Novel Negative Selection Marker CD54 Enhances Differentiation of Human Adipose-Derived Mesenchymal Stem Cells

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Abstract

Due to their multi-differentiation potential and immunosuppressive function, mesenchymal stem cells (MSCs) hold huge promise in regenerative medicine. Lack of specific selection markers to isolate MSCs renders their use at risk of fibroblast contamination. The aim of the study was to identify new surface protein markers that can be used for MSC purification during in vitro expansion. With real-time RT-PCR, we demonstrated that primary human dermal fibroblasts expressed CD54 mRNA 10-fold more than early passage human adipose-derived MSCs (AMSCs). Flow cytometry illustrated 66.0% ± 4.1% of dermal fibroblasts strongly expressed CD54 on their surface with a mean fluorescence intensity ratio of 24.0 ± 0.0 compared to 11.0% ± 0.7% and minimal intensity for AMSCs. Evaluation of CD54 sorted AMSCs revealed CD73 expression was 2.2-fold higher in the CD54- versus CD54+ fraction. CD54- AMSCs demonstrated increased adipogenic and osteogenic differentiation potential relative to CD54+ AMSCs. In conclusion, we identified CD54 as a novel selection marker capable of distinguishing MSCs from fibroblasts and thus enhancing MSC osteogenic and adipogenic differentiation potential.

Keywords: Mesenchymal stem cells; Osteogenesis; Adipogenesis; Selection marker; CD 54

Introduction

Mesenchymal stem cells (MSCs) are self-renewing multipotent cells capable of differentiating into several cell lineages including osteoblasts, chondrocytes, and adipocytes [1]. First described by Friedenstein et al. [2], MSCs have successfully been isolated from bone marrow [2,3], adipose [4,5], peripheral blood [6], umbilical cord blood and matrix [7], fetal blood and liver [8], connective tissue of dermis [6,9], and skeletal muscle sources [6]. The multi-differentiation potential of MSC raises a clinical interest to employ these cells for regeneration purposes, for example, in osteogenesis imperfecta. MSCs lack major histocompatibility complex class II antigens and have been shown in vitro to inhibit the activation and/or function of natural killer cells [10], T cells [11-13], dendritic cells [14-16], and B-cells [17]. These immunomodulatory properties have led to clinical trials to assess their therapeutic potential for graft-versus-host disease after hematopoietic transplantation, type I diabetes, and multiple sclerosis. Due to easy access via liposuction, adipose has become the preferred source of MSCs for therapeutic applications.

Irrespective of their source, MSC isolation involves several steps including positive selection via the properties of plastic-adherence and colony formation [18]. Although this eliminates contaminants such as blood and immune cells, a heterogeneous starting population and fibroblast contamination represent disadvantages. Fibroblasts are known to undergo senescence and apoptosis in culture, while surviving cells become immortal and potentially tumorigenic [19]. Thus, identification and elimination of fibroblasts from MSC culture could improve MSC yield and differentiation potential and also prevent tumor formation after MSC transplantation.

However, there are currently no markers which can be used to identify and isolate MSCs. Despite consensus that MSCs are positive for expression of CD73, CD90, and CD105, and negative for expression of hematopoietic cell surface markers CD11a, CD19, CD34, CD45, and HLA-DR [20], expression levels of these markers vary across laboratories due to tissue source or the specific culture conditions used [18]. Perhaps more importantly, fibroblasts also express CD105, CD73, and CD90 on their surface and lack hematopoietic markers [21]. Additionally, fibroblasts and MSCs share an almost identical in vitro morphology, rendering useless physical filtration techniques [22]. Thus, more effective strategies to purify MSCs are needed. In the present study, we compared AMSCs and dermal fibroblasts using real-time RT-PCR and flow cytometry and identified CD54 as a novel negative selection marker that enhances MSC differentiation potential.

Materials and Methods

Isolation and cultivation of AMSCs

AMSCs were isolated from lipoaspirate using a modified method as described [23]. Briefly, lipoaspirate was obtained and washed with equal volume of hank’s buffered salt solution (HBSS; Invitrogen, Grand Island, NY). After gentle shaking, isolated samples were separated into two phases. The lower phase (containing stem cells, adipocytes, and blood) was washed and enzymatically dissociated with 0.075% collagenase type I (Sigma-Aldrich, St. Louis, MO)/HBSS for 1 h at 37°C with gentle shaking. Collagenase was inactivated by adding a 1:10 volume of fetal bovine serum (FBS) to adipose collagenase mixture.

The mixture was centrifuged at 400 g for 10 min at 25°C. The cellular pellet was resuspended in red blood cell lysis buffer (eBioscience, San Diego, CA) and incubated at 25°C for 10 min. The pellet was resuspended in washing medium (HBSS with 2.4% FBS) and...
sequentially passed through 100, 70, and 40 μm mesh filters to remove cell masses. An equal amount of HISTOPAQUE-1077 (Sigma-Aldrich) was added and centrifuged at 400 g for 30 min to separate MSCs. Cells were seeded at 1-2×10^4 cells/cm^2 and grown at 37°C in dulbecco’s modified eagle medium-High Glucose (DMEM, Invitrogen) with 10% FBS and 1% penicillin/streptomycin and equilibrated against 5% CO2 and 95% air.

Media was changed every 2-3 days. At 70-80% confluency, cells were washed with PBS and then detached with 0.25% trypsin-EDTA (Invitrogen). Cells were centrifuged at 300 g for 5 min. After removing the supernatant, cells were then resuspended in DMEM culture media and seeded at approximately 5×10^4 cells/cm^2 counting as passage one. MSCs were harvested at 70-90% confluency for RNA extraction, flow cytometry, or magnetic-activated cell sorting (MACS) at early (≤ 5) passage.

Human adult dermal fibroblasts (ATCC, Manassas, VA) were cultured in DMEM-high glucose (Invitrogen) with 10% FBS and 1% penicillin/streptomycin at 37°C in an incubator with 5% CO2. Cells were harvested at 70-90% confluency for mRNA extraction and flow cytometry.

**Real-time and semi-quantitative RT-PCR**

Harvested cells were homogenized using cell shredders (Qiagen Inc, Valencia, CA). Lysates were collected and total RNA retrieved using a Qiagen RNA isolation mini kit (Qiagen Inc, Valencia, CA). RNA purity was determined using spectrophotometry at 260/280 nm. cDNA was synthesized from 2 μg of purified total RNA using reverse transcriptase (Super Script III kit, Invitrogen).

Real-time PCR was performed using SYBR green PCR core reagents (Applied Biosystems, Foster City, CA) following the manufacturer’s protocol on an ABI Fast Real Time PCR 7900 System (Applied Biosystems, Foster City, CA) following the manufacturer’s protocol. cDNA was used as template in 96-well microplates. Primers were designed using the Primer3 program (Whitehead Institute, Cambridge, MA). The PCR protocols involved activation of DNA polymerase followed by 40 cycles of denaturation at 94°C for 15 s, and annealing and extension at 60°C for 1 min. Each sample was analyzed in triplicate. Reactions without template were used as negative controls. β-actin mRNA was used as an internal control. Standard curves were plotted for each target gene and internal control. RNA quantity was expressed relative to the corresponding β-actin mRNA control. Relative expression levels were calculated using the standard curve method recommended by Applied Biosystems.

**Flow cytometry**

1×10^5 cells were collected, washed, blocked with 10% goat serum (Abcam, Cambridge, MA) and diluted in PBS containing 3% BSA (Sigma-Aldrich) for 20 min at 4°C. Cells were then incubated with 2 μg/ml of CD54 antibody (Biology, San Diego, CA) for 1 h at 4°C. Isotype-matched control antibody was used at the same concentration as the primary antibody to evaluate non-specific binding. After 3 washes with PBS, cells were incubated with FITC-conjugated goat anti-mouse secondary antibody for 30 min in the dark at 4°C followed by 3 washes with PBS. Cells were resuspended in PBS containing 3% BSA. Propidium iodide (Vector laboratories, Burlingame, CA) was added at a final concentration of 0.02 μM for live cell gating. Ten-thousand events were acquired with a FACScaliber flow cytometer (Becton Dickenson, Mountain View, CA) and results were analyzed with CellQuest software program (Becton Dickenson). The cutoff level defined by the isotype control antibody was set to less than 1%. The mean fluorescent intensity (MFI) ratio was calculated by dividing the MFI of CD54 antibody by the MFI of the isotype control antibody.

**Magnetic-activated cell sorting**

MACS was performed according to protocols described by Miltenyi Biotech Inc (Auburn, CA). Briefly, 1×10^5 cells were labeled with 2 μg anti-CD54 antibodies in 100 μL PBS, incubated for 1 h at 4°C and then washed with PBS. Cells were centrifuged at 300 g for 10 min. The supernatant was aspirated and cells were resuspended in 50 μL of MACS buffer. 20 μL of goat anti-mouse IgG1 conjugated microbeads were added to cells and incubated at 4°C for 30 min. Cells were then washed, collected, and resuspended in 500 μL of buffer. MACS columns attached to the magnetic sorter were first rinsed with 3 mL of buffer and eluent discarded. Cells were then applied to the columns and washed with 3 mL of buffer 3 times, and eluent collected in a single centrifuge tube labeled as CD54- cells. Columns were then removed from the magnetic sorter and 5 mL of buffer was immediately applied. The eluent was collected in a fresh centrifuge tube and labeled as CD54+ cells.

Sorted cells were cultured in a 6 well plate with 2 mL of DMEM-High Glucose (Invitrogen) with 10% FBS and 1% penicillin/streptomycin. Cells were allowed 1 day to recover before they were harvested for mRNA or DNA isolation and analyzed to either adiogenic or osteogenic differentiation.

**CFU-F assay**

Various adipose-derived MSC (AMSC) lines (sorted and unsorted) were harvested at 70-90% confluency for mRNA extraction and flow cytometry.

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**Table 1:** The sequences of primers used in study.

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were seeded at 156 cells/cm² on 6 well plates in triplicate, cultured for 14 days, and then stained with 0.5% crystal violet (Sigma-Aldrich) in 25% methanol to evaluate clone number.

**MSC differentiation**

Reagents used in adipogenic and osteogenic differentiation assays were purchased from Sigma-Aldrich otherwise indicated. AMSCs used in the adipogenesis assay were seeded at 1.5×10⁶ cells/cm². Adipogenic induction medium was comprised of DMEM with 4.5 G/L glucose, 2 mM glutamine, 10% FBS, 1% penicillin/streptomycin (Invitrogen) containing 0.5 M IBMX, 2.5×10⁻³ M dexamethasone, 1.7 mM insulin, and 140 mM indomethacin. Adipogenic induction medium was applied 1 day after seeding and replaced every 3 days thereafter. Cells were stained with oil red O at day 14.

AMSCs used in the osteogenesis assay were seeded at 2.5×10⁵ cells/cm³. Osteogenic induction medium was comprised of DMEM with 4.5 G/L glucose, 2 mM glutamine, 10% FBS, 1% penicillin/streptomycin (Invitrogen) containing 1 M beta-glycerophosphate, 2.5×10⁻³ M glucose, 2 mM glutamine, 10% FBS, 1% penicillin/streptomycin (Invitrogen) containing 1 M beta-glycerophosphate, 2.5×10⁻³ M dexamethasone, and 10 mg/mL ascorbic acid. Osteogenic induction medium was applied 1 day after seeding and replaced every 3 days thereafter. Cells were stained with oil red O at day 21.

**Statistical analysis of data**

All data are expressed as means ± standard error of the mean (SEM) of 3 independent experiments. Analysis of significance was performed using a 2-tailed student t-test with p<0.05 considered significant.

**Results**

**Expression of CD54 marker in AMSCs and fibroblasts**

*In vitro* expanded human AMSCs displayed typical morphology. RT-PCR data demonstrated AMSCs were positive for expression of CD73, CD90, and CD105, and negative for expression of CD11a, CD19, CD34, CD45, and HLA-DR (data not shown). Figure 1 shows there was no statistical difference in the relative expression levels of CD81, CD109, CD146, CD164, and CD172a in AMSCs versus fibroblasts whereas fibroblasts expressed significantly higher CD49d and 10.3-fold greater levels of CD54. Thus, we selected CD54 for further analysis.

Flow cytometry data illustrated that 88.0% ± 4.1% fibroblasts strongly express CD54 on the cell surface with a MFI ratio of 24.0 ± 0.0 while only 11.0% ± 0.7% of AMSCs showed weak staining (Figures 2a and 2b). MACS was used to sort the expanded AMSCs at early passage with anti-CD54 antibody. Consequently we created two new populations. Semi-quantitative PCR was performed using primers specific for CD73, CD90 and CD105. Fifteen μL of each PCR product was detected by ethidium bromide gel electrophoresis using a 1% agarose gel. The relative expression level was taken as a ratio over the expression of the house keeping gene β-actin.

**Phenotypic and CFU analysis of CD54+ and CD54- AMCS**

We next isolated RNA from CD54+ and CD54- AMSC fractions and analyzed for expression of MSC markers using semi-quantitative RT-PCR. Figure 3 demonstrates that CD73 mRNA expression was 2.2-fold higher in CD54+ versus CD54- cells. Both fractions expressed similar levels of CD90 and CD105.
Clonogenic assays revealed that early passage unsorted AMSCs displayed the highest colony forming capability with 16 colonies per 1,500 cells seeded (Figure 4) whereas CD54\(^-\) and CD54\(^+\) AMSCs produced 14 and 11 colonies per 1,500 cells seeded, respectively.

**Adipogenic and osteogenic differentiation potential of CD54\(^-\) and CD54\(^+\) AMSCs**

After 14 days of adipogenic induction, differentiated adipocytes displayed a round shape with the formation of lipid droplets that accumulated the oil red O stain (Figures 5a-5c). Control cells were negative for oil red O staining (Figures 5d-5f). CD 54\(^-\) AMSCs showed the highest accumulation of oil red O with 46.7% ± 5.0% positive staining. Positive staining was observed in 28.7% ± 6.4% of unsorted AMSCs and 16.2% ± 6.7% of CD54\(^+\) AMSCs (Figure 6).

Osteogenic differentiation potential was assessed next. After 21 days of induction, CD54 and unsorted AMSCs stained with alizarin red showed a more dense extracellular matrix than did CD54\(^+\) cells (Figure 7).

**Discussion**

MSCs hold great promise in regenerative medicine but excitement has been tempered due to the possibility of fibroblast contamination [25]. Distinguishing MSCs and fibroblasts is currently not possible as both adhere to plastic and express similar levels of CD73, CD90 and CD105. The aim of our study was to identify a novel selection marker capable of separating MSCs from fibroblasts resulting in enhanced MSC multipotency.

We focused on CD markers because their cell surface localization allowed for their potential use in sorting procedures. Previous studies from several groups reported qualitative differences in expression of CD49d, CD54, CD81, CD109, CD146, CD164, and CD172a by MSCs and fibroblasts [6,21,26-28], but none of these studies confirmed their utility as selection markers. In the present study, mRNA expression levels of these CD markers were further analyzed and validated by real-time PCR, a more quantitative method. We found dermal fibroblasts express 10-fold more CD54 mRNA and 5-fold more CD54 cell surface protein than AMSCs. To our knowledge, this is the first quantitative comparison of CD54 mRNA and protein expression in MSCs and fibroblasts. This finding is in line with a previous report that 70-100% of human adult fibroblasts positively stained with anti-CD54 antibody using flow cytometry [21]. However, a recent study showed the only 25% of human adult dermal fibroblasts were CD54\(^+\), in line...
with AMSCs [29]. This discrepancy may be explained by the difference in the experimental design, because endothelial growth medium supplemented with 10% FCS and bFGF was used to culture dermal fibroblasts [29], whereas in our study we used DMEM supplemented with 10% FBS.

CD54, also called inter-cellular adhesion molecule 1, is a transmembrane glycoprotein with 5 extracellular immunoglobulin G-like domains and a short cytoplasmic tail that associates with multiple cytoskeletal linker proteins [30]. CD54 is primarily expressed in endothelial cells and its interaction with lymphocyte function-associated antigen-1 and macrophage antigen-1 is important for leukocyte adhesion and transendothelial migration [30]. It was reported that lymphocyte function-associated antigen-1-dependent monocyte migration across connective tissue barriers was primarily via engagement of CD54 on fibroblasts [31].

The difference in CD54 expression by MSCs and fibroblasts suggests that if MSCs become gradually overgrown by contaminating fibroblasts, expression of CD54 should increase at later passage numbers. To test this hypothesis, we compared CD54 mRNA expression in AMSC cultures of passage 2 and 20. The results confirmed our hypothesis as CD54 expression was substantially increased in AMSCs at passage 20 (data not shown). This finding encouraged us to sort AMSCs with anti-CD54 antibody before cell expansion to generate CD54+ and CD54− populations. We found CD73 expression was 2.2-fold higher in CD54− AMSCs. Cultured CD54− AMSCs expressed higher levels of CD73, an immunosuppressive molecule, and increased differentiation capacity [9,29,34-36]. Our clonogenic assays illustrated CD54− AMSCs formed different cell types, including osteoblast and adipocyte from skin and/or other sources could be treated to enrich AMSCs for early osteogenic and adipogenic progenitors. Therefore, we suggest CD54+ AMSCs would benefit their immunosuppressive effects.

Due to the similarities in morphology and cell surface marker expression, distinction between MSCs and fibroblasts should be based on their differentiation potential. We assessed adipogenic and osteogenic differentiation potential of CD54 sorted and unsorted AMSCs and found that differentiation capacity was enhanced in CD54− AMSCs relative to CD54+ cells. Our data suggest CD54 may be utilized to enrich AMSCs for early osteogenic and adipogenic progenitors. Although CD54+ cells underwent adipogenic and osteogenic differentiation, the differentiation efficiency was extremely low. The reason why these fibroblast-like cells possess the differentiation capabilities remains unknown. Recently, several papers revealed that fibroblasts derived from skin and/or other sources could be treated to differentiate into several cell types, including osteoblast and adipocyte [9,29,34-36]. Our clonogenic assays illustrated CD54− AMSCs formed slightly less colonies than other populations. This may be due the higher growth rate of fibroblasts (48 h per subculture) relative to AMSCs (72 h per subculture) under our culture conditions (data not shown). It is also possible that the higher seeding density is disadvantageous in MSC expansion. Such phenomenon were previously described [37,38].

Conclusion

Herein, we demonstrated that dermal fibroblasts express 10-fold more CD54 mRNA and 5-fold more CD54 protein on their surface than AMSCs. Cultured CD54− AMSCs expressed higher levels of CD73, an immunosuppressive molecule, and increased differentiation capacity into adipocytes and osteoblasts. In conclusion, we identified CD54 as a novel selection marker for distinguishing MSCs from fibroblasts. CD54 may allow for enrichment of MSCs with enhanced multipotency and immunosuppressive properties, both advantageous features for therapeutic applications.

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References


