Mesenchymal Stem Cells Require MARCKS Protein for Directed Chemotaxis *In Vitro*

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Mesenchymal stem cells (MSCs) reside within tissues such as bone marrow, cord blood, and dental pulp and can differentiate into other mesenchymal cell types. Differentiated MSCs, called circulating fibrocytes, have been demonstrated in human lungs and migrate to injured lung tissue in experimental models. It is likely that MSCs migrate from the bone marrow to sites of injury by following increasing chemokine concentrations. In the present study, we show that primary mouse bone marrow mesenchymal stem cells (BM-MSCs) exhibit directed chemotaxis through transwell inserts toward increasing concentrations of the chemokines complement component 5a, stromal cell-derived factor-1 α , and monocyte chemoattractant protein-1. Prior research has indicated that myristoylated alanine-rich C kinase substrate (MARCKS) protein is critically important for motility in macrophages, neutrophils, and fibroblasts, and here we investigated a possible role for MARCKS in BM-MSC directed chemotaxis. The presence of MARCKS in these cells as well as in human cord blood MSC was verified by Western blotting, and MARCKS was rapidly phosphorylated in these cells after exposure to chemokines. A synthetic peptide that inhibits MARCKS function attenuated, in a concentration-dependent manner, directed chemotaxis of BM-MSCs, while a missense control peptide had no effect. Our results illustrate, for the first time, that MARCKS protein plays an integral role in BM-MSC-directed chemotaxis *in vitro*.

Keywords: mesenchymal; MARCKS; chemotaxis; chemokines; phosphorylation

Mesenchymal stem cells (MSCs) are found in diverse tissues such as bone marrow, cord blood, and dental pulp (1). MSCs can be induced by extracellular stimuli to differentiate into osteoblasts, chondroblasts, adipoblasts, fibrocytes, fibroblasts, myofibroblasts, tenoblasts, and neuroblasts (1). It recently has been suggested that MSCs can alleviate, via paracrine responses, lung injury caused by bleomycin exposure (2), and it has been postulated that MSCs interfere with fibroblast conversion to myofibroblasts that produce extracellular matrix (3). Although the majority of reports indicate that MSCs migrate to sites of tissue injury and ameliorate damage from multiple etiologies (4, 5), recent reports have suggested that, in cases of *chronic* injury, MSCs may actually exacerbate damage and contribute to fibrosis (6). Whatever role MSCs might be playing at sites of injury, it is clear that the cells migrate from the bone marrow, transit through the vasculature, and arrive at the affected tissues (7).

CLINICAL RELEVANCE

Adult mesenchymal stem cells (MSCs) are being used with increasing frequency in laboratories and clinics as potential treatments for injury and tissue regeneration. The fundamental mechanisms through which MSCs migrate to sites of injury remains undefined. Here we have shown that myristoylated and alanine-rich C-kinase (MARCKS) is required for directed chemotaxis by MSCs and offers a potential target for enhancing or blocking the effects of migrating MSCs.

To our knowledge, there are no data available on molecular mechanisms that control stem cell migration.

Directed cellular chemotaxis is a complex process wherein an attractant (chemokine) binds to a specific membrane receptor, thus activating signal transduction pathways (8). Subsequently, these pathways induce actin polymerization at the leading edge of the polarized cell, with the ultimate result of movement toward increasing concentrations of chemokines (9–11). Myristoylated alanine-rich C-kinase substrate (MARCKS) is a ubiquitous multi-functional protein that is activated after being phosphorylated by protein kinase C (PKC) (12). MARCKS has previously been shown to bind F-actin filaments (13) and has been identified at the leading edge of polarized cells (12, 14), and disruption of MARCKS can affect cell movement *in vitro* (15). In a number of *in vivo* and *in vitro* studies, a synthetic peptide identical to the N-terminus of MARCKS, named the MANS (myristoylated N-terminal sequence) peptide, has been shown to affect MARCKS function, resulting in disruption of MARCKS-dependent vesicular transport within cells as well as cell migration (16–18). Here, we sought to determine if MARCKS also is involved in directed chemotaxis of MSCs.

The results indicate that primary mouse bone marrow mesenchymal stem cells (BM-MSCs) migrate toward chemotactic agents known to be involved in inflammation and/or stem cell recruitment. Western blotting identified MARCKS protein in MSC cell lysates, and MARCKS phosphorylation was induced by exposure to the chemotactic agents. Finally, treatment of these cells with the MANS peptide, but not a missense (RNS peptide) control, attenuated, in a concentration-dependent manner, directional chemotaxis. As clinical trials using MSCs increase (19), it will be important to understand how these cells migrate to sites of injury and ultimately whether or not their presence is beneficial to the repair process.

MATERIALS AND METHODS

Cell Culture

Murine monocyte-macrophage cell J774A.1 cells were propagated in DMEM supplemented with 10% fetal bovine serum and supplements.

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Normal human lung fibroblasts (NHLF) (Lonza, Walkersville, MD) were cultured in Fibroblast Basal Medium 2 (Lonza). BM-MSCs from C57Bl/6J mice (1) (Institute for Regenerative Medicine, College Station, TX) were propagated in complete Iscove's Modified Dulbecco's Medium (cIMDM) as described (1). Human cord blood mesenchymal stem cells (Adult Stem Cell Core, University of Vermont, Burlington, VT) were propagated in complete Alpha Modified Dulbecco's Medium (α MEM) as described previously (20). All cells were incubated at 37°C in a humidified 5% CO₂ atmosphere.

Chemotaxis Assays

Following 12 hours of incubation in serum-free (SF)-IMDM, 1.5×10^5 BM-MSCs were introduced to the upper chamber of 24-well 5- μ m pore Transwell inserts coated with rat tail collagen. Optimum pore size was determined empirically (data not shown). Lower chambers contained SF-IMDM with either: chemotactic agents (50 ng/ml monocyte chemoattractant protein [MCP]-1; 0.05, 0.5, 5, 10, 25, 50, 100 ng/ml stromal cell-derived factor [SDF]-1 α ; 25, 50, 100, 200 ng/ml complement component [C]5a), 0.001% bovine serum albumin (BSA), cIMDM, or SF-IMDM. To examine the role of MARCKS in migration, cells were pre-incubated with 0.5, 5, 50, or 100 μ M MANS (16, 17) or 50 or 100 μ M RNS randomized sequence peptide (16) for 15 minutes at 37°C before the migration assay.

After 3 hours of incubation, inserts were removed, and the inner surface rinsed and wiped to remove nonmigrated cells. Cells on the outside of the inserts were formalin-fixed and stained, and the inserts were attached to glass slides. Cells were counted in 15 random fields at $\times 400$ magnification per insert. The mean number of cells from 30 or 45 fields per experimental condition was calculated.

Western Blotting

Protein concentrations from BSA, and whole cell lysates from J774A.1 cells, BM-MSCs, CB-MSCs (21), and NHLF were determined, then the proteins denatured. Five or 20 μ g of each sample was resolved electrophoretically, then transferred to nitrocellulose. Proteins were probed with α -MARCKS, α -phospho-MARCKS, or α -GAPDH antibodies with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunolabeled proteins were visualized using chemiluminescent substrate, and band images captured on film and analyzed by densitometry using Image J software (NIH, Bethesda, MD).

Phospho-MARCKS Assay

A quantity of 1×10^6 BM-MSCs were loaded into 6-well plates and incubated in cIMDM for 24 hours. Before experimentation, the cells were incubated overnight in SF-IMDM. BM-MSCs were exposed to medium alone, 100 nM phorbol 12-myristate-13-acetate (PMA), 0.001% BSA, 50 ng/ml C5a, 25 ng/ml SDF-1 α , 5 ng/ml SCF, 50 ng/ml C5a plus 50 μ M MANS, or 50 ng/ml C5a plus 50 μ M RNS in duplicate wells. Cells were harvested at 0, 0.5, 1, and 3 minutes into PBS with PhosSTOP (Roche, Indianapolis, IN), then placed in SB+I buffer and denatured at 95°C for 10 minutes. Equal concentrations of whole cell lysate proteins were resolved electrophoretically, transferred to nitrocellulose, and probed with α -GAPDH, α -Phospho-MARCKS, or α -MARCKS antibodies. Immunolabeled proteins were visualized and analyzed as described above.

Statistical Analysis

Unpaired two-tailed *t* tests were performed in conjunction with F-tests for all statistical analyses using Graphpad Prism v.4. Symbols signify statistical significance, where **P* < 0.05, ***P* < 0.001, and ****P* < 0.0001 (Figures 1 and 4). All values are expressed as means $\pm$ 1 SEM.

RESULTS

Mouse BM-MSCs Display Directed Migration toward Chemotactic Agents

The well-described chemotactic agents SDF-1 α , MCP-1, and C5a were used to examine the response of primary BM-MSCs isolated from mouse bone marrow aspirates (1) in a Transwell system (Figure 1A). Serum-starved BM-MSCs displayed no chemotaxis and low random migration (2.33 ± 0.90 MSCs/field) after 3 hours. Serum-rich cIMDM, used here as a positive control, provoked a statistically significant chemotactic response (42.80 ± 2.20 MSCs/field; *P* < 0.0001) well above the random movement control. Similarly, SDF-1 α (38.07 ± 2.80 MSCs/field; *P* < 0.0001); MCP-1 (25.20 ± 3.17 MSCs/field; *P* < 0.0001) and C5a (23.33 ± 2.32 MSCs/field; *P* < 0.0001) induced significant increases in directed chemotaxis by BM-MSCs compared with the random movement control (Figure 1A). Stem cell factor

Figure 1. Primary mouse bone marrow mesenchymal stem cell (BM-MSC) chemotaxis toward varying concentrations of chemokines. A quantity of 1.5×10^5 BM-MSCs in serum-free Iscove's Modified Dulbecco's Medium (SF-IMDM) were loaded into 5- μ m pore Transwell inserts for chemotaxis assays with wells containing medium supplemented with 50 ng/ml chemokines (A) or increasing concentrations of stromal cell-derived factor (SDF)-1 α (B) or complement component (C)5a (C). SF-IMDM was the random movement control, and complete medium (cIMDM) was the positive control. Each bar represents the mean number BM-MSCs $\pm$ SEM within 45 randomly chosen microscopic fields (magnification: $\times 400$). The three chemokines (but not stem cell factor [SCF]) induced statistically significant degrees of directed chemotaxis (A) compared with the negative control at 3 hours (§), and SDF-1 α (B) and C5a (C) exhibited concentration-dependent chemotaxis, with peak concentrations of 25 ng/ml and 50 ng/ml, respectively (**P* < 0.05, ***P* < 0.001, ****P* < 0.0001).

(SCF) induced no directed chemotactic responses in this system (data not shown).

BM-MSCs Migrate toward Chemokines in a Concentration-Dependent Manner

Once BM-MSC chemotaxis was observed, cells were exposed to increasing concentrations of the chemotactic agents SDF-1 α and C5a to identify the concentration range that induced maximal chemotactic activity. As illustrated in Figure 1, the optimal concentrations were 25 ng/ml for SDF-1 α and 50 ng/ml for C5a. BM-MSCs did not exhibit chemotaxis to SDF-1 α concentrations above 50 ng/ml or below 10 ng/ml (Figure 1B), nor to C5a concentrations above 100 ng/ml or below 25 ng/ml (Figure 1C).

MARCKS Protein Is Expressed in BM-MSCs

To determine if mouse BM-MSCs express MARCKS protein, we examined via Western blot whole cell lysates from BM-MSCs, human cord blood mesenchymal stem cells (HCB-MSCs), J774A.1 cells, and normal human lung fibroblasts (NHLF). MARCKS protein was identified in each of the cell lysates (Figure 2A).

MARCKS protein was examined using equal concentrations of whole cell lysates harvested after 3 hours of exposure to chemokines, MANS, or RNS (Figure 2B). GAPDH was used as an internal control for protein concentrations per lane in the Western blots. No statistically significant concentration differences were detected by densitometry of the immunolabeled MARCKS band from any treated whole cell lysate (data not shown).

Exposure of BM-MSC to Chemokines Induces Phosphorylation of MARCKS

BM-MSCs were exposed to 0.001% BSA, 5 ng/ml stem cell factor (SCF), 50 ng/ml MCP-1, 25 ng/ml SDF-1 α , 50 ng/ml C5a, or 100 nM of the protein kinase C (PKC) activator, 12-phorbol-13-myristate acetate (PMA) for 30 seconds, 1 minutes, or 3 minutes to determine effects on phosphorylation of MARCKS, a necessary step in MARCKS activation (12). Serum-starved and BSA-exposed MSCs did not show MARCKS phosphorylation above baseline levels (time 0 control). PMA, which induces MARCKS phosphorylation via activation of PKC (12), served as a positive control. MSCs exposed to PMA showed a steady increase in phosphorylated MARCKS over time (Figure 3). Exposure to the chemotactic agents SDF-1 α and C5a induced a strong and rapid phosphorylation of MARCKS at the 30-second time point (Figure 3), with levels of phosphorylation declining at 1 and 3 minutes (Figure 3). Neither chemokine affected levels of total MARCKS protein in the cells (data not shown). However, stem cell factor (SCF), suggested to be an attractant to MSCs *in vivo* (22), was not chemotactic in our system and did not induce MARCKS phosphorylation above baseline at any of the observed times (Figure 3). Neither the MANS or the RNS peptide affected MARCKS phosphorylation alone or in BM-MSCs exposed to SDF-1 α or C5a (data not shown).

MANS Peptide Attenuates Directed Chemotaxis of BM-MSC

Once MARCKS was identified in BM-MSCs, we examined the effects of the MANS peptide on BM-MSC chemotaxis (Figure 4). When BM-MSCs were pre-incubated for 15 minutes with 100 μ M MANS peptide before chemotaxis assays using the optimal concentrations of C5a (50 ng/ml) and SDF-1 α (25 ng/ml), chemotaxis was reduced to levels equivalent to random migration induced by serum-free medium containing 0.001% BSA (Figure 4A). This

Figure 2. Western blot of MARCKS protein in whole cell lysates (A) and lysates of cells treated with chemokines (B). Each well was loaded with either 5 μ g protein (MW, BSA) or 20 μ g whole cell lysates, resolved on 10% Bis-Tris gels, transferred to nitrocellulose, and immunolabeled with (A, B) α -MARCKS or (B) α -GAPDH. The lane labels represent: MW, dual color molecular weight ladder (Bio-Rad Laboratories, Hercules, CA); BSA, bovine serum albumin fraction V; J774A.1, monocyte-macrophage J774A.1; NHLF, normal human lung fibroblast; BM-MSC, bone marrow mesenchymal stem cell; CB-MSC, cord blood mesenchymal stem cell; SF-IMDM, BM-MSCs in serum-free medium; cIMDM, BM-MSCs in complete medium; SDF-1, BM-MSCs exposed to 25 ng/ml SDF-1 α ; C5a, BM-MSCs exposed to 50 ng/ml C5a; MCP-1, BM-MSCs exposed to 50 ng/ml MCP-1; MANS, BM-MSCs exposed to 50 ng/ml C5a and 100 μ M MANS peptide; RNS, BM-MSCs exposed to 50 ng/ml C5a and 100 μ M RNS peptide. MARCKS protein resolved at 80 kD. GAPDH is the loading control and resolved at 36 kD. MARCKS clearly is present in these cells, and exposure to the control media, chemotactic agents, and peptides does not affect MARCKS protein levels.

inhibition of chemotaxis was highly significant statistically ($P < 0.0001$) and was not due to a toxic response, as determined by assays using a Cyto Tox-One kit (Promega, Madison, WI) (data not shown). The response to the MANS peptide was concentration-dependent, with significant attenuation of migration at MANS concentrations as low as 5 μ M. The control RNS peptide, at any concentration used, did not affect chemotaxis in response to the same concentrations of C5a and SDF-1 α (Figure 4B).

DISCUSSION

In the studies reported here, we show that primary murine bone marrow mesenchymal stem cells exhibit directed chemotaxis in response to the chemotactic agents C5a, SDF-1 α , and MCP-1. In addition, we show for the first time that chemotaxis of BM-MSCs is dependent on MARCKS protein and can be disrupted by exposing the cells to a peptide that specifically interferes with MARCKS function. It is interesting to note that BM-MSCs are sensitive to stem cell-specific chemokines (SDF-1 α), as well as potent chemotactic agents (C5a, MCP-1) that also attract fibroblasts, neutrophils, and macrophages (23). These findings suggest BM-MSCs can respond to a broad range of chemokines that could be present at sites of tissue damage and may be involved in the attraction of MSCs to these sites.

MSCs are widely viewed as beneficial for repair or resolution of tissue injury. In animal studies using radiation-, bleomycin-, or LPS-induced lung injury, MSCs are attracted to the sites of injury (3, 4, 24), and, after arriving at these sites, it appears that MSCs exert beneficial or protective effects (4, 19). Further, MSCs appear to be immunosuppressive, reducing the risk of

Figure 3. Chemokine-induced phosphorylation of MARCKS protein. Equal concentrations of 20 μ g/well BSA, untreated BM-MSCs, BM-MSCs exposed to 0.001% BSA (wt/vol) or BM-MSCs treated with chemokines were resolved on 10% Bis-Tris gels, transferred to nitrocellulose, probed with either α -GAPDH antibodies (loading control) or monoclonal α -phospho-MARCKS antibodies, detected by chemiluminescence, and compared by densitometry. Phospho-MARCKS and GAPDH bands are normalized to GAPDH concentration and an intensity control present on each blot. Samples were measured as intensity per mm^2 and graphed. Digital images of phospho-MARCKS and GAPDH bands are shown below the graph. MARCKS is rapidly phosphorylated upon treatment with the positive control (PMA) and the chemotactic agents. SCF, which did not induce directed chemotaxis in our system, apparently did not induce comparable phosphorylation.

transplanted MSCs producing further damage through rejection (25). Conversely, studies of some chronic organ injuries suggest that MSCs may have a detrimental effect, with the potential to worsen fibrosis and contribute to tissue scarring (26). Timing of MSC arrival to injured tissues appears to be critical, with acute injuries perhaps being affected positively (2, 4) by MSCs, while more chronic damage appears to elicit deleterious effects from arriving MSCs (26). Relevant to this concept, a recent paper from our laboratory (27) shows that human and mouse MSCs

synthesize and release Wnt isoforms that influence mesenchymal cell proliferation as well as TGF- β that induces target cells to enhance collagen expression. We did report some differences between the human cord blood and bone marrow MSCs in these studies (27), but did not note any differences in chemotactic potential (data not shown here). In instances of chronic inflammation and injury, it may be beneficial that MSCs and subsequent paracrine activity be limited or delayed. Clearly, defining mechanisms by which MSCs migrate to sites of injury

Figure 4. Effect of MANS peptide on BM-MSC migration. A quantity of 1.5×10^5 primary mouse BM-MSCs were mock exposed, exposed to 100 μ M MANS peptide (MANS), or exposed to 100 μ M RNS control peptide (RNS) at 37°C for 15 minutes, then loaded into 5- μ m pore Transwell chambers. The lower well contained SF-IMDM supplemented with: no chemokines, 0.001% BSA, 20% serum, 25 ng/ml SDF-1 α , or 50 ng/ml C5a (A). A quantity of 1.5×10^5 Mouse BM-MSCs were mock-exposed, exposed to increasing concentrations (500 nM to 50 μ M) MANS peptide, or 50 μ M RNS peptide at 37°C, then loaded into 5- μ m Transwell chambers. The lower well contained SF-IMDM containing: no chemokines (SF-IMDM), 20% serum (cIMDM), or 50 ng/ml C5a (C5a) (B). Each bar represents the mean of 45 randomly selected fields at $\times 400$ magnification $\pm$ SEM. MANS peptide attenuates MSC migration in a concentration-dependent manner. RNS peptide did not inhibit MSC chemotaxis. Statistical significance was determined by two-tailed unpaired *t* tests, where $\times \times \times P < 0.0001$ when compared with 50 ng/ml C5a (solid circle), $\wedge \wedge \wedge P < 0.0001$ when compared with 25 ng/ml SDF-1 α ($\dagger$), or $***P < 0.0001$ when compared with the negative control (SF-IMDM) at 3 hours ($\ddagger$).

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can be critical with respect to development of potential stem cell therapies.

Chemotaxis is a complex orchestration of enzymes, phosphorylases, and proteins under careful regulation to induce eukaryotic cell polarization and directional movement (8). Generally, chemokines induce cellular signaling by extracellular binding to receptors in the seven-transmembrane receptor family (8). Based on the number of receptors triggered, combined with receptor-desensitizing activities by β -activins, cells move along the chemotactic gradient toward the origin of the agents (11, 28). The chemokine receptors, along with other cellular signals such as Wnts (29), induce G proteins attached to receptor cytoplasmic domains to cleave PIP₂, starting cell signaling cascades that induce cell polarization, transcription, and translation of numerous proteins, release of cells from the extracellular matrix, and cytoskeletal reorganization leading to directional movement (8). One of the products of PIP₂ cleavage, diacylglycerol (DAG), activates PKC, the major enzyme class responsible for MARCKS phosphorylation (30). Once activated, MARCKS can be involved in numerous cellular mechanisms for migration, including actin binding (15), vesicle transport (31), binding of intracellular PIP₂ and calmodulin (13), integrin regulation (32), phospholipase D activation (33), and potential roles in dynamic adhesion (34).

To our knowledge, this is the first demonstration that MARCKS is a prominent protein in human and mouse primary MSCs, and that MARCKS plays an essential role in regulating MSC directed chemotaxis. The inhibitory effects of the MANS peptide on BM-MSC chemotaxis was not due to toxicity or cell death nor inhibition of MARCKS phosphorylation or MARCKS expression. Thus, the question remains as to the precise mechanism whereby MARCKS regulates MSC-directed chemotaxis. It is known that under normal, nonstimulated conditions, MARCKS is localized to the inner face of the plasma membrane in cells, and phosphorylation releases MARCKS from the membrane into the cytoplasm, where it can carry out its cell-specific activities (12). Accordingly, our results show that the chemotactic agents C5a and SDF-1 α induce a rapid MARCKS phosphorylation by 30 seconds after exposure (Figure 3). Interestingly, SCF, which has been speculated to act as a chemoattractant for mesenchymal cells *in vivo* (22), did not induce chemotaxis by BM-MSCs. Inasmuch as SCF is a potent factor in inflammatory cell attraction and maintenance of lung airway inflammation (22, 35), these findings were surprising. SCF also did not affect MARCKS phosphorylation, providing additional evidence that phosphorylation of MARCKS is an important component of stem cell migration. The fact that PMA induced MARCKS phosphorylation in MSCs but did not affect directed migration (data not shown) indicates that MARCKS phosphorylation by itself is necessary but not sufficient to induce MSC-directed chemotaxis.

The synthetic MANS peptide used in these studies is identical to the myristoylated 24-amino acid domain on the N-terminus of MARCKS, and has been shown previously to attenuate degranulation in airway epithelium (30) and leukocytes (17) and also to inhibit migration of inflammatory cells *in vitro* (18).

It was interesting to note that the RNS, used as a negative control for the effects of MANS, itself stimulated some chemotaxis (Figure 4). Stimulation of other cell activities by RNS has been seen previously and could simply be a nonspecific response to a peptide. Nonetheless, RNS does serve as a useful negative control for MANS in a number of experimental settings (17, 18, 30). Since the function of the MARCKS amino terminus is thought to center on membrane binding, the inhibitory effects of MANS on MSC migration could be related to its interfering

with MARCKS interaction with the cytoskeleton at the leading edge of the membrane in migrating cells. While the actual mechanism by which the MANS peptide inhibits MSC-directed chemotaxis remains to be determined, controlling cell migration provides a potentially useful tool to regulate MSC movement to sites of injury *in vivo*. The studies reported here are the first to demonstrate that MARCKS protein is an integral component in the intracellular machinery by which MSCs migrate in response to chemoattractants.

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