Characterization of Adipose Tissue Mesenchymal Stromal Cell Subsets with Distinct Plastic Adherence

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Mesenchymal stromal cells (MSCs) established by *in-vitro* adherence culture have been widely utilized for various cell therapeutic trials, but potential heterogeneity that can be caused by preparation methods are poorly characterized. In this study, we show that at least two distinct subsets of MSCs with different adherence to plastic surface exist in human adipose tissue-derived stromal vascular fraction (SVF); while 69% of total colony forming units in SVF adhere to the surface before 3 hrs of plating, 13–17% of colonogenic cells adhered to the surface at later period of 15 hr to 1 week after plating. Of note, the late adherent MSCs exhibited higher self-renewal of colony forming cells and higher proliferating potential with comparable level of osteogenic or adipogenic differentiation potential to the early adherence subsets. Moreover, late adherent cells exhibited distinct pattern of paracrine secretome including higher level secretion of cytokines than the early adherent subsets. Taken together, these results suggest the possibility that distinct adherence properties of MSCs can be another parameter of clonal heterogeneity in the subpopulations of adipose tissue MSCs and that it can be an important factor for optimization of MSC preparation for cell therapeutic trials.

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are non-hematopoietic adherent cell populations that can be obtained from various tissues including bone marrow (BM), adipose tissue, or placental tissue. These MSCs exhibit multi-lineage differentiation potential towards diverse types of tissues including bone, cartilage, adipose tissues [1,2]. Recent studies have shown that the primary mode of action for MSCs is the establishment of a regenerative microenvironment in response to tissue injury, thereby stimulating the regeneration of hematopoietic stem cells, neuronal stem cells, and other tissue-specific stem cells [3-5]. Principal function of the MSCs to establish regenerative microenvironment are exerted via paracrine secretion of various growth factors that can stimulate angiogenesis and inhibit apoptosis and fibrosis [6].

While the MSCs can be derived from various types of tissues, studies have shown that MSCs from diverse sources nevertheless share several common surface phenotypes: these include the

expression of CD90 (Thy-1), CD166 (SB10/ALCAM), CD73 (SH3), CD105 (SH2, endoglin), and the primitive subpopulation of MSCs have been identified by specific surface markers such as CD271 or CD146 [7,8]. On the other hand, studies have shown that considerable extent of heterogeneity can be caused in the subsets of MSCs depending on the methods of preparations or *in-vitro* expansion culture conditions culture [9,10], suggesting the need for optimization of process for MSC preparations for cell therapy.

Of note, most of MSC cultures are established by adherence to the plastic surface, but various approaches have been described to obtain the adherent populations. For example, traditional MSC populations were prepared by plating total mononuclear cells of BM or stromal vascular fraction (SVF) of adipose tissue in the plastic culture dish and by harvesting total adherent cell populations. On the other hand, recent studies reported a protocol where murine MSCs can be prepared from BM by 3 hr short-term plating of total BM cells thus harvesting the early adherent cell population [11,12]. In contrast, Song et al. [13] have reported preparation of clonal MSCs by serial fractionation of the BM mononuclear cells. However, it has not been clear yet whether these differences in the preparation methods of the MSCs could be related to the difference in their characteristics in MSC function or in their cell therapeutic function.

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Therefore, in the current study, we have isolated MSCs with respect to their adherence to the plastic surface and examined the characteristics of early and late adherence populations of MSCs. Our results reveal the presence of early and late adherent population with distinct cellular characteristics, pointing the need for consideration for cell therapeutic preparation of MSCs.

MATERIALS AND METHODS

Isolation of stromal vascular fraction cells from lipo-aspirates and cell culture

Aspirated abdominal subcutaneous adipose tissue was obtained from female donors undergoing liposuction surgery. This study was approved by the Institutional Review Boards of Seoul St. Mary's Hospital and all samples were obtained after obtaining informed consent. The raw lipoaspirates were chopped with scissors and washed with sterile phosphate-buffered saline (PBS). The lipoaspirates were digested in PBS containing 0.1% collagenase type I (Sigma Aldrich, St. Louis, MO, USA) for 1 hour at 37°C on a shaker. The collagenase was inactivated with an equal volume of Dulbecco's modified Eagle's Medium (DMEM) high glucose (GE lifescience hyclone™, South Logan, UT, USA)/10% fetal bovine serum (FBS) (Thermo Hyclone™, South Logan, UT, USA) and 1% penicillin/streptomycin. Mature adipocytes and lipids were separated from pellets by centrifugation (1300 rpm, 3 min). The pellets were filtered through a 100 mm mesh (Millipore, MA, USA) to remove connective tissue and centrifuged again (1300 rpm, 3 min) to obtain SVF cell pellets. 1.5×10^6 cells of SVF were seeded to two 150 mm-culture-dishes (FALCON®,

Corning, NY, USA) for different time intervals. After each time point, the non-adherent supernatant was transferred to another dish for plating (Fig. 1). The adherent cell cultures were incubated until colonies were formed.

Colony-formation assay

For colony formation (CFU-F) in *in vitro* cultured MSCs, MSCs were plated at a density of 1000 cells per 100 mm dish, and after incubation for 14 days, the number of colonies containing >50 cells was counted after staining with crystal violet (Sigma, St. Louis, MO, USA) in methanol.

Flow cytometry analysis

Flow cytometry analysis of MSC surface markers was performed as described [14]. After cultured until 4 passages, each group of stromal vascular fractions (SVFs) was stained with monoclonal antibodies, anti-human CD31-phycoerythrin (PE), CD45-allophycocyanin, CD73-PE, CD90-fluorescein isothiocyanate (FITC), CD105-FITC, CD140-β (PDGFR-β)-PE, CD146-PE (BD Pharmingen, San Diego, CA, USA), SSEA-3-IgM (Millipore corporation, Temecula, CA, USA) and SSEA-4-biotin (R&D systems, Minneapolis, MN, USA) and analyzed using LSR II (Becton Dickinson, USA) and BD FACSDiva™ software (BD Bioscience).

Osteogenic and adipogenic differentiation

Osteogenic differentiation was induced by DMEM supplemented with 10% FBS, 10 nM dexamethasone, 0.2 mM ascorbic acid and 10 mM β-glycerophosphate as described [2]. After 28

Figure 1. Schematic illustration of the process for isolation of mesenchymal subsets in human adipose tissues by difference in plastic adherence time. Stromal vascular fraction (SVF) was isolated from the aspirated fat tissue by conventional enzyme-digestion methods. 1.5×10^6 cells of SVF were plated onto culture-dishes for 3 hours and non-adherent cells were transferred to a new dish for adherence for additional 12 hours (15 hr), non-adherent cells at the point were then transferred for 1 week culture in new dish.

days, the mineralization of the extracellular matrix was determined by Alizarin Red S staining, which was quantified by dye elution using cetylpyridinium chloride (Sigma) and measurement of the absorbance at 550 nm. For adipogenic differentiation,

confluent cells were incubated in DMEM containing 10% FBS, 1 μ M dexamethasone, 0.5 mM isobutyl-1-methylxanthine, 100 μ M indomethacin and 10 μ g mL⁻¹ insulin [15]. After 14 days, cells were fixed with 4% formaldehyde and stained with

Figure 2. Isolation of mesenchymal colonogenic cells by difference in plastic adherence time. (A) Light microscopy findings of the cells attached at different time points at initial adherence and after expansion culture. Representative images of phase-contrast light microscopy are shown ($\times 100$). (B) Quantitative comparisons of CFU-F numbers contained in each fraction of adherent cells. The numbers of mesenchymal colonies (CFU-F) that were derived from each adherent populations are shown along with total numbers of CFU-F obtained by plating whole SVF in the dish ($n=6$, $*p<0.05$). CFU-F: colony forming units-F, SVF: stromal vascular fraction, SPT: supernatant.

Figure 3. Comparisons of biological characteristics of early and late adherent mesenchymal cell population. (A) Common surface phenotype of each mesenchymal fraction. Shown are the summary of the flowcytometry analysis for indicated markers. (B) Comparisons of expression levels for indicated surface markers for subsets of MSCs ($n=3$, $*p<0.05$). (C) Differences in cell size and cell density of mesenchymal subsets as determined by forward scatter (FSC) and side scatter (SSC) in flowcytometry. Representative flow cytometry plots for each subset (3 hr, 15 hr, 1 week point) are shown in colored line in comparison to the plots for SVF (gray shaded). SVF: stromal vascular fraction, SSEA: stage specific embryonic antigen, PDGFR- β : platelet-derived growth factor- β , MSCs: mesenchymal stromal cells.

Oil Red O to visualize lipid droplets. The number of differentiated adipocytes was subsequently determined by dye extraction using 4% Nonidet P40 in isopropyl alcohol followed by spectrophotometry at 520 nm.

Secretome analysis

After culturing of adipose tissue-derived mesenchymal stromal cells (ADSCs) in serum-containing medium, ADSCs were washed with PBS twice and cultured in serum-free medium for 24 hours. The supernatant was transferred to tubes and centrifuged (1500 rpm, 5 min) to remove suspended cells. The secretome analysis was performed by RayBio™ Custom C-Series Human Cytokine Array according to their protocols for the RayBio Human Cytokine Antibody Array (RayBiotech, Norcross, GA, USA). Relative intensities of obtained spots were measured by Image J and corrected by background subtraction. Results of duplicate readings were averaged.

Statistical analysis

Data are expressed as the mean $\pm$ standard error and analyzed by the Student's t-test. The level of significance was set at $p < 0.05$.

RESULTS

Fractionation of MSCs with different kinetics of adherence

To see if subpopulations of mesenchymal colonogenic cells in adipose SVF exhibit difference in the time for adherence to plastic surface, we first plated the SVF in the dish for different time and collected the adherent populations (Fig. 1). The total SVFs were plated in the dish for 3 hrs and non-adherent populations were transferred to another dish for additional 12 hrs (15 hr) or 1 week culture period and maintained until colony formation in each dish.

As shown in Figure 2A most of the adherent cells in SVF adhered to the plastic dish within 3 hr of plating period, but minor populations of SVFs adhered to the surface at 15 hr or 1

Figure 4. Distinct biological function of early and late adherent MSCs. (A) Comparisons of proliferative activity of each distinct adherent population. Each indicated fractions of adherent MSCs were subjected to serial subculture for 50 days. Shown are the accumulated numbers of cell growth ($n=3$, $*p < 0.05$ for difference between early and late 1 week subpopulation). (B) Comparisons for colonization and self-renewal potential of CFU-F. Primary CFU-F numbers were determined by plating 1000 MSCs in dish until colonization. The CFU-F in each fraction was subjected to the secondary colony formation assay. Self-renewal of CFU-F was determined by the numbers of secondary colony derived from each primary colony. Shown are the mean numbers of colonies in each colonization assay along with self-renewal of CFU-F ($n=3$, $*p < 0.05$). MSCs: mesenchymal stromal cells, CFU-F: colony forming units-F, SVF: stromal vascular fraction.

week time point and showed proliferative activity reaching confluence in the dish (Fig. 2A, lower panel). When each fraction was measured for total content of colony forming cells, 69% of CFU-F that can be obtained from total SVF fraction was obtained from 3 hr time point of plating, but 13–17% of CFU-Fs were obtained from 15 hr fraction or 1 week fraction (Fig. 2B). These results show that the colonogenic mesenchymal progenitor cell pools exhibit heterogeneity in the plastic adherence and that, while most of CFU-Fs exhibit early (<3 hr) adherence, minor population of CFU-Fs exhibit delayed (15 hr or 1 week) adherence to the plastic surface.

Heterogeneity of MSC subpopulation with adherence time

Next, to see if there is any distinction in the characteristics of each MSC fraction obtained by difference in adherence time, we examined the surface phenotype and their proliferation or differentiation properties between the groups. When examined for surface markers, all the fractions of MSCs exhibited comparable expression of surface markers for CD105, CD90, CD73,

SSEA4 and absence of CD45 and CD31 (Fig. 3A). In contrast, each fraction of MSCs exhibited notable difference in the expression level of CD146, SSEA3, and PDGFR- β as reflected by difference in the mean fluorescence intensity (Fig. 3B). Similarly, each subset of MSCs exhibited a distinction in their physical properties (size and density) as reflected by forward scatter and side scatter in flow cytometry (Fig. 3C).

Functional distinction between early and late adherent MSC subpopulation

Next, to further examine functional difference of each distinct adherent population, we next determined to compare the proliferative activity and self-renewal of mesenchymal colonies from each group MSC. When each group MSCs were subjected to the serial sub-culture for 50 days *in-vitro*, the difference in their proliferative activity was seen; the late adherent MSC populations (15 hr or 1 week fraction) exhibited higher proliferative activity than the MSCs established from 3 hr plating or from plating of total SVF fraction (Fig. 4A). Moreover, when examined for frequency of CFU-F in each fraction, late adherent cell pop-

Figure 5. Comparisons for multi-lineage differentiation of MSC subsets. Each indicated MSC subsets were subjected to adipogenic (A) and osteogenic (B) differentiation. Shown are the representative images of differentiation and quantitative measurement of mineral and lipid droplets ($n=3$, $p>0.05$) (magnification: $\times 50$). SVF: stromal vascular fraction, MSC: mesenchymal stromal cell.

ulation (15 hr or 1 week) exhibited higher frequency of CFU-F in the primary and in the serial secondary colony formation (Fig. 4B), thus exhibiting higher self-renewal of mesenchymal colonies than for total SVF or early (3 hr) adherent populations. These results show that while most of the mesenchymal CFU-F resides in the early adherence (3 hr) fraction, a distinct subset of

colony forming cells exists in the late adherent fractions exhibiting a higher self-renewal and proliferative potential than early adherent populations.

In contrast, when the osteogenic and adipogenic differentiation was examined for each fraction of MSCs, no significant difference was seen among each MSC fraction, as reflected by

Figure 6. Comparisons for secretome of MSC subsets. Secretome of each indicated MSC subset were analyzed by performing immunoblot for indicated factors using protein array blot. (A) Representative protein blots for each indicated subset of MSCs are shown. Blots for bFGF, IL-1RA and IL-6 are marked. (B) Quantitative comparisons for secretion level of each indicated cytokines or growth factors. Shown are the relative secretion levels of the factors relative to the level in whole SVF cultures and their mean value (n=2). Note, higher levels of cytokine levels for late (15 hr or 1 week) compared to the levels in 3 hr fraction. MSC: mesenchymal stromal cell, bFGF: basic fibroblast growth factor, BMP: bone morphogenic protein, EGF: epidermal growth factor, ENA-78: epithelial neutrophil-activating protein-78, IGF: insulin-like growth factor, IL: interleukin, IP: interferon-gamma inducible protein, LIF: leukemia inhibitory factor, MCSF: macrophage colony-stimulating factor, MIF: macrophage migration inhibitory factor, MIP: macrophage inflammatory protein, MP3: macrophage activating protein, MMP: matrix metalloproteinase, PDGF: platelet-derived growth factor, SCF: stem cell factor, SDF: stromal cell-derived factor, TGF: tumor growth factor, TIMP: tissue inhibitor of metalloproteinase, SVF: stromal vascular fraction.

comparable levels of minerals or lipid droplet contents after terminal differentiation (Fig. 5). These results show that the late adherent MSC population represents distinct subsets of adipose tissue MSCs with higher proliferation/self-renewal but comparable level of multi-lineage differentiation potential to the major early adherent populations.

Distinct secretome pattern of early and late adherent MSC subpopulations

Next, to see if potential distinction exists in the secretory activity of bioactive factors among the MSC subpopulations, we compared the secretome in the protein array blot between the early and late adherent population. As shown in Figure 6, for most of the secretory factors examined, the late adherent (15 hr or 1 week) MSCs exhibited higher level secretion of the factors than early (3 hr) adherent populations, including higher level of IL-6, IL-1RA or basic fibroblast growth factor. These results show that the early and late adherent populations also exhibit difference in the paracrine secretion pattern with distinct levels of cytokine secretion. Taken together, our results show that while most of the ADSC can be quantitatively harvested by short-term incubation for 3 hrs, minor subpopulation of adipose-derived MSCs with higher proliferation, self-renewal and higher secretory activities can reside in the late adherent cell populations.

DISCUSSION

MSCs have been obtained by *in-vitro* culture of adherent stromal cell populations contained in various tissues including BM, adipose tissues or placenta. These culture-expanded MSCs from various types of tissues share certain extent of biological properties such as common surface markers and paracrine secretion of bioactive peptides in the injured tissues, facilitating the establishment of regenerative microenvironment in the tissue injury condition [6]. Recently, the *ex-vivo* expanded MSCs has been widely used in a variety of cell therapeutic trials that includes regeneration of damaged cardiovascular [16], neural [17] and muscular-skeletal tissues [18-21] as well as for the suppression of allogeneic immune reactions [22,23] for facilitation of hematopoietic engraftment [14,24].

However, despite many of common properties in MSCs such as surface phenotype and their biological characteristics, significant heterogeneity was also observed among the subpopulation of MSC preparation in the morphology, proliferation and differentiation potentials [25-27] as well as in their cell size or self-renewing potential [25,28].

While the cellular bases for such heterogeneity of MSCs are not clear yet, it has been pointed that initial cell populations in BM or adipose tissue SVF are heterogeneous [29] and signifi-

cant extent of heterogeneity can be caused by difference in the methods of preparation and *in-vitro* culture [9,10].

So far, MSCs have been prepared in a conventional adherence to the plastic surface. Recently, a guideline protocol was suggested for efficient preparation of MSCs in the murine BM model [11,12], where BM mononuclear cells are selected by adherence to the plastic dish for 3 hrs after plating. On the other hand, other study showed that human BM-derived MSCs can be established by serial subfraction culture methods exhibiting considerable variations in epitopes, differentiation potential, and cytokine secretions [13], suggesting the possibility that adherence to the plastic surface could constitute another parameter for heterogeneous subpopulation of MSCs.

In consistence to the possibility, our study show that at least two distinct subpopulations (early and late) exist in the adipose tissue SVF, and that these subsets can be separated by difference in their adherence time to the plastic surface. Our study showed that, while most of the mesenchymal CFU-F in adipose SVF were contained in the early adherent fraction, distinct subsets of MSCs can be obtained from late adherent populations in the SVF of human adipose tissues.

Of note, the subsets of MSCs with distinction in plastic adherence exhibited a series of distinct functional characteristics, i.e., the late adherent populations exhibited higher self-renewal and proliferative activity than the early adherent mesenchymal subsets. Moreover, these late-adherent MSC subsets also exhibited distinct cytokine secretion pattern from earlier adherent subsets, thus indicating that their paracrine activity for cell therapeutic action could be distinct. Thus, our study points to the possibility that the MSC populations can have different cell therapeutic outcomes caused by the cellular heterogeneity depending on the preparation of the mesenchymal culture. In coherence to the possibility, previous studies showed that the MSCs isolated by different methods exhibited differences in surface epitope, differentiation potential and immune modulating activities [30] as well as difference in their therapeutic function for immune suppression [31].

However, it should be also mentioned that, while we have separated late adherent cells by collecting the cells non-adherent at 3 hr, but adherent during 15 hr to 1 week time points, we observed that significant extent of individual variations exist in the time for late adherence. Nevertheless, our study can provide an alternative model of MSC preparation in a manner that the late adherent cell populations excluding the 3 hr-adherent population (i.e., 15 hr to 1 week) are selectively expanded for higher proliferation and secretory activities of expanded cell products. Further studies on more numbers of individual fat tissues are necessary to set more detail cut-off time point for MSC subsets.

Taken together, our study show that the human adipose tissue

SVF harbors heterogeneous subsets of MSCs that are distinct in their adherence kinetics and biological characteristics, and suggest that the optimization of the preparation methods are warranted for standardization of the MSC quality and their therapeutic outcomes in the cell therapeutic trials.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was approved by the Institutional Review Boards of St. Mary's Hospital and Seoul St. Mary's Hospital (KC11 TNSI 0186).

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