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Therapeutic Angiogenesis Using Autologous Bone Marrow Stromal Cells: Improved Blood Flow in a Chronic Limb Ischemia Model

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Background. **We evaluated the effect of autologous marrow stromal cells (MSCs) on neovascularization and blood flow in an animal model of chronic limb ischemia.**

Methods. **Chronic hind limb ischemia was created by ligating the left common iliac artery of male Lewis rats. Three weeks after ligation, 5.0 million LacZ$^+$MSCs (n = 10) or culture medium (n = 10) were injected into the anteromedial muscle compartment of the left thigh. At 4 and 6 weeks after injection, half the animals (n = 5) from each group underwent femoral artery ultrasonic blood flow measurements of the ischemic and nonischemic limbs to obtain a flow ratio. The animals also underwent angiography and measurements of blood vessel density and arteriolar density. Qualitative histologic assessment of the limb muscles was performed.**

Results. **LacZ$^+$MSCs were found to differentiate into endothelium (F VIII$^+$), vascular smooth muscle (positive α-smooth muscle actin), skeletal muscle (positive desmin), and adipocytes. Ischemic hind limbs where MSCs were implanted had greater vascular density and arteriolar density than control limbs ($p < 0.001$). Femoral artery flow index (left femoral artery flow/right femoral artery flow) was 0.89 ± 0.12 and 0.90 ± 0.06 for rats injected with MSCs measured at 4- and 6-weeks, respectively, compared with 0.50 ± 0.15 and 0.50 ± 0.10 for the control rats ($p < 0.001$). Angiography demonstrated reconstitution of the left femoral artery in rats that received MSC implantation through pelvic and abdominal wall collateral formation.**

Conclusions. **Local MSC implantation induces a neovascular response resulting in a significant increase in blood flow to the ischemic limb. Marrow stromal cells are also capable of spontaneously regenerating the various components of muscular tissues.**

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Marrow stromal cells (MSCs) are pluripotent adult-type stem cells that have been shown to differentiate into different cellular phenotypes, including osteocytes, chondrocytes, and cardiomyocytes [1, 2]. Several studies have suggested that these mesenchymal stem cells undergo milieu-dependent differentiation, and as such have the potential to participate in organ repair and regeneration [3, 4]. Previous studies from our laboratory have demonstrated that these cells have the potential to induce a neovascular response in murine matrigel angiogenesis assay [5]. In the present study, we explored the ability of autologous MSCs to not only induce new blood vessel growth but also improve blood flow in an animal model of chronic limb ischemia.

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Material and Methods

Animals

Male Lewis rats (250 to 275 g) were obtained from Charles River Laboratories (Laprairie Co, Quebec, Canada). These isogenic rats were used as donors and recipients of MSCs to simulate autologous implantation. All animals were studied using guidelines published in "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication 85-23, 7th edition, revised 1996).

Harvest and Culture Expansion of Bone Marrow Stromal Cells

We sacrificed male Lewis rat and harvested bone marrow by flushing femurs and tibias with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 U/mL penicillin/streptomycin. We plated whole marrow in tissue culture dishes and 5 to 7 days later discarded the nonadherent hematopoietic cells and maintained the adherent bone marrow stromal cells at 37°C with 5% CO_2.

Generation of LacZ$^+$ Marrow Stromal Cells

Marrow stromal cells were labeled with retroviral particles carrying the LacZ gene that codes for the prokaryotic

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β-galactosidase enzyme. The retroviral particles were derived from GP+AM12-nlsLacZ cell line. These cells produce 1.6×10^5 infectious particles/mL as determined by titer assay on NIH 3T3 cells. Marrow stromal cells transduction was performed twice per day for 3 consecutive days (with 6 μg/mL lipofectamine). Following LacZ transduction, 85% to 90% of all MSCs expressed detectable β-galactosidase activity as assessed by X-gal staining, which served as a cellular marker for the presence of MSCs.

Creation of Hind Limb Ischemia and Marrow Stromal Cell Implantation

Thirty minutes before anesthesia, each rat received the narcotic analgesic buprenorphine (Temgesic, 0.01 mg/kg subcutaneously, Reckitt and Colman Pharmaceuticals Inc, Wayne, NJ). Anesthesia was induced by 4% isoflurane in gas (30% O_2 and 70% air). After induction the rat was transferred to a servo-controlled, heated table and body temperature was maintained at 37°C. The isoflurane was reduced to 2% delivered by a facemask. Using sterile technique, the abdomen was opened through a lower midline incision, the aortic bifurcation was identified and the left common iliac artery was ligated using 5-0 silk at its midpoint as described by Rochester and coworkers [6]. Anesthesia was discontinued and animals were allowed to recover completely before replacing them in their cages. No mortality, paralysis, gangrene, or ulcers occurred in any group. Three weeks after ligation, 5.0 million $LacZ^+$MSCs (MSC group, n = 10) or culture medium (control group, n = 10) were injected into the anteromedial muscle compartment of the left thigh.

Experimental Protocol for Angiogenesis Assessment

At 4 and 6 weeks after the creation of hind limb ischemia, 5 rats from the treatment group and 5 rats from the control group were placed under isoflurane anesthesia as described above with body temperature maintained at 37°C on a servo-controlled, heated table. The trachea was intubated followed by ventilation using Harvard rodent ventilator (Harvard Apparatus Co, Inc, South Natick, MA) delivering 3 mL tidal volume at 70 breaths per minute. A cannula (PE50) was inserted into the right femoral vein. Constant infusion delivered 1% of body weight per hour, which contained 2% bovine serum albumin in normal saline. During the 1-hour postsurgical equilibration period, inspired anesthetic concentration was titrated to the minimum concentration that precluded a response when the tail was pinched. The following measurements and tests were performed:

BLOOD FLOW MEASUREMENT. Blood flow was measured in both femoral arteries using Transonic Systems Inc (Ithaca, NY) T106 transient time ultrasound flowmeter (R1 probe). The flow probe was placed around the proximal part of the femoral artery and blood flow measurement was taken after a 1-hour equilibration period.

HIND LIMB ANGIOGRAPHY. Under isoflurane anesthesia, the abdomen was opened and a cannula was inserted into the abdominal aorta with the tip below the renal arteries. The cannula was used to infuse 0.5 mL of Hypaque-M 60% contrast media (Nycomed Image AS) in 2 seconds. Images were taken using OEC 9600 mobile C-arm equipped with a 12-inch image intensifier and digital subtraction angiography capabilities. Images were acquired at a frame rate of 4 frames/sec with image magnification set at maximum.

MUSCLE SPECIMENS RETRIEVAL AND PROCESSING. After blood flow measurements and angiography, rats were killed with pentobarbital overdose. The rat systemic circulation was flushed through the left ventricle with 20 mL of cold (4°C) phosphate-buffered solution followed by 20 mL of cold 2% paraformaldehyde. Muscle specimens obtained from both hind limbs were cut into thin slices and placed in 2% paraformaldehyde at 4°C for 24 hours followed by incubation in X-gal staining solution, which consisted of 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6 0 \cdot 3H_2O$, 0.01% sodium deoxycholate, 2 mmol/L $MgCl_2$, 1 mmol/L EGTA, and 1 mg/mL X-gal made in wash solution (phosphate-buffered saline with 0.02% NP40). The X-gal solution was adjusted to pH of 8.0 for maximum specificity of the staining as described before [7]. After 16 hours, we fixed the specimens in 10% formalin and embedded them in paraffin. Sections were cut at 3 to 4 μm.

IMMUNOHISTOCHEMICAL STAINING. Random sections from each specimen were deparaffinized and standard three-steps immunohistochemical staining was performed using the following primary antibodies (on separate sections): polyclonal rabbit anti-human factor VIII, monoclonal mouse antihuman desmin (both from DAKO Corp, Carpinteria, CA), and monoclonal mouse antihuman α-smooth muscle actin (Sigma, St. Louis, MO). All antibodies are known to cross-react with the corresponding rat antigens. Universal biotinylated antimouse and rabbit secondary antibody (Ventana Medical Systems, Tucson, AZ) was used followed by avidin-peroxidase complex binding. The reaction was developed with diamino benzidine substrate.

MICROSCOPY AND VASCULAR DENSITY. All sections were examined with an Olympus BX60 microscope. Digital images were transferred to a computer equipped with Image Pro software (Media Cybernetics). In H&E-stained sections, we considered as blood vessels only tubular structures with patent lumen that were lined with endothelium. In sections stained with antifactor VIII antibody, we considered as blood vessel only tubular structures that were factor VIII+. For vascular density measurements using sections stained with antifactor VIII, we counted the number of blood vessels in 10 random high power fields (magnification ×400) selected using the systemic sampling with random start technique. We calculated the average of the 10 high power fields (hpf) and expressed the vascular density as blood vessels/hpf. Arterioles were identified by the presence of smooth muscles in their wall in sections stained with anti α-smooth muscle actin and were counted in the same technique.

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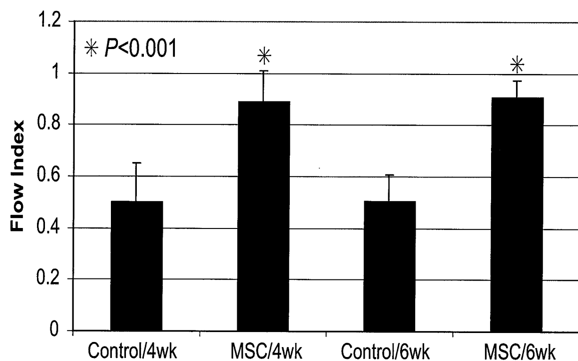

Fig 1. Comparison of femoral artery flow index in the treatment (marrow stromal cell [MSC]) and control subgroups at 4 and 6 weeks after intramuscular injection.

Statistical Analysis

All data are expressed as the mean ± SD. Statistical analysis was carried using the SPSS version 10.0 (SPSS Inc.). A *p*-value of less than 0.05 was considered to be statistically significant. Analysis of variance was used to compare the means of the different groups of animals followed by Bonferroni multiple comparison test.

Results

Changes in Femoral Artery Flow

To account for animal-to-animal and minute-to-minute variation in hemodynamics, we used the femoral artery flow index (FAFI) to standardize the femoral artery flow measurements and allow for comparisons between different animals.

$$\text{FAFI} = \text{Left femoral artery flow/Right femoral artery flow.}$$

FAFI has no units and equals 1 in normal rats in which the left common iliac artery was not ligated (data are not shown). Marrow stromal cell implantation resulted in significant increase in the femoral artery flow index (Fig 1). FAFI was 0.89 ± 0.12 and 0.90 ± 0.06 for rats injected with MSCs measured at 4 and 6 weeks, respectively, compared with 0.50 ± 0.15 and 0.50 ± 0.10 for the control rats ($p < 0.001$).

Angiography

Hind limb angiography demonstrated complete occlusion of the left common iliac artery at the site of ligation in both the treatment and control groups (Fig 2). Angiography also showed absence of flow in the left femoral arterial system in the control rats. In rats that received MSCs implantation, there was reconstitution of the left femoral artery by collateral vessels derived from the abdominal wall and the contralateral pelvic arteries.

Vascular Density

About a fourfold increase in vascular density was found in the left hind limb muscle specimens of rats that received MSCs implantation (Fig 3). Vascular density in left limb muscles was 4.5 ± 2.0 and 5.4 ± 0.7 for rats injected with MSCs and measured at 4 and 6 weeks, respectively, compared with 1.2 ± 0.6 and 1.1 ± 0.3 for control rats.

Arteriolar Density

Marrow stromal cell implantation was associated with increased arteriolar density (about threefold) in the treated ischemic hind limbs (Fig 4). The arteriolar density in the left limb muscles was 1.6 ± 0.4 and 1.7 ± 0.5 for rats injected with MSCs and measured at 4 and 6 weeks, respectively, compared with 0.5 ± 0.1 and 0.4 ± 0.2 for control rats.

In Vivo Differentiation of Marrow Stromal Cells and Vasculogenesis

$LacZ^+$MSCs identified by their indigo blue color when stained with X-gal were found to undergo in vivo phenotypic differentiation into endothelial cells that participate in the formation of new blood vessels (Fig 5). These $LacZ^+$MSCs express the endothelial markers factor VIII (Fig 6A, 6B) and CD34 (data not shown). Both markers are not expressed by MSCs before implantation, as found by immunohistochemical and fluorescent-activated cell sorting analysis (data not shown). $LacZ^+$MSCs also differentiate into vascular smooth muscles and express α smooth muscle actin (Fig 6C, 6D). In vivo differentiation into other components of muscular tissue was noted, including skeletal muscle fibers (Fig 6E) with expression of desmin (Fig 6F) and adipocytes (Fig 6G).

Comment

Autologous MSCs are desirable for cell therapy as they are abundant, easy to harvest, and easy to culture expand to achieve sufficient numbers for therapeutic purposes. They are also relatively easy to modify genetically. Although several studies have demonstrated the potential of MSCs to differentiate into different cell types [1, 2], from a therapeutic point of view functional improvement

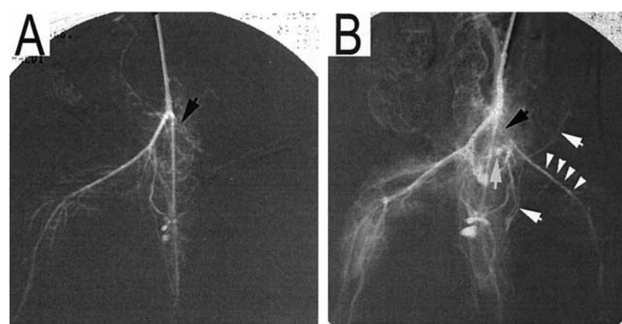

Fig 2. Angiograms of the pelvic and hind limb arteries 4 weeks after intervention. (A) Control rat angiogram showing no flow of contrast distal to the site of left common iliac artery ligation (arrow). *(B) Angiogram of rat that received marrow stromal cell implantation showing the site of left common iliac artery ligation* (black arrow)*. Reconstitution of the left femoral artery* (arrowheads) *by pelvic and abdominal wall collaterals* (white arrows) *is visible.*

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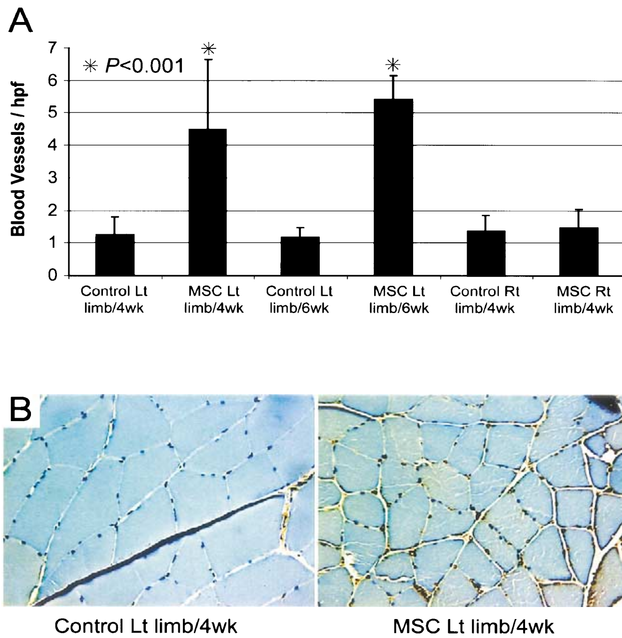

Fig 3. (A) Comparison of the vascular densities in muscle specimens obtained from either left or right limbs in the treatment (marrow stromal cell [MSC]*) or control group at 4 and 6 weeks after intervention.* (hpf = *high power field;* Lt = *left;* Rt = *right.) (B) Immunohistochemical staining for the endothelial marker factor VIII in muscle specimens from ischemic left hind limbs clearly demonstrates marked increase in blood vessels in ischemic muscle tissues where MSCs were implanted compared with controls. (Magnification ×100 before 46% reduction.)*

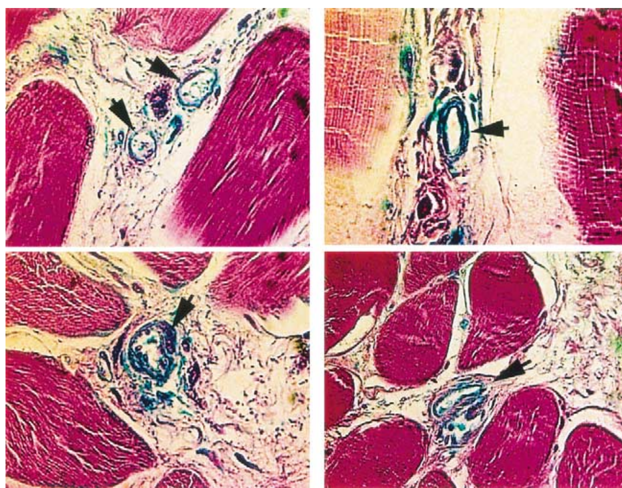

Fig 5. Histologic sections of muscle tissues where marrow stromal cells (MSCs) were implanted stained with X-gal to identify the LacZ⁺MSCs and counterstained with eosin. Arrows point to several blood vessels where LacZ⁺MSCs (indigo-blue color) *are actively participating in the formation of new blood vessels (ie, vasculogenesis) with histologic features suggestive of endothelial differentiation. (Magnification ×200 before 43% reduction.)*

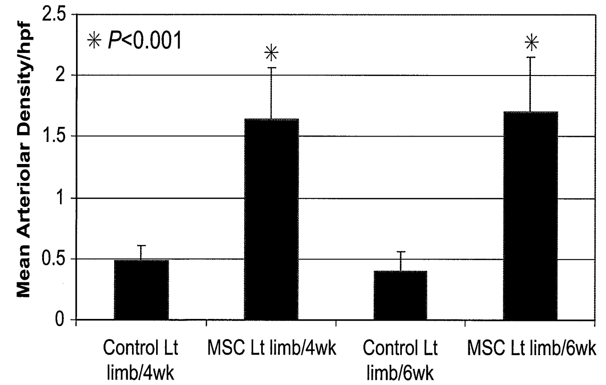

Fig 4. Comparison of the arteriolar densities in muscle specimens obtained from either left or right limbs in the treatment (marrow stromal cell [MSC]*) or control group at 4 and 6 weeks after intervention. Arterioles were identified as blood vessels with smooth muscle in their walls as demonstrated by immunohistochemical staining with anti-α smooth muscle actin.* (hpf = *high power field;* Lt = *left.)*

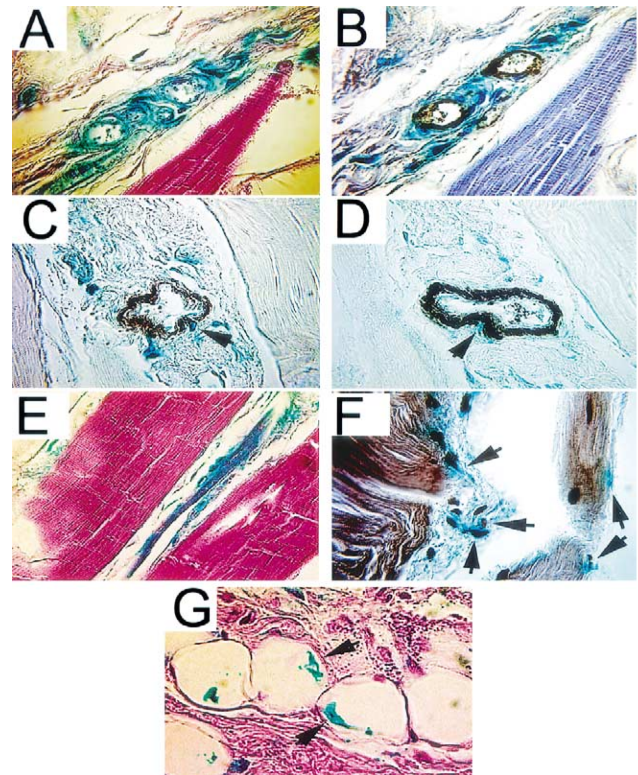

Fig 6. Histologic sections of muscle tissues in which marrow stromal cells (MSCs) were implanted. (A) X-gal- and eosin-stained section showing LacZ⁺MSCs forming new blood vessels and express factor VIII as shown in (B), which is a consecutive section that was stained with X-gal and anti-factor VIII antibodies. (Magnification ×200 before 35% reduction). (C) and (D) sections stained with X-gal and anti-α smooth muscle actin that show LacZ⁺MSCs (arrows) *differentiating into vascular smooth muscle and expressing α smooth muscle actin. (Magnification ×400 before 35% reduction.) (E) X-gal- and eosin-stained section showing LacZ⁺MSCs differentiating into skeletal muscle fibers. (Magnification ×200 before 35% reduction.) (F) Section stained with X-gal and antidesmin showing LacZ⁺MSCs* (arrows) *expressing desmin. (Magnification ×200 before 35% reduction.) (G) X-gal- and eosin-stained section showing LacZ⁺MSCs* (arrows) *differentiating into adipocytes. (Magnification ×400 before 35% reduction.)*

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is important. In this study, we hypothesized and demonstrated that MSCs not only induce a neovascular response when placed in ischemic tissue, but also improve blood flow to that tissue.

We implanted MSC locally in a rat hind limb ischemia model as described by Rochester and colleagues [6] as opposed to a coronary ischemic model for a number of reasons. The purpose of the study was to demonstrate a proof of principle, namely that MSCs can improve blood flow to an ischemic organ. Whether the ischemia was myocardial, cerebral, or limb was not crucial for this study. What was crucial was that the model be simple. Indeed, we found that the hind limb ischemia model of the rat is easy to perform, is associated with a low procedural mortality, results in a stable and predictable reduction in blood flow (around 50% of normal), and allows for an easy method for the determination of blood flow using ultrasound of the femoral artery. Larger animal models are generally needed for coronary ischemia and are associated with higher costs and increased mortality; they also require a more elaborate method of evaluating blood flow such as radioactive or colored microspheres [8]. One should not assume, however, that the improved blood flow after MSC implantation found in this study can be generalized and assumed to occur in the rat coronary circulation or, more importantly, in the human situation and this is clearly a limitation of the study. Our findings do at least support the concept that MSCs can be used to improve blood flow in ischemic tissue.

Our flow data demonstrate that MSC-related neovascularization produces a physiologically significant increase in the femoral artery blood flow index from a base line of 50% in controls to 90% in the treated limb. The mechanisms by which local implantation of MSCs improve blood flow are probably multifactorial, as the neovascularization which we observed was of three types: angiogenesis, vasculogenesis, and collateral vessel formation.

Angiogenesis

Host-derived vascular density increase significantly in the treated hind limb as compared with control limbs. The local angiogenic effect due to MSC implantation may occur in part due to MSC-mediated vascular endothelial growth factor (VEGF) production. We have previously shown in a matrigel model [5] that the host-derived angiogenic response following MSC implantation can be reduced by 80% by blocking VEGF receptors using monoclonal antibodies, suggesting an important role for VEGF. In addition, in unpublished data, MSCs exposed to hypoxic conditions upregulate VEGF-mRNA expression and hypoxic inducible factor. Other growth factors may be upregulated (fibroblastic growth factor or transforming growth factor) but await further study and evaluation. The impact of an increase in local small vessel vascular density to overall blood flow is, however, probably minor [9]. Nevertheless, the increased density may act by reducing the overall resistance to flow through an increase in total vascular cross-sectional area.

Vasculogenesis

Marrow stromal cells appeared to participate in forming arteriole-like vessels and were found to express endothelial markers when lining the vessel wall lumen and express smooth muscle markers when incorporated into the vessel wall. This finding is not surprising because MSCs are pluripotent cells and have been shown previously to undergo differentiation into many cell types including adipose, chondrocytes, and myocardial cells. In a previous study we have shown that MSCs can undergo phenotypic change and express endothelial markers such as CD31 and VEGF receptor [5]. Therefore, in addition to stimulating a neovascular response, some cells actually appear to participate and become incorporated into the new blood vessel formation as well as surrounding adipose and skeletal muscle. This observation is consistent with those of others who describe this phenomenon as "milieu-dependent" differentiation [1]. The role of these newly created arteriolar-like structures to blood flow is not entirely known, but arteriogenesis may allow for sufficiently large vessels to improve local blood flow if they also communicate with an inflow source of blood [9].

Collateral Vessel Formation

Improved collateral vessel formation in the treated group was evident by angiography and occurred from the abdominal wall and contralateral pelvis and this improvement appears to be directly responsible for the improved blood flow to the treated limb. How these collaterals develop from an area that is remote from the local effects of MSC- mediated angiogenesis is not entirely clear. Normally, collateral formation that occurs following arterial occlusion, as in arteriosclerosis, is due to the recruitment of naturally preexisting small vessels that increase in size mainly due to a local increase in shear stress with resulting invasion of monocytes, cytokines, and growth factors (fibroblast growth factors and to a lesser extent VEGF); tissue ischemia per se is not thought to be an important stimulus for collateral formation [10]. In addition, the normal collateral formation which occurs after vessel occlusion is not always visible on an angiogram. The explanation for the robust collateral formation demonstrated in this model maybe due to one or a combination of the following: (1) The improved flow to the ischemic limb correlates with the local finding of an increase in vascular density of the ischemic treated limb suggesting that an increase in total vascular cross-sectional area might reduce the local resistance to flow thereby improving collateral formation. (2) Local injection of MSCs may act as a potent signal "sink" due to the increased secretion of growth factors as previously described as well as other unidentified agents, which together stimulate collateral formation. (3) The local implantation of MSCs may have an as-yet unidentified systemic effect such as vasodilatation (perhaps VEGF mediated), which would promote collateral blood flow. Clearly, future studies are needed to elucidate the mechanisms involved to explain the collateral formation that occurs after local injection of MSCs.

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We propose the use of autologous MSC implantation for potential use as cellular therapy for tissue ischemia. The ability of MSCs to promote collateral formation and actively participate in blood vessel formation and surrounding tissue repair suggests that they may have multiple therapeutic benefits in treating patients with limb or myocardial ischemia. The biological mechanisms underlying these observations need further research to identify the appropriateness and safety of MSC as cellular therapy in anticipated human treatment.

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