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In vitro Expansion of Mesenchymal Stem Cells using 3-D Matrix Derived from Cardiac Fibroblast

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Abstract: Mesenchymal stem cells (MSC), nonhematopoietic progenitor cells are capable of differentiating into multiple lineage of the mesenchyme such as bone, cartilage, tendon, fat, heart, muscle, and brain, *in vitro* and *in vivo*. Although autologous MSC have some advantageous factors, MSC have very poor replicative capacity. Previous studies indicated that growth factors but also cell seeding density made an effect on expansion of MSCs. In this study, we examined the growth rate and adhesion of MSC in tissue culture coated with a basement membrane-like extracellular matrix. When self-renewal of MSC were compared between 2-D and 3-D, the growth rate of MSCs on 3-D matrix was highest among plastic, Fibronectin- and 3-D matrix plate but also attachment of MSCs on 3-D matrix was 6-fold greater than that on plastic. MSCs plated into 3-D matrix showed colocalization of $\alpha5$ integrin and αV integrin. This study may reflect that the extracellular matrix had greater effect on the expansion of MSCs because this distinctive in 3-D matrix adhesion differ in localization and function from classically described *in vitro* adhesion.

Keywords:

1. Introduction

It has been known that stem cells resided in adult tissue and organs have the capacities of self-renewal and multipotential differentiation. Especially, mesenchymal stem cells (MSC), nonhematopoietic progenitor cells are capable of differentiating into multiple lineage of the mesenchyme such as bone, cartilage, tendon, fat, heart, muscle, and brain, *in vitro* and *in vivo*.[1,2] Adult stem cells keep a quiescent state before they are stimulated by tissue damage and remodeling. [3,4] Stimulation of signals make adult stem cells re-enter the cell cycle to replenish the stem cell pool and to generate progenitor cells, which then induce various differentiated cell type for tissue regeneration and homeostasis. There are some factors such as cytokines, growth factors, adhesion molecules and extracellular matrix components in the stem cell microenvironment to play important roles in stem cell fate determination, functioning as the driving forces for stem cells to switch from a self-renewal to a differentiation stage.[5-9]

Although autologous MSC have some advantageous factors such as no teratocarcinoma formation, no immune rejection, and no ethical problems, MSC have very poor replicative capacity and short proliferative longevity compared with ES cells, which have an unlimited proliferative life span (period before the cells reach growth arrest in culture) and consistently high telomerase activity.[10,11] Related with cellular replication, the term of self-renewal is defined as the ability to generate identical copies of themselves through mitotic division over extended time periods (even the entire lifetime of an organism).

Previous studies indicated that growth factors but also cell seeding density made an effect on expansion of MSCs. Namely, higher population doublings(i.e.>50 PDs) have been achieved as a result of the supplementation of specific

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growth factors [e.g., fibroblast growth factor-2(FGF-2)] and higher expansion profiles of MSCs can be attained when plated at low density (1.5-3 cell/cm^2) but not at high density (12 cell/cm^2), resulting in a dramatic increase in the fold expansion of total cells (2,000-fold vs. 60-fold expansion, respectively).[12,13] Enhanced expression of telomerase in MSC induces markely augmentation of their proliferative life span and MSC with a high telomerase activity showed osteogenic potential.[14]

Thus, an important challenge in regenerative medicine is to improve the replicative capacity of MSC as well as to keep multipotential differentiation, thereby to obtain a number of MSC sufficient to repair large defects. In this study, we examined the growth rate of MSC in tissue culture coated with a basement membrane-like extracellular matrix. To this aim, we compared self-renewal of MSC between in 2-D and 3-D.

2. Material and Methods

2.1. Isolation and Culture of MSCs

Isolation and primary culture of MSCs from the femoral and tibial bones of donor rats were performed. We collected bone marrow-derived MSC from aspirated the femurs and tibias of 4-weeks aged Sprague-PMEM-Dawley male rats (about 100g) with 10 ml of MSC medium consisting of Dulbecco's modified Eagle's medium-low glucose supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-penicillin & streptomycin solution. Mononuclear cells recovered from the interface of Percoll-separated bone marrow were washed twice and resuspended in 10 % FBS-DMEM, and plated at 1×10^6 cells/100 cm^2 in flasks. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 48 or 72 h, nonadherent cells were discarded, and the adherent cells were thoroughly washed twice with PBS. Fresh complete medium was added and replaced every 3 or 4 days for about 10 days. To further purify MSCs, we used Isolex magnetic cell selection system (Nexell Therapeutics Inc. CA, USA). Briefly, collecting cells above incubated the mixed culture with anti-CD34 monoclonal antibody. Following washing to remove the unbound antibody, Dynabeads M-450 coated with sheep anti-Mouse IgG, which recognized the murine-derived anti-CD34 antibody, were mixed with the cell suspension. A magnetic field was applied to the chamber, enabling the CD34+ cell-bead complexes to be separated magnetically from the rest of the cell suspension. This remaining CD34-negative fraction was further propagated. The cells were harvested with 0.25% trypsin and 1 mM EDTA for 5 min at 37°C, replated in 100 cm^2 plate, again cultured for about 10 days.

2.2. Preparation of cardiac fibroblast derived 3-D matrix; Cardiogel

Cardiogel was prepared using method of Cukierman, *et al.*[15] with minor modification. 1.5 % gelatin solution was added to the culture surface to be used for fibroblast-derived 3D matrix deposition and incubated for 1 hour at 37°C. 4×10^5 cells were seeded in pre-coated 60-mm dish and cultured for 24 hours. After 24 hours, medium was changed every other day. Once the cultures became confluent, plate was rinsed gently with 2 ml PBS by touching the pipette against the dish wall rather than at the bottom of the dish. Treatment of the cultures with pre-warmed extraction buffer (0.5 %(v/v) Triton X-100, 20 mM NH_4OH in PBS) resulted in cell lysis, exposing the extracellular matrix adhering to the substrata of tissue culture dishes. The substratum was washed five times with PBS. The matrix-coated plates were covered with at least 3 ml PBS supplemented with 100 U/mL penicillin, 100 mg/ml streptomycin, and 0.25 μg/ml Fungizone(Fig. 1).

2.3. Immunocytochemistry

Cells were grown on 4-well plastic dishes (SonicSeal Slide, Nalge Nunc, Rochester, NY, USA). Following incubation, the cells were washed twice with PBS and then fixed with 4% paraformaldehyde in 0.5 ml PBS for 30 min at room temperature. The cells were washed again with PBS and then permeabilized for 30 min in PBS containing 0.2%

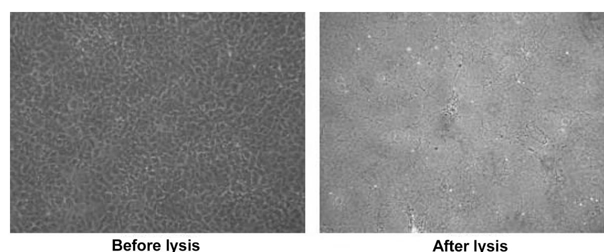

Figure 1. Preparation of cardiac fibroblast derived 3D extracellular matrix: Cardiogel. Cardiac fibroblasts were plated in 60Ø gelatin pre-coated plates at a density of 1×10^6cells per well. Cells were cultured at 37°C until full density. Then, these were lysis with detergent.

triton. The cells were then blocked in PBS containing 10% goat serum and then incubated for 1 hr with mouse polyclonal antibody (ICAM-1, CD34, 71, 90, 105, 106). The cells were rewashed three times for 10 min with PBS and incubated with FITC-conjugated goat anti-mouse antibody as the secondary antibody for 1 hr. Photographs of cells were taken under fluorescence by immunofluorescence microscopy (Olympus, Melville, NY, USA). All images were made by using an excitation filter under reflected light fluorescence microscopy and transferred to a computer equipped with MetaMorph software ver. 4.6 (Universal Imaging Co.).

2.4. Flow cytometry

To verify the nature of cultured MSCs, cells were labeled against various surface and intracellular markers and analyzed by flow cytometry. MSCs were retrieved with a standard trypsinization technique. Cells were washed in phosphate buffered saline (PBS) and fixed in 70% ethanol at 4°C for 30 min with agitation. Cells were washed twice in PBS and resuspended at 2×10^6 cells/ml in blocking buffer (1 % BSA, 0.1% FBS) containing the following antibodies conjugated with fluorescin isothiocyanate (FITC) or Texas Red: CD14, CD34, and CD90. The labeling reaction mixture was agitated for 20 min at room temperature. Cells were washed twice and then labeled with anti-rabbit-FITC conjugated IgG and anti-mouse-Texas Red (Jackson ImmunoResearch Laboratories, Inc.PA, USA) for 20 min at room temperature in the dark. After two more washes, flow cytometric analysis was performed on a FACSCalibur system (Becton Dickinson, CA, USA) using CellQuest™, software with 10,000 events recorded for each sample.

2.5. Proliferation assay

MSCs were plated in triplicate wells of each plate at a density of 1×10^4 per well. Cell expansion rate of each plate was determined by the MTT assay. After the incubation period, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma, St. Louis, MO, USA) was added to each well to a final concentration of 0.5 mg/mL and was incubated at 37°C for 3 hr to allow MTT reduction. The formazan crystals were dissolved by adding dimethylsulfoxide (DMSO) and absorbance was measured at the 570 nm with a spectrophotometer.

2.6. Assays for Cell Adhesion

Four-well plates (Nunc, Rochester, NY) were coated with Fn (Sigma) for 24 hours at 4°C or cardiogel. Fn was dissolved in PBS (pH 7.4) to yield a final concentration of 10 µg/ml, and a volume of 150 µl was added to the individual wells. The plates were then blocked with 10 mg/ml BSA (Sigma) in PBS for 1 hr at 37°C. Suspensions of 2×10^4 viable MSCs were then added to each well and allowed to attach for 30 minutes at 37°C and 5% CO_2. To determine MSC adhesion, plates were carefully washed three times with PBS, and then four separate fields were photographed by phase contrast microscope. The number of attached cells was estimated by microscopic cell counting. Each experiment was performed in triplicate wells and repeated at least three times.

2.7. Statistical analysis

Results were expressed as mean±SEM. Statistical analysis as performed by student's t-test. Relationships were considered statistically significant when p value was less than 0.05.

3. Result

3.1. Isolation and Characteristics of MSCs

MSCs were first isolated from mixed cultures with hematopoietic cells based on their attachment on the culture plate. The isolated MSCs were further purified using bead targeting the hematopoietic marker CD34, yielding 3×10^6 cells within 2 weeks of culture with 95 % purity. The MSCs retained a fibroblastic morphology through repeated passages, and their identity was confirmed by immunocytochemistry and FACS analysis. The cultured MSCs expressed CD71, CD90, CD105, CD106, and ICAM. They expressed neither the hematopoietic marker CD34 nor CD14(Fig. 2).

3.2. Cell proliferation of MSCs during *ex vivo* expansion

To determine whether *in vitro* extracellular environment were associated with expansion of cells, we plated bone marrow derived MSCs on plastic plate, fibronectin-coated plate and 3-D matrix. Preparing of fibronectin-coated plate, plate were coated with fibronectin (Sigma, MO, USA) for 24 hours at 4°C. Fibronectin was solubilized in PBS (pH 7.4) to yield a final concentration of 10 µg/mL. The growth rate of MSCs on 3-D matrix was highest among them and thus the cumulative cell number in the cultures on 3-D matrix was 3-fold greater than that on plastic at 96 hrs(Fig. 3).

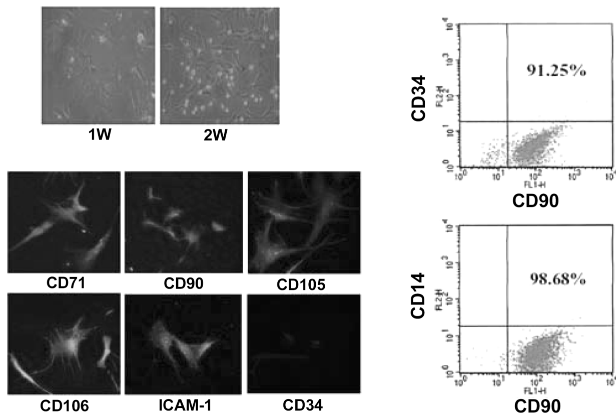

Figure 2. Characterization of isolated MSCs. Bone marrow derived mesenchymal stem cells were primarily cultured in PVC plate during 1week and 2week. Flat and spindle-like cell shape. At 2weeks, the MSCs were positive for CD71, CD90, CD105, CD106, and ICAM-1 and were negative for CD34 by immunocytochemistry. To verify MSCs, cells were analyzed CD14, CD34, and CD90 by flow cytometry.

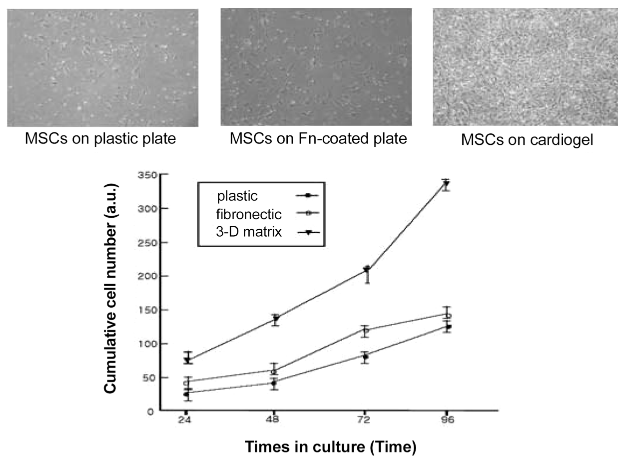

Figure 3. Cell proliferation of MSCs during *ex vivo* expansion. MSCs cultured on each 3 different plates; plastic plate, Fn-coated plate, or cardiogel (3-D matrix). Differs significantly from the cell number in cultures on plastic plate, Fn-coated plate, or cardiogel dishes on time, 24~96 hour. In process of time, MSCs were best proliferated in 3-D matrix than plastic plate or Fn-coated plate. Data denote the means±S.E.M. of 2~3 replicate measurements in three different cell cultures.

3.3. Cell attachment of MSCs to the indicated substrates

To determine the effect of the indicated substrates in adhesion of MSCs, we performed quantitative adhesion assays with different culture condition. These distinctive in 3-D matrix adhesions differ in structure, localization, and

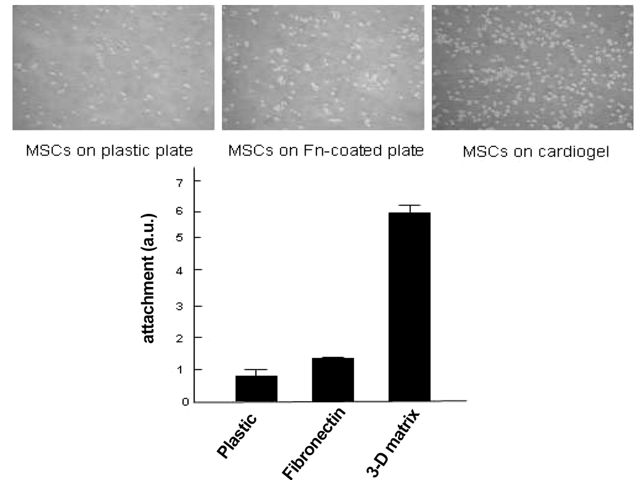

Figure 4. Cell attachment of MSCs to the indicated substrates. MSCs were plated on plastic, Fn-coated plate cardiogel for 3 hours. Four separate fields were photographed by phase contrast microscope. The number of attached cells was estimated by microscopic cell counting. Above all, MSCs of 3-D matrix were best attached to the plate over 6-fold as compared with the other plates. Data denote the means±S.E.M. of 2~3 replicate measurements in three different cell cultures.

function from classically described in 2-D adhesions. As shown in Figure 4, the cell adhesion to cardiogel(3-D matrix) was significantly increased in MSCs as compared with adhesion to Fn-coated plates (2-Ds). The adhesive difference was over 6-fold greater when prepared on cardiogel as compared with the Fn-coated flat plates.

3.4. Difference of focal or fibrillar adhesion between fibronectin- and 3-D matrix plate

It has been known that adhesion between cells and extracellular matrix has a critical role in cellular physiology. Focal and fibrillar adhesions have distinct molecular compositions. Focal adhesions characteristically contain integrin αv as well as plaque proteins such as paxillin, vinculin, and FAK, whereas fibrillar adhesions are composed prominently of α5 integrin and tensin.[16] To examine the difference of adhesion between 2-D and 3-D, the immunocytochemistry method was applied to MSCs cultured in 2-D or 3-D plate. In 3-D matrix plate, we found that αv and α5 integrin colocalized to unusual cell-matrix attachments parallel to fibronectin-containing extracellular fibers(Fig. 5) rather than localizing separately to classical focal and fibrillar adhesions, respectively.

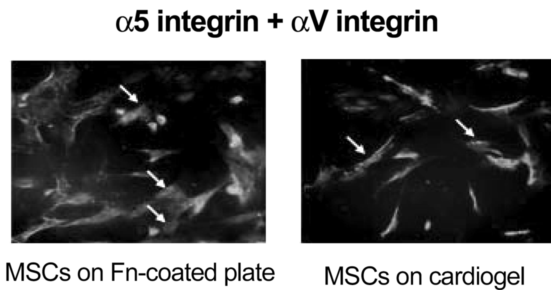

Figure 5. Difference of focal or fibrillar adhesion between Fibronectin- and 3-D matrix plate. Confocal images of indirect immunofluorescence staining of an MSCs on Fn- and 3-D matrix. α5 integrin (green) and αv integrin (red) colocalize in MSCs in 3-D matrix (yellow in merged image indicates overlap of red and green labels), but not on a 2D substrate (Fibronectin).

4. Discussion

The present study demonstrates that the actions of ECM can be attributed to its effect on proliferation of stem cells. The results indicate that expansion and adhesion of MSCs on 3-D matrix, cardiogel is greater than MSCs cultured on plat and fibronectin coated plate.

In general, ECM plays a critical role in organ maintenance, and reconstruction following injury because stem cells which have multi-potential to regenerate injured tissue, reside on the basement membrane in the epithelium and some other tissues. These observations suggest that the basement membrane and/or some other ECMs have an effect on the proliferation of stem cells. This study showed that ECM derived from cardiac fibroblast markedly increased the growth rate and adhesion of MSC(Fig. 3, 4). The mechanism by which 3-D matrix stimulates MSC proliferation and adhesion has not been known but our result give a clue that adhesion between cells and extracellular matrix might be related to expansion and adhesion of MSCs (Fig. 5).

Cell-matrix adhesions the cell surface structures that mediate cell interactions with extracellular matrix (ECM) is composed of focal adhesions and other adhesive structures. Focal adhesions are integrin based structures that mediate strong cell-substrate adhesion and transmit information in a bidirectional manner between extracellular molecules and the cytoplasm.[17-22] A second structure termed the fibrillar adhesion functions in generating extracellular fibrils of fibronectin.[23] It has been known that these structures make an effect on cell adhesion, migration, signaling, and cytoskeletal function. As shown in Figure 5, colocalization of focal (integrin αv) and fibrillar (integrin α5) adhesion in 3-D matrix could make an effect on expansion of MSCs because the only difference of 2-D and 3-D matrix is the localization of focal and fibrillar adhesion.

In conclusion, the ECM had greater effects on the proliferation of MSC. MSCs expanded on the ECM should be useful for regeneration of large tissue defects and repeated cell therapies, which require a large number of stem or progenitor cells. Therefore, we have provided new insight into physiological effect of ECM so a better understanding of the role of ECM in the expansion of MSCs may provide strategies to improve outcome.

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