Stimulation of Skin and Wound Fibroblast Migration by Mesenchymal Stem Cells Derived from Normal Donors and Chronic Wound Patients

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ABSTRACT

Chronic wounds continue to be a major cause of morbidity for patients and an economic burden on the health care system. Novel therapeutic approaches to improved wound healing will need, however, to address cellular changes induced by a number of systemic comorbidities seen in chronic wound patients, such as diabetes, chronic renal failure, and arterial or venous insufficiency. These effects likely include impaired inflammatory cell migration, reduced growth factor production, and poor tissue remodeling. The multifunctional properties of bone marrow-derived mesenchymal stem cells (MSCs), including their ability to differentiate into various cell types and capacity to secrete factors important in accelerating healing of cutaneous wounds, have made MSCs a promising agent for tissue repair and regeneration. In this study we have used an in vitro scratch assay procedure incorporating labeled MSCs and fibroblasts derived from normal donors and chronic wound patients in order to characterize the induction of mobilization when these cells are mixed. A modified Boyden chamber assay was also used to examine the effect of soluble factors on fibroblast migration. These studies suggest that MSCs play a role in skin wound closure by affecting dermal fibroblast migration in a dose-dependent manner. Deficiencies were noted, however, in chronic wound patient fibroblasts and MSCs as compared with those derived from normal donors. These findings provide a foundation to develop therapies targeted specifically to the use of bone marrow-derived MSCs in wound healing and may provide insight into why some wounds do not heal. Stem Cells Translational Medicine 2012;1:221–229

INTRODUCTION

Wound healing is a dynamic process consisting of a continuum of four phases: homeostasis, inflammation, proliferation, and tissue remodeling or resolution [1]. This regenerative process is mediated by the presence of fibroblasts, new blood vessels (created through a process of angiogenesis), and chronic inflammatory cells (consisting predominantly of macrophages) in the wound bed. One possible method to increase the efficiency of the repair process and minimize undesirable outcomes, for example, scar formation, would be to manipulate the cell(s) that control this overall process. Earlier studies in cell-based therapies for diabetic wound healing focused mainly on using fibroblasts; however, recent studies have explored the benefits of bone marrow-derived mesenchymal stem cells (MSCs) [2–5].

The in vitro scratch assay is a simple and straightforward method to measure the rate of cell migration in an in vitro wound model [6]. Other in vitro techniques have disadvantages in primarily focusing only on a single cell type, which does not permit the analysis of cellular interactions [7]. Oberringer et al. reported an in vitro wound-healing model based on coculture of multiple cell types [8]. Following this concept, we have used a coculture scratch/migration assay to evaluate the contribution and interaction of dermal fibroblast and MSCs in the wound-healing process. This model will also allow a concurrent analysis of how human MSCs influence human fibroblast migration, whether mediated through soluble factors, cellular specific interactions, or a combination of both [9, 10]. We have used the scratch wound assay wherein a confluent monolayer of human MSCs and/or human dermal fibroblasts was scored with a sterile tip and the cells were observed using fluorescent microscopy as they repopulated the scratch area.

We also made use of a modified Boyden chamber assay to further examine evidence of soluble factors and/or cell-to-cell interactions between MSCs and dermal fibroblasts. The modified procedure entails the measurement of fibroblast migration toward MSCs in a defined environment. The measurement of the rate of
migration is used to elucidate the role of soluble chemotactic agents present in a given system. Taken together these two methods should provide valuable insights into the interaction between MSCs and dermal fibroblasts in the wound-healing process.

It has been reported that MSCs could act as a chemoattractant for macrophages and endothelial cells [11]. We provide evidence to suggest that MSCs induce the migration and possible recruiting of dermal fibroblasts to the wound site by both local microenvironment and paracrine signaling.

**MATERIALS AND METHODS**

**Cell Lines**

293T/17 cells, transformed human embryonic kidney cells expressing simian virus 40 (SV40) T antigen, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, http://www.atcc.org). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; high glucose, GLUTAMAX) supplemented with 10% heat-inactivated fetal bovine serum (FBS) without antibiotics so as not to interfere with packaging cell transfection or the masking of low-level contamination.

**Mesenchymal Stem Cell Isolation**

We isolated and expanded human MSCs from fresh whole bone marrow obtained from normal donors and chronic wound patients. Normal donor bone marrow was acquired from AllCells LLC (Emeryville, CA, http://www.allcells.com). Chronic wound patient bone marrow was obtained under an institutional review board (IRB) approved protocol (IND# BB IND 13201). Chronic wound patients had wounds of >2 years’ duration without evidence of healing and were not diabetic. MSCs were isolated by the plastic adherence method. A minimum of three separate batches of MSCs derived from different chronic wound patients were used for all comparisons made. Briefly, bone marrow mononuclear cells were isolated by low-density centrifugation using Ficoll-Paque Premium (d = 1.077) according to the manufacturer’s protocol. The mononuclear cells were collected at the interface, washed three times in phosphate-buffered saline (PBS) supplemented with 2% FBS, and resuspended in MSC medium (a-minimum essential medium [a-MEM], 20% MSCs, qualified FBS, and 1% Pen/Strep/glutamine). Initial cultures were seeded between 2–3 × 10^5 cells/cm² in tissue culture-treated dishes. After 48–72 hours the nonadherent cells were removed, the culture flasks were rinsed once with PBS, and fresh medium was added to the flask. The cells were grown until 80% confluence was reached and then passaged by Trypsin-EDTA detachment. Cells were split at a 1:4 ratio into new tissue culture flasks. Low passage stocks of transduced cells were cryopreserved until use.

The cryopreserved cells were thawed at 37°C and immediately cultured in a-MEM supplemented with 20% fetal bovine serum and 1% penicillin and streptomycin at 37°C in 95% humidified air and 5% CO₂. Up to fourth passage cells were used in this experiment.

**Differentiation of Human MSCs**

Human MSCs were analyzed for their capacity to differentiate into adipogenic or osteogenic lineages. Human MSCs cultured in MesenCult without any differentiation additive were used as negative control.

After second passages human MSCs were seeded at 3 × 10^5 cells/cm² in six-well plates fed with MesenCult medium and allowed to adhere for 24 hours. The induction into adipogenic or osteogenic differentiation the MesenCult Basal medium was replaced with particular medium in each case. For adipogenic differentiation the medium included MesenCult Basal medium (human) with adipogenic stimulatory supplements (catalog number 05401, 05403; StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com). After 4 weeks in culture, adipogenic differentiation was analyzed by the accumulation of lipid vacuoles using Oil Red staining [12, 13].

For osteogenic differentiation the medium includes MesenCult MSC Basal medium supplemented with ascorbic acid, β-glycerophosphate, osteogenic stimulatory supplements, and dexamethasone (catalog nos. 05401, 07157, 05406, 05405, and 05407; StemCell Technologies). After 6 weeks in culture, osteogenic differentiation was evaluated by alizarin red staining to visualize calcium deposits.

**Dermal Fibroblasts**

Normal human adult dermal fibroblasts were provided as a kind gift from Dr. Jie Li, Department of Dermatology, University of Miami Miller School of Medicine. Chronic wound patient fibroblasts were collected under an IRB approved protocol (IND# BB IND 13201) from wounds of >2 years’ duration without evidence of healing despite standard of care and advanced wound care treatments. When the fibroblasts reached 80%–90% confluence, the cells were expanded at a ratio of 1:4. Dermal fibroblasts were maintained at 37°C in 5% CO₂, and passages 2–6 were used for all experiments. Dermal fibroblasts were cultured in mesenchymal complete expansion medium when seeded for coculture with either mesenchymal stem cell or control inserts and for the duration of the experiment.

**Plasmids and Cloning**

The packaging construct pCMVD8.9, the heterologous vesicular stomatitis virus G glycoprotein (VSV-G) envelope expressing construct pCMV-G, and the gene transfer vector FUW were kind gifts from Dr. David Baltimore’s laboratory [14]. FUW is a self-inactivating lentiviral construct containing the human ubiquitin promoter.

The genes for red fluorescent protein mCherry and yellow fluorescent protein were obtained from the pmCherryN1 and pEYFP vectors, respectively (Clontech, Palo Alto, CA, http://www.clontech.com). Puromycin resistance (puromycin-N-acetyl transferase) under the control of an internal ribosome entry site (IRES) of the encephalomyocarditis virus was obtained from pIRESpuro3 (Clontech). These sequences were used as sources of the genes for polymerase chain reaction (PCR) cloning and creation of our lentiviral vector constructs.

The first vector created was the introduction of the IVS (synthetic intron)/IRES/puro3 (conferring puromycin resistance) sequence into the FUW gene transfer vector. The IVS sequence, also obtained from pIRESpuro3, is known to enhance the stability of the mRNA. This construct, FUIPW, became the backbone for subsequent gene transfer vectors, as puromycin selection was highly desirable for selection of transduced cells. Using the remaining 5’ BamHI and 3’ Hpal in the multiple cloning site, both of which are upstream of the IVS IRES puromycin sequence, we
inserted sequences coding for EGFP, mCherry (red), and EYFP (yellow). These vectors became designated as FUGIPW, FUmCherryIPW, and FUH2BmCherryIPW, respectively, and will code for the soluble form of these fluorescent proteins with puromycin selection.

In our experimental designs we wanted to develop a convenient method for marking stem cells that would allow us to study cell fate phenomena. To this end, we proposed to use our lentiviral system to deliver fusion protein coding sequences of the two previously mentioned reporter genes with nuclear localization signals garnered from the sequence for histone 2B. This sequence was amplified from normal human cDNA omitting the inherent stop codon and ligated in-frame to the full sequence for the corresponding fluorescent fusion partner. The clone includes the addition of the amino acid sequence RDPPLEVAT in the middle that contains an XhoI restriction site to facilitate easier shuttling of additional fusion partners. The resultant fluorescent fusion protein would then be sequestered entirely in the nucleus of the transduced cells. In this way potential “donor” cells could be tracked independently of “target/helper” cells that were transduced with a different fluorescent fusion protein. These vector constructs became designated FUH2BmCherryIPW (red fusion) and FUH2BmCherryIPW (yellow fusion). All proteins are expressed as S’ H2B fusions to the fluorescent counterpart. Combining this lentiviral technology with these localized reporter genes all with the capability of puromycin selection allowed us to expand virtually 100% transduced cells. In addition we were able to perform quantitative real-time PCR to characterize virus titers, integrated provirus, and mRNA levels based on the unique sequence of the woodchuck hepatitis virus posttranscriptional regulatory element present in all constructs.

**Lentiviral Vector Particle Production**

Lentiviral vector particles were produced using an optimized protocol based on a calcium phosphate transfection of our three-protocol self-inactivating lentiviral system into 293T/17 cells. On the day prior to transfection, 293T/17 cells were seeded at 1.14 × 10⁵ cells/cm² in advanced DMEM (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 10% FBS, supplemented with 1% l-glutamine, 0.01 mM lecithin, 0.01 mM cholesterol, and 1% chemically defined lipids. Cells were incubated at 37°C with 5% CO₂. On the day of transfection, warm advanced DMEM (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) with additives at 0.143 ml/cm² was added for a final concentration of 25 mM cholesterol, and 1% chemically defined lipids to 37°C. Meanwhile medium was replaced in culture vessels with the 2% FBS advanced DMEM with additives at 0.143 ml/cm². After 20 minutes of incubation, 1:100 dilution of 2.5 mM chloroquin was added for a final concentration of 25 μM. After mixing well, the suspension was added dropwise to cells randomly to disperse. The dish was tilted dish back and forth and side to side to mix and then incubated for 5 hours at 37°C, 5% CO₂, with humidity. After 5 hours of incubation, the medium was carefully aspirated from the dishes. Dishes were washed carefully with 15 ml of PBS, while being careful not to disturb the adherent cells. Culture medium (2% FBS advanced DMEM with additives) was replaced at 0.228 ml/cm² and incubated for 48 hours prior to collecting the supernatant. Supernatant was centrifuged at 2,000 rpm for 5 minutes to remove cellular debris. The supernatant was then filtered using a prewetted (with DMEM 10% FBS) low protein binding 0.45-μm filter Corning number 430768.

**Concentration of Lentiviral Supernatant**

Supernatants containing the virus were concentrated by flocculating with polybrene (catalog no. H9268; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) and chondroitin sulfate (Sigma catalog no. C4384) [Landa’ zuri]. Briefly, polybrene and chondroitin sulfate (PB/CS) were added to the supernatant to a final concentration of 80 μg/ml and incubated in a 37°C water bath for 20 minutes. After incubation the supernatants were centrifuged at 10,000g for 5 minutes. Supernatants were aspirated, and the resulting pellet was vigorously resuspended in ice-cold Hanks’ balanced saline solution until no clumps were observed to a final volume of 1/100 of the original. Aliquots of the virus were stored at −80°C until use.

**Cellular Transduction Using Lentivirus**

Early passage (second and third) MSCs were transduced with H2BmCherry. Early passage (second and third) fibroblasts were transduced with H2BEYFP. Briefly, cells were grown to 80% confluence at early passage and then passaged into new tissue culture vessels with medium containing the PB/CS complexes. The virus was allowed to remain in the cultures until the cells were fed. When cells reached 80% confluence, they were harvested and either used immediately in the experiments or cryopreserved until use.

**Flow Cytometry Analysis**

Both normal and chronic wound patients’ (primary) cultured MSCs were characterized by flow cytometry. Prior to fluorescence-activated cell sorting (FACS) analysis, MSCs were washed and resuspended in PBS and 2% fetal bovine serum (Akron Biotec, Boca Raton, FL, http://www.akronbiotech.com). Cells were blocked with human FcR blocking reagent (MACS, Milteny Biotec GMBH, Bergisch Gladbach, Germany, http://www.miltenyibiotech.com) to increase the specificity of antibody labeling and improve the purity of target cells. Immunostaining was performed using the markers recommended for confirmation of human MSCs (HuMSCs): CD90 anti-human (BioLegend, San Diego, http://www.biologend.com), CD105 anti-human (BioLegend), CD44 mouse anti-human (BD Biosciences, San Diego, http://www.bdbiosciences.com), CD73, and HLA-class I anti-human (BioLegend) conjugated with fluorochromes [15]. Cell concentration was in the range of 5 × 10⁵ to 1 × 10⁶ cells/ml. The antibody concentration was used as a company recommendation from previous titration for optimal staining. Cells were incubated with antibody between 15 and 20 minutes on ice, protected from light. The samples were washed with PBS and 2% FBS by centrifugation at 500g for 5 minutes and resuspended in 0.5% paraformaldehyde in PBS to be fixed. The panel of antibodies combined analysis samples was performed at BD LSR II Flow Cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). To eliminate overlap between the fluorochromes, we used compensation in multicolor flow cytometric analysis [16].

www.StemCellsTM.com
Scratch Wound Assays

Coculture of Mesenchymal Stem Cell and Dermal Fibroblast

In coculture experiments, the different cells type were prepared to seed in six-well plates. Cells were distinguished by fluorescent tagging, using the lentiviruses mentioned above. Each well was seeded with dermal fibroblasts to a final density of 1 × 10^5 cells per well. MSCs were seeded at different cell percentages (5%, 10%, 20%, 40%, and 80%) compared with fibroblast concentration, and these were maintained at 37°C and 5% CO₂ for 24 hours to permit cell adhesion and the formation of a confluent monolayer. To prevent the influence of cell proliferation, 2 hours prior to scratch the medium was substituted with a fresh serum-free culture medium containing mitomycin at 10 μg/ml. The confluent monolayer was then scored with a sterile pipette tip to leave a scratch of ~0.4–0.5 mm in width. Culture medium was then immediately removed (along with any dislodged cells). The removed medium was replaced with a fresh serum supplemented culture medium (20% FBS). All scratch assays were performed in quadruplicate.

Microscopy, Image Capture, and Analysis

The scratched area was monitored by collecting digitized images at various time points after the scratch was performed until closure was either complete or no longer progressing. Digitized images were captured with an inverted IX81 Olympus microscope (Olympus America, Center Valley, PA, http://www.olympusamerica.com) and ORCA-AG Hamamatsu digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Shizuoka Pref., Japan, http://www.hamamatsu.com) under phase or fluorescence, in which preselected fields were imaged using phase and (red and green) fluorescence microscopy. The digitized images were then analyzed using Image-Pro Plus 5.1 software to count the cells that migrated along the horizontal axis of the image (which equated to the top, middle, and bottom of the field of view).

Modified Dual Boyden Chamber Cell Migration Assay

Dermal fibroblast chemotaxis as measured using a 24-well Boyden chamber cell migration assay (QCM 24-well colorimetric cell migration assay ECM508, Millipore, Billerica, MA, http://www.millipore.com) according to the manufacturer’s protocol. Mesenchymal stem cells were seeded in the bottom chamber at 20%, 40%, and 80% the number of fibroblasts that would be added to the upper chamber and incubated for 24 hours in MSC medium. A cell culture insert with 8-μm pore size and polycarbonate membrane was placed in the well, and 5 × 10^5 dermal fibroblasts were added to the insert (upper chamber) in MSC medium and incubated at 37°C in 5% CO₂ for 24 hours. Control bottom chambers contained MSC medium without cells. Cultures were incubated at 37°C in 5% CO₂ for 24 hours. Cell migration through the membrane was determined by measuring absorbance at 560 nm on SpectraMax Plus microplate reader after staining and extracting cells that had migrated through the membrane at 24 hours.

Characterization of Mesenchymal Stem Cells

In normal and patient cells, FACS analysis showed that MSCs were positive for HLA-class 1, CD90, CD73, and CD105 and negative for CD45, consistent with previous reports [17] (Fig. 1A).

Differentiation of Human MSCs

To further establish that we had isolated MSCs from patients and donors, we examined their differentiation potential by placing cells in culture conditions favorable for adipogenic or osteogenic differentiation. After 4 weeks in lineage-specific culture conditions, the expanded cells from the patient donors were highly differentiated, without evidence of the other lineages.

Adipogenic differentiation was induced in the expanded MSC cultures by treatment with the specific medium described in Materials and Methods. This induction was apparent by the accumulation of lipid-rich vacuoles within cells. Fat globules were seen in MSC culture grown in adipogenic medium, indicating differentiation into adipocytes (Fig. 1B).

The osteogenic differentiation of cultured human MSCs was originated under the influence of dexamethasone, β-glycerophosphate, and ascorbic acid and in the presence of 10% FBS. Biomineralization of isolated HuMSCs in response to these compounds and the resultant calcium phosphate-rich monolayers was visualized by the calcium-binding dye, alizarin red S (Fig. 1B).

Normal Donor Mesenchymal Stem Cells Enhance Normal Donor Dermal Fibroblast Migration

To study the effect of MSCs in dermal fibroblast migration during the wound-healing process, we used a scratch assay method, which has been described as an in vitro procedure to study cell migration [6]. The dermal fibroblasts were labeled with a lentivirus containing the YFP gene fused to the human histone H2B sequence, and MSCs were labeled with the mCherry gene also fused to the human histone H2B sequence. Using two colors allowed us to distinguish fibroblasts from MSCs.

Dermal fibroblasts were plated at 1 × 10^5 cells/well in a six-well plate, and we referred to it as Fib only. MSCs were seeded at relative concentrations of 0% (control), 5%, 10%, 20%, 40%, and 80%, respectively. These cocultures allow us to visualize which cells are mobilized to the scratch. Given that scratch wound assays were performed in the presence of mitomycin C, which inhibits fibroblast and MSC proliferation in cell cultures, we warranted that wound closure is controlled by a migration and not proliferation.

An advantage of these studies compared with others is the use of nuclear-labeled cells, which allows for more exact quantification of migrating cells. (Fig. 2A). The analysis of migration of normal fibroblasts when exposed to normal MSCs outlines a general dose-dependent effect. Increased fibroblast migration was noted when the MSC population was 10% (Fig. 2B). When 20% (or more) of the cells were MSCs, fibroblast migration decreased.

Figure 1. Characterization of MSCs. (A): Surface marker characterization of MSCs by fluorescence-activated cell sorting. MSCs are positive for CD73, CD90, CD105, and HLA-class 1 and negative for CD45. (B): In vitro differentiation of human MSCs (HuMSCs). Cultured HuMSCs from donors were tested for the ability to differentiate in multiple cell lineages, such as adipogenic and osteogenic lineages. Adipogenesis was indicated by the accumulation of neutral lipid vacuole. Stained with Oil Red O (c, d). Osteogenesis was evidenced by the mineral deposition of MSCs cultured in osteogenic medium, indicating early stages of bone formation. Stained with alizarin red S (g, h). Undifferentiated controls for adipogenic (a, b) and osteogenic (e, f) conditions. Abbreviations: MSCs, mesenchymal stem cells.
Dermal Fibroblast Migration from Patients Is Decreased Compared with Normal Fibroblasts

Stem cell-based therapies for cutaneous wound repair have been examined using different approaches including the direct application of MSCs to the wound surface [2, 3, 5, 18–21]. To examine the effect of MSCs on dermal fibroblast migration in normal and chronic wound healing, we developed cocultures between normal fibroblasts and MSCs as well as between normal fibroblasts and normal MSCs as well as fibroblast and MSCs from chronic wound patients.

When we mixed different concentrations of normal MSCs with patient fibroblasts, we found a pattern similar to that seen with normal fibroblast and normal MSCs. The total number of cells migrating to the scratch was, however, dramatically lower compared with normal fibroblasts (Fig. 2B). A similar dose-response was noted when chronic wound patient MSCs were mixed with patient fibroblasts; however, it appeared that 20% MSCs were optimal in inducing migration. The overall migration of chronic wound patient fibroblasts was, however, even lower when cocultured with patient MSCs versus normal MSCs.
Fibroblasts cells were plated in the upper chamber of 8-μm pore size Transwell insert. Cells were allowed to migrate for 24 hours toward chemoattracting 5%, 10%, 20%, and 40% of 5 × 10^5 MSCs present in the lower chamber. Nonmigrating cells in the upper chamber were removed with the aid of a cotton swab, whereas migrated cells adhering to the lower surface of the membrane were stained and measured by absorbance at 560 nm (five randomly selected fields per Transwell insert). (B): Fibroblast migration induced by MSCs as chemoattractant. Normal fibroblast were seeded in the upper chamber; MSCs were seeded in the bottom at different concentrations (5%, 10%, 20%, and 40%). (C): Fibroblast migration induced by MSCs as chemoattractant. Patient fibroblasts were seeded in the upper chamber; MSCs were seeded in the bottom at different concentrations (5%, 10%, 20%, and 40%). Abbreviation: MSC, mesenchymal stem cell.

**DISCUSSION**

The in vitro scratch assay has several advantages, including its simplicity, ability to mimic the migration of cells in vivo, and capacity to isolate variables by testing the interaction between distinct cell types [6]. These are powerful reasons to use this classic method to study wound-closure mechanisms during the healing process. A unique advantage of our scratch assay procedure was the ability to selectively label cell populations with our lentiviral constructs. Recombinant lentiviral technology has been used to durably transduce cells and to establish stable transgenic animals [14, 22–24]. Lentiviruses have particularly distinct advantages in their ability to transduce both dividing and nondividing cells. The constructs used conferred fluorescent tags to cells in an anatomically defined region, the nucleus. Nuclear localization provides additional morphologic identification of labeled cells with the ability to confirm identity by colocalization with nuclear stains if needed. In addition, anatomical features such as clearly visible segregating chromosomes easily identify dividing cells. Using a fusion protein also helps to prevent leakage of signal following fixation or as a result of apoptosis. We have established that fixation with common fixatives such as formalin and paraformaldehyde works well with these constructs.

By marking cells with different colors, we could distinguish and quantify fibroblasts from MSCs during the wound-closure process. This method goes beyond established assays [6] in allowing one to both follow the wound-healing process and track specific cell types.

The assay systems described could become quite useful in examining cellular interactions when designing cell-based therapy trials. Cell-based therapy approaches for wound healing have important advantages over delivering single soluble diffusible factors in that administered cells can respond to the local environment and release multiple factors.

Dermal fibroblasts provide essential functions during wound healing such as wound contraction, extracellular matrix deposition, and tissue remodeling [10]. Other properties such as scarring are, however, less desirable. Coordination of these events is critical when trying to achieve durable wound closure. Several accessory cells have been implicated in the orchestration of tissue repair including mesenchymal stem cells [25–30].

We have investigated the interaction between MSCs and fibroblasts cells in stimulating cell migration. Improved migration of fibroblasts would support the administration of MSCs to wounds to improve healing. If MSCs directly increase migration by purely paracrine mechanisms, one would assume that fibroblast migration would increase with the increasing MSC concentration, as more MSCs would secrete a greater amount of factors. We have found, however, that this is not the case. At lower concentrations, as more MSCs would secrete a greater amount of factors. We have found, however, that this is not the case.
concentrations of MSCs, fibroblasts appeared to be most stimulated to migrate. As the concentration of MSCs increased, fibroblast migration was not improved or decreased. This may be due to variable expression of migratory and attractant cytokines produced. With fewer MSCs, migration-inducing cytokines may predominate in terms of relative concentration or overall effects. As the number of MSCs increase, cytokines that attract fibroblasts and/or inhibit migration may predominate. Direct contact with fibroblasts could also prove to be critical in determining which cytokines MSCs will express. At high density, it is likely that more MSCs will contact one another, perhaps yielding an entirely different microenvironment. It is interesting to note that many reports describe the reparative effect of MSCs administered systemically or directly to damaged tissues despite the fact that very few MSCs can be found in the healing tissue [26, 31, 32]. This may be due to the observation we have made that few MSCs may be better in producing a physiologic effect.

The results presented also support previous observations made by our laboratory [33, 34] that bone marrow cells derived from chronic wound patients have deficiencies. Both scratch assay and Boyden chamber experiments illustrate this by the observation that normal donor MSCs were significantly better than MSCs derived from chronic wound patients in getting normal fibroblasts to migrate. Current methods of characterizing MSCs may not be sufficient to predict the ability of particular MSC cultures to be effective in healing chronic wounds. In this report we have noted that patient and normal donor MSCs appeared equivalent by traditional measures such as FACS analysis and multilineage functional assays although differing significantly in their ability to induce fibroblast migration. Although the specific mechanisms effecting MSCs ability to induce fibroblast migration remain to be elucidated, some clues can be derived from the observations made here.

Fibroblast migration appears to be stimulated by MSCs in a dose-dependent manner in the scratch assay, which is a mixed culture system where MSCs and fibroblasts are able to contact one another and microenvironmental cues may play a role. We also observed (Fig. 2B) that normal donor MSCs are greatly reduced in their ability to stimulate chronic wound patient fibroblast migration when compared with their ability to induce normal fibroblast migration. This limited response could be due to a lack of response to paracrine stimulation by MSCs or to the overall senescent phenotype described in chronic wound fibroblasts [35].

As MSCs and fibroblasts do not contact each other in the Boyden chamber assay, differences in stimulating fibroblast migration appear due, at least in part, to paracrine effects. Deficiencies in specific cytokine production indicate that the healing contribution of MSCs present at distant sites in chronic wound patients would be limited.

There is, however, little difference when looking at the effect of normal versus chronic wound patient MSCs on the migration of chronic wound fibroblasts in the Boyden chamber assay. This is not entirely unexpected because chronic wound fibroblasts have been shown to be unresponsive to cytokines [36] and the increased factors released by normal MSCs may then not be enough to overcome this defect. From the viewpoint of stimulating migration, the findings indicate that both bone marrow and wound fibroblasts are adversely affected in the chronic wound state.

At a concentration of 20% MSCs in the scratch assay (Fig. 2B), normal MSCs were significantly better than chronic wound patient-derived MSCs at getting chronic wound fibroblasts to migrate (p < .01). This observation is in contrast to that seen in the Boyden chamber assay and may reflect the need for cells to contact one another or be in close proximity to best achieve an effect. When MSCs are in close proximity to fibroblasts, the local concentration of secreted factors by MSCs would be effectively much greater. But as mentioned above, this effect would be expected to be greater when the relative number of MSCs increased and is not seen here. There are perhaps other more important factors involved when MSCs and fibroblast are allowed to contact one another at an optimal ratio. The percentage of normal donor MSCs required to achieve peak stimulation of fibroblast migration is also higher for patient fibroblasts (20%) than when stimulating normal fibroblasts (10%) to move. This implies that more normal MSCs may need to achieve a healing effect. These issues are likely to become important when designing clinical protocols for the delivery of MSCs to chronic wounds.

Given the findings of this early study, it appears that there may be an optimal “dose range” for MSCs in stimulating healing in chronic wounds. Perhaps many fewer cells than anticipated will be needed to stimulate healing. It also seems that local administration would be best, particularly when delivering autologous MSCs to a chronic wound patient. Direct administration to wounds may require new approaches and could speak against the delivery of MSCs systemically or in a matrix where they are not able to directly contact cells in the wound bed. Given the limited migration response we observed when using chronic wound patient MSCs, it might also be best to consider the use of MSCs derived from normal healthy donors.

In this report we have developed a modified assay system to initially characterize the interaction of bone marrow-derived MSCs and fibroblasts using materials derived from both normal donors and chronic wound patients. Fibroblast migration appears to be unfavorably altered in chronic wound patients with defects likely in both their wound-bed fibroblasts and bone marrow-derived MSCs. The exact mechanisms of how these defects occur and determining mechanisms to correct them is an area of continuing investigation. The assay systems described here will be helpful in carrying out this work.

**CONCLUSION**

In this study, we describe a modified scratch assay system using lentiviruses to mark cells anatomically and by fluorescence so they can be tracked and their identity determined in a mixed cell population. Using this modified mixed cell scratch assay and Boyden chamber assays, we have demonstrated that bone marrow-derived MSCs induce fibroblast migration in a dose-dependent manner. Although the induction of fibroblast migration appears to be at least in part due to a paracrine effect, local microenvironment factors also appear to be important. We have also observed that MSCs and fibroblast derived from chronic wound patients appear to have deficiencies as compared with their normal counterparts. These findings may become important in the design of future clinical protocols for the treatment of chronic wounds with MSCs.
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AUTHOR CONTRIBUTIONS

L.R.-M.: conception and design, collection and analysis of data, interpretation; D.F.: conception and design, collection and analysis of data, interpretation, manuscript writing; M.S.: design, collection and analysis of data, interpretation, final preparation of manuscript.

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