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Intravenous Application of CD271-selected Mesenchymal Stem Cells during Fracture Healing

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Abstract

Objectives—Circulating mesenchymal stem cells (MSCs) participate in fracture healing and can be used to enhance fracture healing. This study investigated how CD271-selected MSCs travel in circulation and when it is the optimal time to apply MSCs intravenously during fracture healing.

Methods—Based on the expression of CD271, MSCs were isolated from human bone marrow and labeled with cypate, a near infrared fluorochrome. A unilateral closed fracture was created at the femur in immunodeficient mice. The cypate-labeled MSCs were injected into the tail vein of the mice at days 1 and 3 after fracture, and were tracked by near infrared imaging. The mice were euthanized at 3 weeks after fracture. Immunohistochemistry was performed to detect human MSCs at the fracture sites. Migration of CD271-selected MSCs, under the influence of stem cell derived factor-1 (SDF-1), was assessed *in vitro*.

Results—Intravenously injected at day1, but not day 3, after fracture, CD271-selected MSCs accumulated at the fracture sites significantly and that lasted for at least 7 days. All fractures, with or without MSC injections, healed in 3 weeks. Human cells were localized at the fracture sites in mice by immunohistochemistry. CD271-selected MSCs migrated toward the medium contained SDF-1 *in vitro*.

Conclusions—After intravenous injection, CD271-selected MSCs were recruited to fracture sites. The stages of fracture healing influenced the homing of culture-expanded MSCs. In mice, an optimal window of intravenous injection of MSCs was around 24 hours after fracture.

Clinical Relevance—Intravenous application of MSCs may serve as a practical route to deliver stem cells for the treatment of fracture non-union and delayed union.

Levels of evidence—Level I

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Introduction

Mesenchymal stem cells (MSCs) are multipotent and responsible for tissue repair and regeneration, such as fracture healing. Characterizations of MSCs, however, have been carried out mostly *in vitro*. Conventionally, MSCs are selected by their capacity of plastic-adhesion in tissue culture. As a result, the isolated MSCs are a heterogeneous population comprising of a mixture of uncommitted stem cells and committed progenitor cells. While obtaining pure MSCs is still to be realized in the future, CD271 (low-affinity nerve growth factor receptor) has emerged as a cell surface marker for MSC enrichment. Immunohistochemically, CD271 was localized specifically in the bone marrow stroma where MSCs reside, but not hematopoietic stem cells.[1] The selected CD271-positive MSCs are homogeneously small round cells that are capable of differentiating into adipogenic and osteogenic lineages and supporting hematopoietic precursors.[2]

In many orthopaedic procedures, bone marrow, from which the original MSCs were isolated, is often supplemented locally to augment bone healing. This approach, however, gives little consideration of integrating the functionality of the implanted MSCs and the local cellular milieu. Ideally, the implanted MSCs should be naturally incorporated into the process of osteogenesis, which consists of a series of spatial-temporal controlled events at the fracture site. Circulating MSCs, including those defined by CD271, have been identified in peripheral blood.[3] In response to tissue injury, the circulating MSCs are recruited to the injury/fracture site and participate in tissue repair, including fracture healing. Stem cell-derived factor 1 (SDF-1), a chemokine, plays a central role in the recruitment of circulating MSCs. It is selectively up-regulated in the tissue by ischemia or injury, including bone fracture.[4]

Supplementation of MSCs via circulation is a novel approach to enhance fracture healing. To realize this strategy, it requires that 1) the circulating MSCs respond to the chemotactic molecules, such as SDF-1, and 2) a sufficient SDF-1 gradient is present at the fracture site. However, culture expansion alters the phenotype and homing capacity of MSCs significantly. Normal fracture healing is a cascade of spontaneous tissue transformations and a constant evolution of chemokine gradients, which affects stem cell recruitment. The purpose of this study was to investigate how culture-expanded CD271-selected MSCs travel in circulation and when it is the optimal time to apply MSCs systematically during fracture healing. In this study, CD271-selected MSCs were intravenously injected into a mouse fracture model and the homing of these cells to fracture sites was tracked with near infrared imaging and immunohistochemistry.

Materials and Methods

1. MSC Isolation: Using human bone marrow for this study was approved by Saint Louis University and Medstar Health Research Institute Institutional Review Board. Bone marrow reaming during intramedullary fixation of tibial fracture was collected. After filtered to remove bone debris and fatty materials, the remaining bone marrow was diluted with phosphate buffered saline (PBS). To the top of 15 ml Ficoll-Paque density gradient media (Sigma-Aldrich Co, St. Louis, MO) in a 50-

ml conical tube, 35 ml of diluted bone marrow was added and centrifuged at 400 g for 40 minutes at 20°C. Mononuclear cells of bone marrow were collected at the inter-phase of plasma and Ficoll-Paque solution. The cells were incubated with a CD271 antibody conjugated with magnetic microbeads (Miltenyi Biotec Inc., Auburn, CA) and passed through a magnetic cell separator. The CD271 positive cells were collected and were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum, with 5% carbon dioxide in the air at 37°C. Medium was changed twice a week. Cells were used after three passages in the following studies.

2. The animal model of fracture and tracking of CD271-selected MSCs: Immunodeficient male mice (Taconic Corp., Hudson, NY) at 8 weeks of age were used for this study (approved by the Washington University Institutional Animal Care and Use Committee). The animals were anesthetized with intraperitoneal injection of a cocktail of ketamine/xylazine. A unilateral closed fracture was created at the mid-shaft of femur, using 3-point bending. Femoral fracture was confirmed with radiography. The fracture was not stabilized to eliminate any interference with natural fracture healing and potential artifacts of near infrared imaging. A preliminary study showed that intravenously injected MSCs accumulated at the bone openings of intramedullary fixation (data not shown). Fracture healing was monitored with radiography.

   CD271-selected MSCs were incubated with 5 μM cypate, a near infrared fluorochrome synthesized by Dr. Achilefu's laboratory,[5] for 45 minutes. The cells were trypsinized and counted. MSCs (2×10^6) were suspended in 200 μl PBS for intravenous injection. Before injection, cypate labeling of the cells were assessed under a fluorescent microscope equipped with a near infrared filter. Under anesthesia, the cypate-labeled MSCs in 200 μl PBS or 200 μl PBS only were injected via tail vein into mice which had unilateral femoral fracture. Mice were divided into four study groups: two groups of mice underwent MSC injections after 1 or 3 days of fracture; another two groups of mice were injected with PBS only, to serve as study controls, after 1 or 3 days of fracture. Each group consisted of 5 mice.

   Whole-body near infrared images of mice were taken, under isoflurane inhalation anesthesia, on both prone and supine positions immediately after MSC injection as well as at 24 hours, 48 hours, and 7 days after MSC injection. The images were analyzed using Pearl Cam Software (LI-COR Biosciences, Lincoln, NE) for fluorescent intensity at the fracture sites. For the same mouse, the fluorescent area on the fractured femur was encircled and then an identical area on the contralateral non-fractured femur was also encircled. The mean intensity of each femur was recorded.

3. Histology and immunohistochemistry: Mice were euthanized at 3 weeks post fracture. The previously fractured femurs were dissected and processed for histology. Tissue sections were stained with hematoxylin and eosin (H&E). Immunohistology was performed, using a human-specific vimentin monoclonal

antibody (Dako Inc., Carpinteria, CA), which does not cross react with mouse vimentin, to detect cells of human origin on the mouse tissue sections.

4. Migration of CD271-selected MSCs under the influence of SDF-1: After starving in serum-free medium overnight, CD271-selected MSCs (passage 3) were trypsinized and plated in trans-wells (1.5×10^5 per well), whose porous bottom membrane has a pore size of 8 μm (Millipore Inc., Burlington, MA). The trans-wells with CD271-selected MSCs were placed in the wells of a 24-well plate, which contained SDF-1 (100 ng/ml; Sigma-Aldrich) supplemented medium. The control wells contained the same medium but not SDF-1. The cells were incubated for 8 hours and the trans-well membranes were fixed with methanol. After staining with hematoxylin, migrated cells on the outer surface of the membrane were counted under a microscope. The experiment was performed in triplicate.

Statistical analyses

Data are expressed as mean ± standard deviation. The intensity of near infrared imaging at the femoral fracture sites and the corresponding site on the contralateral non-fractured femurs in the same group of mice were compared by paired t test. The number of CD271-selected MSCs that were migrated through the membrane of trans-wells, which either contained or were free of SDF-1, was analyzed with t test for the effect of SDF-1 on the migration of CD271-selectd MSCs. Statistical significance was set as $p < 0.05$.

Results

After incubation with cypate, nearly 100% of CD271-selected MSCs were labeled fluorescently. All mice survived and tolerated intravenous injections of MSCs. As near infrared images showed, immediately after intravenous injections, CD271-selected MSCs traveled to the lungs and largely accumulated in the lungs in a few minutes. Immediately after MSC injection, no fluorescence was detected in the limbs, with or without fracture, in both prone and supine positions (Fig 1A). Intensive accumulation of fluorescent signals in the liver was seen for as long as 7 days after the injection.

When MSCs were intravenously injected at days 1 and 3 after fracture, their distribution or homing at the fracture sites demonstrated different patterns. When MSCs were injected after one day of fracture, intensive fluorescent signals were detected after 24 hours in the tissues around the fracture sites. The increased fluorescent intensity at the fracture sites was statistically different from the contralateral non-fractured femurs in 48 hours post injection and that remained on the 7^{th} day ($p<0.05$). In 24 hours post injection, the distribution of fluorescence at the fracture sites was not limited in the femur but extended into a broad area of tissues surrounding the fracture (Fig 1B–D). The fluorescent area was reduced over time and almost exclusively limited to the fracture sites in 7 days after injection. The fluorescent intensity over the femur was unchanged on the fracture side, but significantly reduced on the non-fracture control side after 7 days.

When MSCs were intravenously injected three days after fracture, significant accumulation of MSCs at the fracture sites was found 24 hours post injection, but not 48 hours and 7 days post injection i.e. 5 and 10 days post fracture, respectively.

Radiographs and histology confirmed fracture healing in all the mice in 3 weeks (Fig 1E and F). There was no noticeable difference between the MSC- and PBS-injected mice in terms of callus formation and callus size.

On tissue sections of mice which received injections of human MSCs, immunohistochemistry using an antibody specifically against human vimentin detected positive cells in the fibrous tissues underneath the periosteum that was around the fracture sites (Fig 1G). No human-vimentin positive cells were found in bony callus.

When SDF-1 was present in the culture medium, CD271-selected MSCs migrated across the porous membrane as many as three times more than in the SDF-1 free medium ($p < 0.05$, Fig 1I).

Discussion

Circulating MSCs are found in various models of fracture healing.[6] In a clinical study, circulating MSCs were detected in peripheral blood between 39 and 101 hours after fracture.[7] Growing evidence suggests that circulating MSCs participate in fracture healing. Recruitment of circulating MSCs to fracture sites is achieved through interactions between a chemokine gradient built up in the tissues surrounding the fracture site and the chemokine receptors expressed by MSCs.[8] Translating this mechanism to enhance fracture healing as a stem cell therapy, however, is not straight forward because in part of the heterogeneity of MSCs.

The properties of MSCs/progenitor cells vary depending on tissues of origin and isolation methods,[9] and prolonged tissue culture may modify MSC phenotypes.[10] It is unclear, however, to what extent their homing capacity is altered during fracture healing. Intramedullary reaming is often performed during fracture fixation and joint replacement. In this study, CD271 was used as a marker to select MSCs from bone marrow reaming materials. This method obtained relatively uniform MSCs.[2, 11] In this study, MSCs were used within three passages to prevent extensive phenotypical modification during tissue culture. Cypate labeling of CD271-selected MSCs was highly efficient and avoided sophisticated cell manipulation such as transfection, which potentially changes the properties of stem cells. In this study, CD271-selected MSCs were intravenously injected into mice at different time-points after femoral fracture. Near infrared imaging used in this study was able to capture a distribution pattern of CD271-selected MSCs that was very similar to one obtained with real-time imaging of a gamma camera:[12] MSCs were detected quickly in the lung and liver after intravenous injection, and remained in liver for a much longer time. Without fracture, bone marrow derived MSCs delivered via vein, artery and peritoneal cavity minimally homed to bone, [12] which is consistent with the observations of this study on the non-fractured femurs.

Osteogenic MSCs/progenitors were mobilized into circulation at the early stage of fracture healing.[6] Since CD271-selected MSCs are relatively homogeneous,[2,11] their homing to fracture site was more dictated by the stages of fracture healing than the cellular property of MSCs. When CD271-selected MSCs were intravenously injected after one day of fracture and tracked with near infrared imaging, increased density of florescence was seen at the fracture sites of femurs. After fracture, traumatic inflammation is accompanied with the formation of a local chemokine gradient. The data of this study suggest that an effective chemo-attractive environment is formed as soon as 24 hours after fracture. Circulating MSCs were attracted to the fracture site, when injected after 3 days of fracture, but that lasted less than 48 hours. This may indicate a decrease in the chemokine gradient as fracture healing progresses. Localization of human cells in the tissues around the fracture sites in mice that were injected with human CD271-selected MSCs by immunohistochemistry is evident that the intravenously applied MSCs participated in fracture healing. The exact role of those recruited circulating MSCs in fracture healing, however, may prove to be more complex as human cells were not found in bony callus.

On the near infrared images of mice which received CD271-selected MSCs one day after fracture, the fluorescent signals presented in an extended area around the fracture sites. During fracture, tissues around bone such as muscles are also significantly damaged. The responses of those tissues to injury or repairing of those soft tissues is part of fracture healing. The images of this study revealed that muscles around fracture are important for fracture healing by attracting circulating MSCs to the adjacent areas of fracture. SDF-1 is expressed in most of the organs but only selectively up-regulated by ischemia or injury.[13] CXCR4, the receptor of SDF-1, is expressed by stem/progenitor cells in fracture healing.[14] As a result of the up-regulation of local SDF-1, CXCR4-expressing stem/progenitor cells are recruited to the injured tissue for angiogenesis and repair. Migration of CD271-selected MSCs toward SDF-1 added in culture medium demonstrated that these cells are capable of response to SDF-1 and indicated that SDF-1 may be responsible for the homing of intravenously injected CD271-selected MSCs to fracture sites.

It is noteworthy that this study used a regular fracture model in mice, which heals well. This study provided the insight of CD271-selected MSCs traveling to fracture sites. Whether the intravenously injected MSCs improve fracture healing is a question to be tested in fracture non-union models in future studies. An increased recruitment of circulating MSCs, however, has been demonstrated to increase bone formation during fracture healing.[15]

This study demonstrated that during fracture healing there is an optimal time for the tissues at fracture sites effectively recruiting intravenously injected MSCs. Fracture healing, and the integration of bone graft and implants may benefit from intravenous delivery of MSCs for enhanced bone regeneration.

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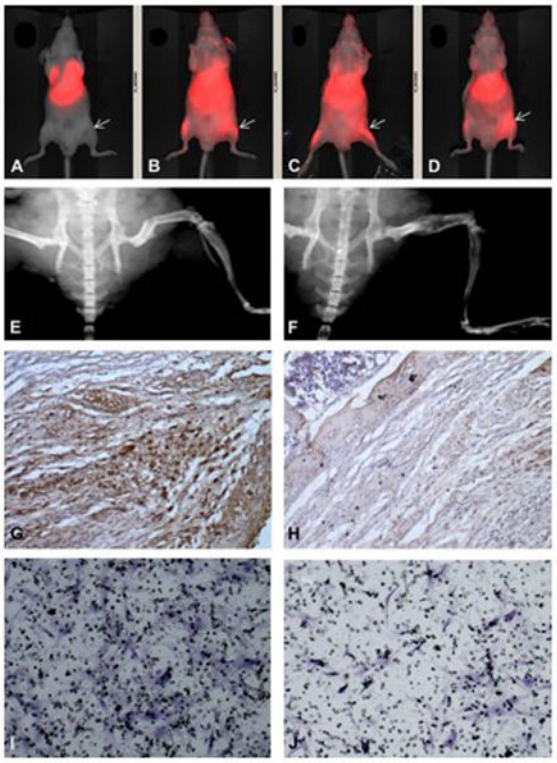

Figure 1.

Whole body near infrared images taken immediately after intravenous injection of cypate-labeled MSCs (A), and after 24 hours (B), 48 hours (C) and 7 days (D), show gradual accumulation of MSCs at the femoral fracture site (indicated with a white arrow). Radiographs show a closed femoral fracture created by 3-point bending (E) and healed fracture after three weeks (F). Immunohistochemistry using an antibody specific to human vimentin shows positive cells (brown) around the fracture site (G), but not in the negative control staining (H). When MSCs were cultured in trans-wells, there were more MSCs migrated toward SDF-1-containing medium (I) than the control medium (J; hematoxylin staining; black dots are membrane structure).