MSC-GLP) included (i) naïve MSC encapsulated using the same method as described above (MB-MSC), (ii) barium cross-linked alginate microbeads of a similar diameter without cellular components (MB) and (iii) injection vehicle (buffered saline).

Hindlimb ischemia model
Following anesthesia (2,2,2 tribromo ethanol, 0.3gm/kg, i.p.), unilateral limb ischemia was induced in 7-8 weeks old male CD-1 mice (Harlan, UK), using a refined procedure which consists of ligation (with a 6-0 silk suture) in 2 points and electro-coagulation of the upper part of the femoral artery. Then, mice received an application of either MB-MSCs-GLP, MB-MSCs (40µL/mouse consisting of ~12,000 microbeads), MB alone or vehicle onto the perivascular fascia between the femoral artery and vein (Online Supplemental Figure 2).

Study design
Feasibility
A pilot study was performed in mice with limb ischemia given vehicle or MB-MSC-GLP (n=5 mice/group). Endpoints of this feasibility study were (i) beads retention at the delivery site (at 1 and 7 days post-delivery) and (ii) capillary and arteriole density (at day 7). The location of the injected microbeads with respect to the femoral artery and vein was identified by staining the sections with Haematoxylin-Eosin (H&E). Capillary and arteriole density was assessed as described below.

Efficacy
In the main series of follow-up experiments, four groups of animals (n=12/group) were randomized to blinded therapy (vehicle, MB, MB-MSC or MB-MSC-GLP). Animals were followed until programmed sacrifice at day 21, unless occurrence of foot necrosis required early euthanasia. Endpoints of the efficacy study were: (i) local clinical outcome, (ii) superficial blood flow, (iii) muscular blood flow, (iv) muscular
oxygen tension (pO$_2$), (v) neovascularization and (vi) muscular levels of angiogenic factors.

**Clinical outcome**: The occurrence of necrotic toes was assessed by daily inspection and recorded. Animals with necrosis extending to the whole foot were sacrificed with an excess of anesthesia.

**Superficial blood flow**: Foot blood flow was measured immediately after ischemia and then at 3, 7, 14 and 21 days (n=12 mice/group) by using a laser Doppler perfusion imaging system (Moor Instrument, UK).

**Muscular blood flow**: Blood flow to the ischemic and contralateral adductor muscle was measured at 21 days (5 mice/group), by the use of intra-cardially adducted fluorescent microspheres (0.1µm in diameter) as previously described. Muscular blood flow was expressed in ml/min/g of tissue.

**Muscular pO$_2$**: Adductor muscle pO$_2$ was measured at day 21 in all survived mice by the OxyLite E Tissue Oxygenation System (Oxford Optronix Ltd., UK) as previously described. Data were expressed in mmHg.

**Neovascularization**: At 21 days after ischemia, terminally anesthetized mice (n=6 at each time point) were perfused with 4% paraformaldehyde. Ischemic muscles were removed en bloc with femoral artery and vein for histological analysis. Muscle cryosections with thickness of 5µm were used for immunohistochemical analysis unless specified. The capillary and arteriole densities of ischemic adductor muscles were assessed using isolectin-B4 and α-smooth muscle actin (Sigma-Aldrich, UK) staining respectively as previously described. Counts of 15 random microscopic fields were averaged and expressed as the number of capillaries and arterioles per mm$^2$. Arterioles were further characterized according to their luminal size (n = at least 5 mice in each group).

In addition, to identify total and functional blood vessels surrounding the femoral artery and vein, whole mount preparations of perivascular zone were processed in mice (n=5 per group), which received an intravenous injection of the endothelial cell marker isolectin-B4 (Invitrogen, UK). Sections with thickness of 60µm were post-fixed with acetone at -20°C for 10min and air-dried at room temperature for 30min. Sections were permeabilized with 1% triton-X 100, followed by blocking of non-specific antigens. Next, the sections were incubated for 3h at room temperature with streptavidin Alexa Flour 568 (1:100, Invitrogen) to detect isolectin-B4 incorporated in vivo by endothelial cells and anti-mouse α-smooth muscle cell actin antibody conjugated with Cy3 (Sigma chemicals, 1:400) to detect vascular smooth muscle cells. Serial z-stack images of the muscle were generated using Leica SP5 AOBS confocal laser scanning microscope.

**Muscular levels of angiogenic factors**: An additional series of mice (n=5 per group) were sacrificed at 7 days after ischemia to investigate the expression of the GLP transgene as well as of angiogenic factors at mRNA and protein level in skeletal muscles. RNA from snap frozen mouse muscles were isolated with miRNeasy kit (Qiagen, UK), according with manufacturer’s indications. cDNA was prepared from 500ng RNA by Transcription Kit (Qiagen, UK). Primers pairs for recombinant GLP qPCR were forward: GTGAGCTCTTATCTGGAAGGC, reverse: AGATAAGAGCTCACATCGCTGG (Sigma). qPCR was performed using Sybr green (Qiagen, UK) reagent on LighCycler 1.5 (Roche). The activation of angiogenic genes was evaluated using murine RT-profiler PCR angiogenesis array (Qiagen, UK). RNA was extracted from ischemic muscles using TRIzol (Invitrogen, UK). One microgram of total RNA was reverse transcribed and resulting cDNA was amplified in a light cycler (Roche 480, UK). Data were analyzed using the software package from
Qiagen and expressed as fold-changes to control. Fold change of ≥ 2 was considered significant. In addition, levels of human angiogenic proteins in ischemic muscles were determined using a cytometric human angiogenesis array (BD Biosciences, UK). This allowed us determining the persistence of functionally-active human cells in recipient muscles. Briefly, tissue proteins were extracted using conventional RIPA (Radio-Immunoprecipitation Assay) buffer and incubated with mixed capture beads for 1h at 25°C. Afterward, 50μL of mixed phycoerythrin detection reagent were added. After incubation for 1h at 25°C in the dark, the complexes were washed twice and analyzed using the BD Canto II flow cytometer (BD Biosciences, UK). Data analysis was carried out using the accompanying FACSDiva and FCAP Array software (BD Biosciences). Tissue chemokine content was normalized to protein concentration. Analysis of biological networks activated or repressed by MB-MSCs in ischemic muscles was conducted by using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) a biological database and web resource of known and predicted protein-protein interactions.

Verification of benefit in relation to administration route and encapsulation

In order to verify the therapeutic activity of encapsulated MSC in relation to the administration route, in separate experiments, the above cell preparations or vehicle were injected in the ischemic adductor muscle (n=7 mice per group).

In addition, to determine if encapsulation is relevant for achievement of therapeutic benefit by allogeneic cells, non-encapsulated MSC were transplanted peri-vascularly or intra-muscularly in immunocompetent mice with unilateral limb ischemia (at the same dosage used in studies of encapsulated MSC, 7 to 9 mice per group). In order to allow retention around the occluded femoral artery, cells were delivered in a suspension of growth factor-reduced Matrigel (BD Bioscience). Controls received Matrigel only (20μL). Additional mice (n=5 per group) were sacrificed at 7 days post-cell therapy for assessment of human angiogenic factors in ischemic muscles.

Statistical analysis

Experiments for the main study were randomized and blinded and the groups only decoded after the completion of all analyses. Results are expressed as mean ± standard error. Analysis of laser Doppler blood flow recovery was performed using repeated measures Two-Way ANOVA followed by pair-wise comparison using the Holm-Sidak method. The effect of treatments on microsphere blood flow and histological and molecular endpoints was analyzed using one-way ANOVA, followed by pair post-test comparison using Tukey test. When the normality test failed, differences between groups were analyzed using Kruskal-Wallis test. A P value of <0.05 was considered statistically significant. In addition, the Cohen’s d test (difference between means / pooled standard deviations) was used to determine the scale of magnitude for differences or changes of means. This test was used to objectively determine if a statistically significant difference was of biological importance. The results of Cohen’s d are considered to be small (<0.5), medium (0.5 – 0.8), or large (>0.8).
References


