Molecular Imaging of the Paracrine Proangiogenic Effects of Progenitor Cell Therapy in Limb Ischemia

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Background—Stem cells are thought to enhance vascular remodeling in ischemic tissue in part through paracrine effects. Using molecular imaging, we tested the hypothesis that treatment of limb ischemia with multipotential adult progenitor cells (MAPCs) promotes recovery of blood flow through the recruitment of proangiogenic monocytes.

Methods and Results—Hind-limb ischemia was produced in mice by iliac artery ligation, and MAPCs were administered intramuscularly on day 1. Optical imaging of luciferase-transfected MAPCs indicated that cells survived for 1 week. Contrast-enhanced ultrasound on days 3, 7, and 21 showed a more complete recovery of blood flow and greater expansion of microvascular blood volume in MAPC-treated mice than in controls. Fluorescent microangiography demonstrated more complete distribution of flow to microvascular units in MAPC-treated mice. On ultrasound molecular imaging, expression of endothelial P-selectin and intravascular recruitment of CXCR1-positive monocytes were significantly higher in MAPC-treated mice than in the control groups at days 3 and 7 after arterial ligation. Muscle immunohistology showed a >10-fold-greater infiltration of monocytes in MAPC-treated than control-treated ischemic limbs at all time points. Intravital microscopy of ischemic or tumor necrosis factor-α–treated cremaster muscle demonstrated that MAPCs migrate to perimicrovascular locations and potentiate selectin-dependent leukocyte rolling. In vitro migration of human CD14+ monocytes was 10-fold greater in response to MAPC-conditioned than basal media.

Conclusions—In limb ischemia, MAPCs stimulate the recruitment of proangiogenic monocytes through endothelial activation and enhanced chemotaxis. These responses are sustained beyond the MAPC lifespan, suggesting that paracrine effects promote flow recovery by rebalancing the immune response toward a more regenerative phenotype. (Circulation. 2013;127:710-719.)

Key Words: angiogenesis ■ echocardiography ■ molecular imaging ■ peripheral arterial disease ■ stem cells

The immune response plays an important regulatory role in postnatal modification of the vascular system that occurs in response to ischemia. Inflammation and vascular remodeling share common signaling pathways, and there is evidence that certain inflammatory cells such as monocytes can promote arteriogenesis in ischemic tissues.1,3 In particular, a sub-set of proangiogenic monocytes may serve as a source for both proangiogenic cytokines and growth factors and may participate in requisite remodeling of the extracellular matrix.1,4,5

Clinical Perspective on p 719

In recent years, there has been growing interest in using stem cells and differentiated progenitor cells as a treatment option for the growing number of patients with severe ischemic coronary artery disease and peripheral arterial disease who are not candidates for revascularization procedures. Understanding the mechanism by which these cells promote vascular remodeling is particularly important because clinical trials have varied considerably in their results.6 Regardless of whether they are incorporated into the native tissues, stem cells are able to act through local paracrine effects by secreting proinflammatory growth factors, cytokines, and chemokines.7,8 In animal models of myocardial infarction, improvement in ventricular function with allogeneic mesenchymal stem cell therapy has been linked to increased monocyte but not T-cell infiltration.9 Yet, little is known about the mechanisms of monocyte recruitment with stem cell therapy or its effect on vascular remodeling. In this study, we hypothesized that progenitor cell therapy enhances vascular remodeling in part by upregulation of monocyte recruitment in ischemic tissue and rebalancing toward a
more reparative immune response. To test this hypothesis, cell therapy with xenogeneic circulating multipotential adult progenitor cells (MAPCs) was performed in a murine model of limb ischemia. Noninvasive contrast-enhanced ultrasound (CEU) perfusion and molecular imaging were used to evaluate in vivo temporal changes in blood flow, endothelial activation (P-selectin expression), and intravascular recruitment of a proangiogenic subset of monocytes. For the latter, targeted imaging of the fractalkine receptor (CX3CR-1) was performed to specifically detect a population monocytes (Ly-6C(lo), CX3CR-1hi, arginase-1-positive) that have been implicated in some studies as being immunomodulatory and proangiogenic in mice.

Intravital microscopy and cell migration assays were used as complementary techniques to evaluate the effect of MAPCs on endothelial-leukocyte interaction and monocyte chemotaxis.

### Methods

#### Hind-limb Ischemia

The study was approved by the Animal Care and Use Committee at Oregon Health & Science University. All studies except optimal administration were performed in C57Bl/6 mice (Jackson Laboratories) at 8 to 10 weeks of age (n=77). Mice were anesthetized with inhaled isoflurane (1.0%–1.5%). Unilateral hind-limb ischemia was produced by ligation of the distal common iliac artery and the origin of the epigastric artery through a midline abdominal incision using sterile technique. Mice were then recovered, and buprenorphine HCl (0.2 mg/kg IM) was administered for analgesia.

#### MAPC Administration

Human MAPCs (Athersys Inc, Cleveland, OH) that were isolated and expanded as described previously were stored in liquid nitrogen until the day of use. These cells are positive for CD29, CD49c, CD90, and major histocompatibility complex class I; were negative for CD11b, CD3, CD45, CD80, and CD106; and have been shown to produce endothelial tube formation in vitro and in vivo. After thawing and washing, cell viability was assessed by Trypan blue exclusion. One day after arterial ligation, the proximal ischemic hind limb was injected with 1×10⁶ MAPCs suspended in 25 μL saline (n=28); control animals received either sham saline injection (n=18) or no injection (n=22). Injections were made into the deep portion of the proximal hind-limb adductor muscles with high-frequency (40 MHz) ultrasound guidance (Vevo 770, VisualSonics Inc). To image the spatial distribution of the cell injectate in the first 5 treated animals, 1×10⁶ nontargeted microbubbles were added to the cell suspension. Immediately after injection, ultrasound imaging was performed with both high-frequency (40 MHz) 2-dimensional imaging and contrast-specific low-power imaging (see below). Sequential short-axis planes were acquired from the inguinal fold to the knee with 0.25-mm adjustments in the elevational plane direction. High-frequency anatomic and contrast ultrasound image sets were digitally coregistered and 3-diensional rendered (OsiriX version 3.5) to evaluate the spatial distribution of the injection.

#### Microbubble Preparation

Nontargeted lipid-shelled decalfluorobutane microbubbles were prepared by sonication of a gas-saturated aqueous suspension of 2 mg/mL disteroylphosphatidylcholine and 1 mg/mL disteroylphosphatidylethanolamine-PEG (2000). For molecular imaging, biotinylated microbubbles were prepared by adding 0.4 mg/mL disteroylphosphatidylethanolamine-PEG (2000)-biotin. Biotinylated rat anti-mouse antibodies against P-selectin (RB4.03.34) or CX CR-1 (sc50030, Santa Cruz) were conjugated to the microbubble surface via a streptavidin linkage as previously described. Microbubble concentration was measured by electrozone sensing (Multisizer III, Beckman Coulter).

### Flow Cytometry

Microbubble attachment to monocytes was assessed by flow cytometry using bone marrow cells collected from the long bones of either wild-type mice or NR4A1 nuclear receptor–green fluorescent protein reporter mice (kindly provided by Dr Kristin A. Hogquist, University of Minnesota). This nuclear receptor has been shown to regulate monocyte differentiation to the CX3CR-1hiLy6C(low) or patrolling monocyte phenotype. Fcγ receptors were blocked (2.4G2, BD Biosciences), and cells were stained with a phycoerythrin-labeled anti-mouse CD115 monoclonal antibody (AF98, Biolegend) to identify monocytes. For wild-type animals, FITC-labeled anti-mouse Ly-6C (AL.21, BD Biosciences) was used to discriminate monocyte subsets. Cells were analyzed by flow cytometry (FACScalibur BD Biosciences) either alone or after exposure to pressure-deflated CX3CR-1–targeted or control microbubbles labeled with 1,1ʹ,3,3ʹ-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate (DiD). Positive interaction was determined by fluorescent intensity beyond the 95% exclusion gate for control experiments with nontargeted microbubbles.

#### Perfusion Imaging

CEU perfusion imaging of the ischemic limb was performed at days 3, 7, and 21 after ligation with a linear-array transducer at 7 MHz (Sequoia 512, Siemens Medical Systems). The nonlinear fundamental signal component for microbubbles was detected with multipulse phase-inversion and amplitude-modulation imaging at a mechanical index of 0.18 and a dynamic range of 55 dB. Blood pool signal (IB) was measured from the left ventricular cavity at end diastole during an intravenous microbubble infusion rate of 1×10¹⁰ min⁻¹. The infusion rate was then increased to 1×10¹¹ min⁻¹, and the proximal hind-limb adductor muscles were imaged in 3 transverse planes between the inguinal fold and the knee. Images were acquired at a frame rate of 2 Hz immediately after a brief high-power (mechanical index, 1.9) destructive pulse sequence, and time-intensity data were fit to the following function:

\[ y = A \left(1 - e^{-t/\beta}\right) \]

where y is intensity at time t, A is the plateau intensity, and the rate constant $\beta$ represents the microvascular flux rate. Skeletal muscle microvascular blood volume was quantified by the following:

\[ A / (1.06 \times I_B \times F \times 1.1) \]

where 1.06 is tissue density (g/cm³), F is the scaling factor (10) that corrected for the different infusion rate for measuring IB to avoid dynamic range saturation, and 1.1 is a coefficient to correct for murine splanchnic attenuation measured a priori. Microvascular blood flow was quantified by the product of microvascular blood volume and $\beta$. On day 21 only, CEU was performed both at rest and during electrostimulated (5 mA) contraction of the adductor muscle group at 2 Hz. Because of the limited number of times that jugular cannulation for CEU could be performed for each animal, a separate group of 6 C57Bl/6 mice underwent perfusion imaging only at baseline and day 1 after ligation to determine the extent of initial ischemia.

#### Molecular Imaging

Molecular imaging of P-selectin and CX CR-1 was performed on the same days as CEU perfusion imaging (3, 7, and 21 days after ligation). Intravenous injections of targeted microbubbles (1×10⁷) were performed in random order. Low-mechanical-index (mechanical index, 0.18) images were acquired 8 minutes after each injection, and the signal from retained microbubbles alone was determined as previously described by subtracting signal from the few remaining circulating microbubbles. This was accomplished by digitally subtracting averaged frames obtained >10 seconds after destroying microbubbles within the beam volume with high-mechanical-index (1.2) imaging. Data were averaged for 2 adjacent but not overlapping short-axis planes. All retained microbubbles within the limb were destroyed between injections by high-power continuous imaging.
Fluorescent Microangiography

Functional microvascular angiography was assessed in 3 mice from each treatment group at days 7 and 21. For these studies, mice received heparin (1000 U/kg) followed by 200 μL of a 0.5-μmol/L solution of FITC-labeled Lycopteresin esculentum lectin (Sigma Aldrich) by intravenous route. Five minutes after injection, the descending aorta was cannulated through an abdominal approach, and perfusion fixation of the lower limbs was performed by infusion of 4% formalin at a perfusion pressure of 90 to 100 mm Hg. Tissues were embedded in egg albumin-gelatin matrix, and 3-dimensional confocal fluorescent microscopy (PW1000, Olympus) was performed on 100-μm sections using a full z-plane depth.

Optical Imaging

Optical imaging was performed to assess MAPC survival and function after injection in 6 immune-competent mice with spontaneous albinism (Crl:CD1) and 6 beige severe combined immunodeficiency (SCID) mice with defective natural killer cells (CB17. Cg-Pkd1<sup>−/−</sup>Cd1<sup>−/−</sup>/Crl). For these experiments, 2x10<sup>6</sup> rat MAPCs stably transfected with firefly luciferase with a lentivirus vector were injected 1 day after arterial ligation. At days 1, 2, 3, and 7 after injection, animals were anesthetized with inhaled isoflurane, and luciferin (150 μg/g) was injected via the intraperitoneal route. Optical imaging (IVIS Spectrum, Caliper Life Sciences) was performed 15 minutes after luciferin injection with medium binning, and data were expressed as photons per second per 1 cm<sup>2</sup>.

Immunohistochemistry

Histology was performed on perfusion-fixed paraffin-embedded sections. Sections were stained with hematoxylin and eosin for evaluation of inflammatory cell infiltration. Immunohistochemistry was performed for monocytes/macrophages with a rat anti-mouse Mac-2 monoclonal antibody (M3/38, eBioscience) with an Alexa Fluor-488-labeled secondary antibody (Invitrogen). The spatial extent of positive staining was quantified by a pixel intensity threshold program (NIH Image-J) and expressed as a percent of the total muscle area per section. Histological evaluation of the spatial relation between MAPCs and monocytes was performed by Mac-2 staining in muscle injected with MAPCs prelabeled with dioctadecyl-tetramethylindocarbocyanine perchlorate (DiI). Rabbit primary antibodies were used to stain for CXCR-1 (ab20821, Abcam), arginase-1 (LS-B4789, LifeSpan Biosciences Inc), macrophage F4/80 (ab74383, Abcam), and CD31 (SP38, Novus Biologicals), with anti-rabbit secondary antibody detection with Alexa Fluor-555-labeled (Invitrogen) or 3,3′-diaminobenzidine chromagen (Vector Labs).

Intravital Microscopy

Intravital microscopy was performed to further assess the effects of MAPCs on vascular leukocyte recruitment. Mice were randomized to one of the following treatment regimens performed 2 days before intravital microscopy: (1) intrascrotal injection of 1x10<sup>6</sup> MAPCs alone (n=3); (2) intrascrotal injection of 0.5 mg tumor necrosis factor-α (TNF-α) alone (n=6); (3) intrascrotal injection of 0.5 mg TNF-α (Sigma), followed 1 hour later by 1x10<sup>6</sup> MAPCs (n=7); (4) left cremasteric ischemia produced by the isolated interruption of flow to the left internal iliac artery and pudic-epigastric trunk and sham intrascrotal saline injection (n=4); or (5) cremasteric ischemia, followed 1 hour later by 1x10<sup>6</sup> MAPCs (n=5). MAPCs were fluorescently labeled before injection with 5 μmol/L DiI. Two days later, mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride, xylazine, and atropine. A cremaster muscle was exteriorized and mounted on a custom stage, and intravital microscopy (Axioskop2-FS, Carl Zeiss, Inc, Thornwood, NY) was performed with a saline-immersion objective (x40/0.5 numeric aperture). Video recordings of 25 μm needles (diameter, 20–40 μm) were made with a charge-coupled device camera (C2400, Hamamatsu Photonics) within 30 minutes of exteriorization. Centerline red blood cell velocity (V<sub>c</sub>) was measured with a dual-slit photodiode (CircaSoft Instrumentation). Venular diameters (d) were measured offline with video calipers. The distance traveled by individual rolling leukocytes was divided by the elapsed time to calculate rolling velocity. The rolling leukocytes (r<sub>r</sub>) crossing a line perpendicular to the vessel over 1 minute were counted, and leukocyte rolling flux fraction (the percent of leukocytes passing through a vessel that are rolling) was calculated by the following equation:

\[
\text{r}_r = \frac{V_r}{(0.25\pi d^2 \times V_c \times 60 \times C_c)}
\]

where C<sub>c</sub> is the systemic blood leukocyte concentration. In 4 additional mice, the degree of cremasteric ischemia was determined by ligation as described above, placement of a catheter in the carotid artery that was advanced into the aorta, and intra-aortic injection of 4x10<sup>6</sup> fluorescently labeled 15-μm microspheres (DYE-TRAK Persimmon, Triton Technology) over 1 minute. After 10 minutes, both cremaster muscles were removed and weighed. The degree of ischemia was quantified by the ratio of left to right cremaster fluorescent activity normalized to weight.

Monocyte Migration

Conditioned media was collected from MAPCs plated at 1x10<sup>6</sup> cells per well grown under standard conditions for 3 days in the absence of serum. Human CD14<sup>+</sup> monocytes were isolated from whole blood with a magnetic isolation kit (Dynabeads FlowComp, Invitrogen) and were confirmed to be CD14<sup>+</sup> by flow cytometry. Either basal nonconditioned or MAPC-conditioned media (750 μL) was placed in the bottom of 24-well 3-μm-pore Transwell plates (Costar). 250 μL nonconditioned serum-free media containing 1.25x10<sup>6</sup> CD14<sup>+</sup> monocytes was added to the inserts; and monocyte migration to the wells was determined after 1 hour. Experiments were performed in triplicate with monocytes from different donors.

Cytokine Assay

A multicytokine antibody array (RayBio Biotin Label-Based Human Antibody Array 1, Ray Biotech) was used to measure human cytokines from serum-free 3-day MAPC-conditioned medium.

Statistics

Statistical analysis was performed with SAS version 9.1. Comparisons between the treatment cohorts with regard to perfusion, molecular imaging data, and histological analysis were made with 1-way ANOVA or repeated-measures ANOVA for longitudinal differences, and when significant (P<0.05), post hoc analysis with unequal Student t test and Bonferroni correction for multiple comparisons was performed. Intravital microscopy data were nonnormally distributed and were compared by the Mann-Whitney rank-sum test except for leukocyte rolling data, which were compared by an unpaired Student t test or a Kruskal-Wallis test for evaluation of treatment effects. A Fisher exact test was used to analyze differences in proportions.

Results

Microvascular Remodeling and Flow Recovery

Arterial ligation resulted in a dusky color in the ischemic limb, but distal toe necrosis did not occur. Microvascular blood flow in the ischemic hind limb measured by CEU was reduced by ≈75% by arterial ligation (Figure 1A). Three-dimensional rendering of the spatial distribution of the MAPC cell suspension after ultrasound-guided intramuscular injection on day 1 indicated that the suspension had distributed longitudinally in the deep portions of the proximal hind-limb adductor muscle group, extending from the inguinal region to the knee (Figure 1C). Injection of MAPCs on day 1 resulted in a more rapid and complete recovery of microvascular blood flow in the ischemic leg compared with either mice injected with sham saline or nontreated control mice (Figure 1A). Blood flow during contractile exercise on day 21 was also significantly greater in
MAPC-treated compared with sham-treated control animals (2.8±0.5 versus 1.6±0.4 mL·min⁻¹·g⁻¹; P<0.05). Parametric CEU analysis indicated that functional microvascular blood volume was greater in MAPC-treated animals than in controls at days 3, 7, and 21 after ligation, and only in the MAPC-treated group did microvascular blood volume exceed that at baseline (Figure 1B). Microvascular fluorescent angiography performed by in vivo lectin staining illustrated a greater number of capillary units functionally perfused and an increase in the number of small to medium transverse or bridging arterioles in MAPC-treated compared with control groups at days 7 and 21 (Figure 1D). At day 21, muscle capillary density and the capillary-to-myocyte ratio were slightly increased in the ischemic tissue from all groups compared with nonischemic limbs (Figure I in the online-only Data Supplement); however, there were no significant differences between MAPC-treated and untreated ischemic muscle.

MAPC Survival in the Ischemic Limb
Optical imaging of luciferase activity after injection of luciferase-transfected MAPCs indicated that cells remained primarily within the injected limb (Figure 2). Activity declined progressively by 2 orders of magnitude between days 1 and 7, suggesting either loss of cells or loss of protein production. The pattern of decay was not substantially different for immune-competent and natural killer--deficient SCID mice, suggesting that clearance of MAPCs occurs similarly in the presence or absence of immune rejection. At day 7, luciferase activity was not significantly different from background levels in the majority of mice in both groups.

Molecular Imaging of Endothelial Activation and Monocyte CX₃CR-1
CEU molecular imaging of P-selectin was used to assess endothelial response (Figure 3A and 3B). P-selectin-targeted signal in the ischemic limb was significantly greater in MAPC-treated mice compared with both control groups at 3 and 7 days after arterial ligation. In MAPC-treated animals, signal was not confined to the initial distribution of cell injection and instead was uniformly distributed throughout the hind-limb adductor muscle group. P-selectin signal subsequently decreased by day 21 for all groups. There was a trend toward higher P-selectin signal in sham saline-injected versus nontreated control limbs at day 3 that did not reach statistical significance after correction for multiple comparisons. Monocyte CX₃CR-1 signal on CEU molecular imaging in the ischemic limb was significantly higher for MAPC-treated mice compared with control groups at all time intervals (Figure 3C) and was distributed uniformly throughout the muscle. There was no significant decline in CX₃CR-1 signal between days 3 and 21. Preferential attachment of the CX₃CR-1--targeted microbubbles to the intended monocyte population was verified by flow cytometry (Figures II–IV in the online-only Data Supplement). For wild-type CD115-positive monocytes, there was greater attachment of DI-D--labeled CX₃CR-1--targeted microbubbles to LyC₆low
than LyC6high cells, indicated by a fluorescent intensity that was almost 2 orders of magnitude greater, and a higher percent of cells interacting with labeled microbubbles (70% versus 15%). Similarly, for CD115-positive monocytes from transgenic mice expressing green fluorescent protein with the NR4A1 nuclear receptor that promotes the CX3CR-1high Ly6Clow phenotype,14,15 fluorescent intensity from interaction with CX3CR-1--targeted microbubbles was ≈6-fold greater for NR4A1high than NR4A1low cells. There was little attachment to monocytes for control nontargeted microbubbles.

Leukocyte Rolling on Intravital Microscopy
To corroborate the P-selectin expression patterns seen on molecular imaging, intravital microscopy was performed 2 days after intrascrotal injection of MAPCs to assess selectin-dependent leukocyte rolling. When given in the absence of TNF-α pretreatment or ischemia, MAPCs remained attached to the outer surface of the cremaster muscle and did not migrate intramuscularly. With either TNF-α pretreatment or cremasteric ischemia, MAPCs migrated into the muscle, and the majority assumed a perivascular arrangement around almost all venules and many arterioles (Figure 4A and Videos I and II in the online-only Data Supplement). Venular leukocyte rolling flux fraction was higher and leukocyte rolling velocity was slower in animals treated with TNF-α and MAPCs 2 days earlier compared with sham-treated control animals receiving TNF-α alone (Figure 4B and 4C). Extravasated leukocytes were frequently observed in the proximity of perivascular MAPCs (Figure 4D). There was no major difference in leukocyte rolling in venules with high versus low density of MAPCs. For ischemia studies, flow in the ischemic left cremaster muscle was 40±18% of that in the contralateral control cremaster. In the ischemic cremaster muscles, venular leukocyte rolling velocity was slower in muscles treated with MAPCs than in sham-injected controls (the Table). Although leukocyte flux fraction was not significantly different between the groups, MAPC treatment resulted in greater flow velocity, greater flow rate, and hence a higher total number of rolling leukocytes. Together, intravital microscopy data suggest that in the presence of ischemia or a proinflammatory milieu, MAPCs are able to migrate to a perivascular location where they promote leukocyte recruitment and probably migration.

Monocyte Migration and Cytokine Production
Cell migration assays were performed to further examine whether products from MAPCs promote monocyte migration. Migration of human monocytes was much greater in response to MAPC-conditioned media compared with nonconditioned media (151±55 versus 6±3 cells per optical field; P<0.01). The cytokine array from conditioned media indicated the production of several key chemokines involved either directly or indirectly in monocyte chemotaxis and selectin expression, including monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, interleukin-1α, vascular endothelial growth factor-A, and macrophage colony-stimulating factor (Figure V in the online-only Data Supplement), some of which were increased markedly by exposure of cells to cytokines known to be elevated in ischemic tissue.

Histology
On hematoxylin and eosin staining of muscle from the ischemic hind limb at days 3 and 7, there was a immune cellular infiltrate seen in all 3 treatment groups that was greater for the MAPC-treated group than in the control groups (Figure VI in
P-selectin targeted imaging in the transverse-axis plane at day 7 illustrates the spatial distribution of signal in the proximal adductor muscle group. The B-mode 2-dimensional image for the MAPC–treated mouse is provided at the top for anatomic reference (femoral acoustic shadowing at the right of the image). **A**, Examples of P-selectin targeted imaging in control noninjected, sham PBS-injected, and multipotential adult progenitor cell (MAPC)–treated limbs. **B**, Means±SEM signal intensity from contrast-enhanced ultrasound (CEU) molecular imaging with P-selectin–targeted microbubbles in control noninjected, sham PBS–injected, and multipotential adult progenitor cell (MAPC)–treated limbs. **C**, Means±SEM signal intensity from CEU molecular imaging with CX3CR-1–targeted microbubbles. **D**, Microscopy showing attachment of CX3CR-1–targeted microbubbles (dark spheres) to murine homotypic monocyte aggregates (top) and lack of microbubble (fluorescently labeled green with DiO) attachment to a Ly-6C<sup>+</sup> (phycocerythrin-positive) monocytes. Scale bar=10 μm. Number of animals for each group is provided in Table I in the online-only Data Supplement. *P<0.05 vs control and PBS; †P<0.05 vs PBS.

**Figure 3.** Molecular imaging for P-selectin and CX3CR-1. **A**, Means±SEM signal intensity from contrast-enhanced ultrasound (CEU) molecular imaging with P-selectin–targeted microbubbles in control noninjected, sham PBS–injected, and multipotential adult progenitor cell (MAPC)–treated limbs. **B**, Means±SEM signal intensity from contrast-enhanced ultrasound (CEU) molecular imaging with CX3CR-1–targeted microbubbles. **C**, Means±SEM signal intensity from CEU molecular imaging with CX3CR-1–targeted microbubbles. **D**, Microscopy showing attachment of CX3CR-1–targeted microbubbles (dark spheres) to murine homotypic monocyte aggregates (top) and lack of microbubble (fluorescently labeled green with DiO) attachment to a Ly-6C<sup>+</sup> (phycocerythrin-positive) monocytes. Scale bar=10 μm. Number of animals for each group is provided in Table I in the online-only Data Supplement. *P<0.05 vs control and PBS; †P<0.05 vs PBS.

The online-only Data Supplement). On quantitative analysis of monocyte Mac-2 staining, there were pronounced differences between groups. For all 3 post-ligation study points, the area staining positive for Mac-2–positive cells was greater in the MAPC–treated compared with the control groups (Figure 5A and 5B). For all groups, monocyte infiltration peaked at day 7. MAPCs produced a more diffuse rather than focal monocyte infiltration, indicated by the greater number of sections with Mac-2–positive cells in MAPC–treated compared with control muscle (Figure 5C). On immunohistochemistry at the early time intervals, there were more arginase-1–positive monocytes in MAPC–treated than in control ischemic muscle (Figure 5D and 5E). The majority of monocytes stained positive for CX3CR-1 in all treatment groups. Although this did not allow us to confidently differentiate between CX3CR-1<sup>hi</sup> and CX3CR-1<sup>lo</sup> populations, MAPC–treated limbs were characterized by a greater number of cells in the muscle interstitium that stained strongly positive for CX3CR-1 (Figure 5F). Dual fluorescent staining demonstrated that most MAPCs that could be identified at day 3 had CX3CR-1–positive monocytes in proximity (Figure 5G).

**Discussion**

Stem cells and adult progenitor cells have been shown to promote beneficial vascular remodeling in ischemic tissues and in wound healing. One mechanism by which cell therapy potentiates angiogenesis is through their paracrine effects that involve the release of biomolecules that alter the local cellular and molecular environments. In a model of limb ischemia that was intended to reproduce a clinically relevant situation of severe reduction in resting flow, we used a panel of techniques, including in vivo CEU perfusion and molecular imaging, to temporally evaluate how MAPC therapy augments recovery of perfusion and influences the recruitment of monocytes thought to promote arteriogenesis in ischemic tissues. Our results suggest that this population of adult progenitor cells promotes monocyte entry into the skeletal muscle in part via a 2-step process that involves increased endothelial recruitment through P-selectin expression and enhanced monocyte chemotaxis.

Stem and progenitor cells have been shown to have somewhat varied immunomodulatory effects, depending on the leukocyte cell type and local tissue environment. Because MAPCs produce chemotactic and endothelial activating factors, it is not unexpected that under certain conditions MAPCs have been shown to stimulate a monocyte/macrophage response. Noteworthy findings of our study were the degree to which MAPCs augmented monocyte infiltration and how MAPC therapy dramatically altered the spatial characterization of the monocyte infiltrate, producing a much more uniform and diffuse distribution of monocytes than in untreated limbs. Monocytes that stained positively for arginine-1 were preferentially recruited without an obvious increase in granulocyte infiltration on hematoxylin and eosin staining, suggesting that cell therapy may rebalance the cellular immune response toward a more regenerative phenotype. A similar rebalancing of the cellular immune response has been described after injection of conditioned media from bone marrow–derived mesenchymal stem cells in a model of wound healing.

One aim of this study was to define the mechanism of enhanced proangiogenic monocyte recruitment with MAPC therapy. For evaluating endothelial activation, we studied P-selectin because of its important role in initiating the immune response through leukocyte capture and rolling. We used a P-selectin–targeted contrast agent that has been validated in multiple different models of ischemic disease. An advantage of using CEU in this particular study is that targeted microbubble signal reflects only luminal surface expression of P-selectin rather than that which is stored preformed within the Weibel-Palade bodies. We have previously shown that the P-selectin signal in this model of limb ischemia is primarily from endothelial activation, not from platelets. In MAPC–treated limbs, molecular imaging detected a significant increase in P-selectin expression at days 3 and 7. These findings were further substantiated by intravital microscopy in which, in venules of cremaster muscle that either was treated with TNF-α or was rendered ischemic, MAPCs resulted in slower
leukocyte rolling velocity and an increased number of rolling leukocytes, indicative of increased selectin expression. Intravital microscopy also provided convincing evidence that MAPCs promote the recruitment of certain immune cells by their ability to migrate to a perivascular position. Their migratory capacity may also explain why molecular imaging signal enhancement in MAPC-treated limbs was diffuse rather than localized to the region of cell distribution identified by imaging at the time of administration. An interesting and currently unexplained finding was the greater microvascular flow velocity in MAPC-treated ischemic muscles only several days after injection. This finding was corroborated by day 3 CEU perfusion data in ischemic limbs. These data imply a difference in the production of vasoactive compounds that may precede and promote structural vascular remodeling.

For temporal characterization of monocyte recruitment, we targeted microbubbles to CX3CR-1 in an attempt to image a subpopulation of monocytes thought to be proangiogenic and reparative. Flow cytometry demonstrated that these microbubbles preferentially attached to the intended population by binding with greater frequency to either Ly6C\textsuperscript{lo} monocytes or those with high NR4A1 expression. Molecular imaging signal for CX3CR-1 was increased by MAPC therapy and was sustained for at least 21 days. From immunohistochemistry, CX3CR-1 appeared to be almost exclusively from infiltrating mononuclear cells, the vast majority of which were monocytes. Expression of arginase-1 by monocytes in the MAPC-treatment group further supports the idea that cell therapy enhanced the recruitment of Ly6C\textsuperscript{lo} CX3CR-1\textsuperscript{hi} monocytes.

The use of targeted microbubbles for molecular imaging ensured that signal originated from cells that were being actively recruited in the vascular space rather than reflecting cumulative extravasation. This could in part explain why molecular imaging signal for monocytes underestimated the differences between treatment groups seen on histology. However, we believe that the greater treatment-related differences in monocyte recruitment on histology than molecular imaging could also be explained by the 2-step process of MAPCs to increase endothelial cell adhesion molecule expression and to enhance monocyte chemotaxis. The notion that MAPCs enhance monocyte chemotaxis was supported by data from migration assays, intravital microscopy, histology, and MAPC cytokine and chemokine array. Although CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and natural killer cells could also have contributed to the CX3CR-1 signal, the degree of lymphocyte infiltration on histology was very small, suggesting that MAPCs induce the specific recruitment of CX3CR-1\textsuperscript{+} monocytes.

Optical imaging of luciferase activity suggested that MAPCs did not persist beyond a week after injection. Although previous studies suggest that natural killer cells could be responsible for early cell clearance, our optical imaging data and monocyte immunohistochemistry from

Table. Intravital Microscopy Data for Ischemic Cremaster Muscle Venules

<table>
<thead>
<tr>
<th>Vessel diameter, (\mu m)</th>
<th>PBS Control (n=12)</th>
<th>MAPC (n=9)</th>
<th>(P)</th>
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</thead>
<tbody>
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<td>Venular blood velocity, mm/s</td>
<td>1.0±0.5</td>
<td>2.1±1.0</td>
<td>0.002</td>
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<td>Venular shear rate, s(^{-1})</td>
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<td>1196±797</td>
<td>0.09</td>
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<td>Leukocyte rolling velocity, mm/s</td>
<td>38±4</td>
<td>43±6</td>
<td>0.04</td>
</tr>
<tr>
<td>Leukocyte rolling velocity/shear rate, mm/s(^{*})</td>
<td>0.060±0.049</td>
<td>0.029±0.019</td>
<td>0.001</td>
</tr>
<tr>
<td>Leukocyte rolling flux fraction, (x\times10^{-2})</td>
<td>7.2±6.6</td>
<td>5.0±5.3</td>
<td>0.45</td>
</tr>
</tbody>
</table>

MAPC indicates multipotential adult progenitor cell. Data are presented as mean±SD.

*More than 100 observations per group were made for rolling velocity data.
SCID beige mice and immunohistological staining for natural killer cells in wild-type mice (Figure VII in the online-only Data Supplement) indicate that most cell loss was not from immunological “rejection.” An intriguing observation was that certain indicators of immune activation such as enhanced CX3CR-1 signal on molecular imaging and monocyte recruitment on histology persisted late (day 21) despite the evidence on optical imaging that few MAPCs survived at 1 week. However, it is known that the monocyte inflammatory response in ischemic limb tissue is self-amplifying.25 Many monocyte/macrophage cell products are capable of perpetuating endothelial activation and monocyte recruitment. Accordingly, we believe that a sustained monocyte response can be achieved with single administration of a chemokine stimulus such as intramuscular injection of MAPCs. Although we did not specifically test which mediators were directly responsible for initiating these events, we did evaluate the MAPC secretome, which indicated the presence of several key cytokines, CC chemokines, and growth factors (monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, vascular endothelial growth factor, macrophage colony-stimulating factor, and interleukin-1α) that have all been shown to directly or indirectly increase P-selectin expression and/or monocyte chemotaxis.26–30

It should be noted that results from microvascular perfusion in this study alone are important. Muscle CEU perfusion imaging has been used to evaluate proangiogenic therapies in animal models of disease.31 As we have demonstrated, it provides information on perfusion at rest and during stress and can measure the expansion of the microvascular blood volume. CEU blood volume data in conjunction with fluorescent microangiography and capillary histology suggested that the MAPC treatment increased flow primarily through remodeling of the distal arteriolar network, which enhanced distribution of flow to parallel microvascular units.

Several limitations of the study deserve mention. The lack of luciferase activity beyond day 7 on optical imaging may have resulted from altered protein production rather than cell loss, although histology supported the latter. Our data do not provide definitive information on how much of the vascular response to MAPC was attributable to monocyte recruitment. It should also be noted that there is some controversy concerning the identification of proangiogenic monocytes, with some reports suggesting that the

![Figure 5. Monocyte immunohistochemistry. A, Quantitative results of the area staining positive for monocyte Mac-2 from days 3, 7, and 21 (note the different y axis scales; n=3 for each condition with >20 sections per subject analyzed). B, Examples of Mac-2 staining at day 7 from multipotential adult progenitor cell (MAPC)-treated and sham PBS-treated ischemic muscle. C, Percent of sections demonstrating any Mac-2-positive cells (≥80 fields for each condition). Histology from muscle at day 3 illustrating separate examples of arginase-positive (Arg1) cells (green) in MAPC-treated muscle (D) that were largely absent in untreated muscle (E). F, Example of positive CX3CR-1 staining of monocytes from a MAPC-treated limb on day 3. G, Example illustrating colocalization of Mac-2–positive monocytes with a Di-labeled MAPC. *P<0.05 vs both control groups.](http://circ.ahajournals.org/.../by-guest-on-April-23,2017)
“inflammatory subset” of monocytes that are CCR2<sup>high</sup> may also contribute to angiogenesis in response to limb ischemia in mice. The differences in flow and shear conditions could have influenced leukocyte rolling data on intravital microscopy experiments in ischemic muscle. However, data from TNF-α-treated muscle were generated under identical shear conditions and support the idea of enhanced rolling with MAPC therapy. Arguing against P-selectin as a mechanism for selective recruitment for “proangiogenic” monocytes, it has been shown that Ly-6<sup>C</sup>low CX<sub>CR1</sub>-<sup>1</sup>high monocytes have slightly less cell surface expression of P-selectin glycoprotein ligand-1 compared with their more inflammatory counterparts. However, we believe that P-selectin is important for the initial microvascular capture for all monocytes and that selective recruitment of 1 subtype is likely to involve specific chemotactic signals.

**Conclusions**

We have demonstrated that adult progenitor cell therapy with MAPCs potentiates the proangiogenic monocyte response in limb ischemia that may in part explain their ability to promote flow recovery. These data will likely provide justification for future studies to evaluate the molecular mediators that are responsible for monocyte recruitment and to develop models that can test how much of the vascular response is attributable to monocytes.

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**Disclosures**

Drs Ting and Woda and N. Lehman are employees of Athersys, Inc. The authors report no conflicts.

**References**


**CLINICAL PERSPECTIVE**

The number of patients with severe symptoms from coronary and peripheral artery disease who are not candidates for revascularization therapy because of age, comorbidities, and/or diffuse distribution of disease is steadily growing. Proangiogenic cell therapy is a promising therapeutic alternative. However, the exact mechanisms by which stem cells promote vascular remodeling are unknown and are important for optimization of therapy in terms of cell type, dose, and method of administration. In this study, we used contrast ultrasound perfusion to demonstrate that multipotential adult progenitor cells given intramuscularly increased microvascular blood flow and volume in a murine ischemic limb model. On a structural level, the improvement in flow was due to arteriolar remodeling. Using molecular imaging, histology, and intravital microscopy, we showed that in the setting of ischemia, multipotential adult progenitor cells migrate to a perivascular location where they recruit a specific population of monocytic cells that are recognized to be proangiogenic and important in wound healing. This process was accomplished by stimulation of endothelial cell adhesion molecules that participate in leukocyte recruitment and by chemokine signaling of monocytes to migrate into tissue. These results add to the growing body of science indicating that stem cells promote flow recovery more from their paracrine effects on host cells than from their engraftment into new blood vessels and that a specific proangiogenic aspect of the inflammatory response is a mediator of this process.
Molecular Imaging of the Paracrine Proangiogenic Effects of Progenitor Cell Therapy in Limb Ischemia

Jae Choon Ryu, Brian P. Davidson, Aris Xie, Yue Qi, Daogang Zha, J. Todd Belcik, Evan S. Caplan, Juliana M. Woda, Catherine C. Hedrick, Richard N. Hanna, Nicholas Lehman, Yan Zhao, Anthony Ting and Jonathan R. Lindner

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**SUPPLEMENTAL MATERIAL**

*Supplement Table: Number of Animals for Each Treatment Group and Time Interval for CEU and Molecular Imaging Data.*

<table>
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<tr>
<th></th>
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<td>10</td>
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<td>-</td>
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<td>6</td>
<td>6</td>
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<tr>
<td>Ischemia + MAPC</td>
<td>-</td>
<td>-</td>
<td>11</td>
<td>7</td>
<td>10</td>
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Supplemental Figure 1 – Capillary Density at Day 21. Examples from PECAM-1 immunostaining illustrate identification of: (A-C) intermyocyte capillaries, (D and E) small arterioles differentiated based on size, presence of a detectable lumen, and presence of smooth muscle cells, and (F) small venules differentiated based on size. Data illustrate mean (±SEM) capillary density (G), and capillary-to-myocyte ratio (H) for control limbs and ischemic limbs in...
various treatment groups. Data are derived from >15 observations for each condition. P-values are not corrected for multiple comparisons.
Supplemental Figure 2 – Flow Cytometry from Wild-type Mice. Gating of bone marrow-derived cells by forward versus side scatter (upper left) was used to identify the typical range for monocytes. Staining for CD115 was used to define monocytes with further differentiation into Ly6C-high and Ly6C-low populations (upper right). The geometric mean fluorescent intensity (log scale) for DiD intensity, indicating interaction with deflated CX3CR-1-targeted microbubbles for the two different populations according to Ly6C are shown to the right.
Supplemental Figure 3 – Flow Cytometry from NR4A1-GFP Mice. Gating of bone marrow-derived cells by forward versus side scatter (upper left) was used to identify the typical range for monocytes. Staining for CD115 was used to define monocytes with further differentiation into NR4A1-high and NR4A1-low populations (upper right). The geometric mean fluorescent intensity (log scale), indicating interaction with deflated CX3CR-1-targeted microbubbles, for the two different populations according to NR4A1 are shown to the right.
Supplemental Figure 4 – Flow Cytometry from Wild-type Mice Demonstrating Lack of Attachment for Non-targeted Agent. Gating of bone marrow-derived cells by forward versus side scatter (upper left) was used to identify the typical range for monocytes. Staining for CD115 was used to define monocytes with further differentiation into Ly6C-high and Ly6C-low populations (upper right). The fluorescent intensity (log scale) histograms for DiD intensity, indicating interaction with deflated non-targeted (left) or CX3CR-1-targeted microbubbles (right) are illustrated below for gates that included all CD115-positive cells (open gate) or the Ly-6C-low cells which demonstrate selective attachment of the targeted microbubbles.
Supplemental Figure 5 – Monocyte Chemoattractant Cytokine Expression. Assays were performed for basal media (no exposure to MAPC), conditioned media obtained from MAPC cultures (CM), and conditioned mediate obtained from MAPC stimulated with cytomix (10 ng/ml TNF-α, 10 ng/ml IL-1β, and 10 ng/ml IFNγ to simulate an activation environment. Data are expressed relative to positive control provided by the manufacturer.
Supplemental Figure 6. **H&E Staining.** Muscle samples that typify the cellular infiltration seen at Day 3 in the ischemic limb treated with MAPCs or PBS injection.
Supplemental Figure 7 – Investigation of the Role of NK Cells in MAPC clearance. (A and B) Immunohistochemistry for Mac-2 did not show any significant difference in monocyte infiltration in the ischemic leg 5 days after MAPC injection for wild-type C57Bl/6 mice and NK cell-deficient SCID beige mice (SCID/NK-) (n=3 for each species). Immunostaining for the NK cell marker CD335 was negative in ischemic muscle (C). Positive control is shown in Panel D which illustrates a CD335 cell-rich region of the spleen; positive immune cell staining is shown in Panel E which illustrate monocyte cell marker F4/80.
Supplement Videos:

Video 1a – Intravital microscopy (transillumination) of a venule from a cremaster muscle 3 days after treatment with intrascrotal TNF-α and MAPC demonstrating leukocyte rolling.

Video 1b – Intravital microscopy of the same venule as in Video 1a using fluorescent epi-illumination demonstrating the perivascular location of di-I-labeled MAPC.

Video 2a – Intravital microscopy (transillumination) of a venule from a cremaster muscle 3 days after treatment with intrascrotal TNF-α and MAPC demonstrating leukocyte rolling and adhesion.

Video 2b – Intravital microscopy of the same venule as in Video 2a using fluorescent epi-illumination demonstrating the perivascular location of di-I-labeled MAPC.