Original Article

Isolation and Characterization of Synovial Mesenchymal Stem Cells

(chondrocytes / mesenchymal stem cells / synovial membrane / synovial fluid)

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Abstract. Synovial membrane and synovial fluid represent a good source of mesenchymal stem cells. They have been regarded as a promising therapeutic tool for musculoskeletal regeneration. Synovium-derived mesenchymal stem cells have higher expression of CD44 and better chondrogenic potential *in vitro* than mesenchymal stem cells from other tissues. In this study we compared mesenchymal stem cells from synovium and synovial fluid on the base of morphological, immunophenotype and differentiation features. A heterogeneous population of cells with different morphology was obtained after isolation and 4-day cultivation. The mesenchymal stem cell immunophenotype was confirmed by positive expression of CD105, CD90, and CD44 by flow cytometry and cells were negative for CD45. CD105$^+$ cells were selected by immunomagnetic separation after 2–4 weeks of cultivation. The percentage of CD105$^+$ cells in the mesenchymal stem cell population from synovia was between 40–50 % before immunomagnetic separation and increased to 95 % following the immunomagnetic separation. Von Kossa, Alcian blue and Oil Red O staining was used to assess the differentiation potential of synovial mesenchymal stem cells. Long-term cultivation did not affect the morphology and immunophenotype of synovial mesenchymal stem cells. Our results confirmed that immunomagnetic separation based on CD 105 antigen is a suitable method to enrich the subpopulation of CD105$^+$ synovial mesenchymal stem cells.

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Abbreviations: DMEM – Dulbecco's modified Eagle's medium, FACS – fluorescence-activated cell sorting, FBS – foetal bovine serum, MACS® – magnetic-activated cell sorting, MSCs – mesenchymal stem cells, PBS – phosphate-buffered saline solution, SF – synovial fluid, SM – synovial membrane.

Introduction

Mesenchymal stem cells (MSCs) are non-haematopoietic, multipotent progenitor cells capable of differentiating *in vitro* and *in vivo* to mesenchymal lineages, including adipose, bone, cartilage and muscle. MSCs have traditionally been isolated from bone marrow (Friedenstein et al., 1976). MSCs have since been found in many other adult tissues such as skeletal muscles, adipose tissues, synovial membranes (Pittenger et al., 1999; Jo et al., 2007; Jackson et al., 2010). Compared to other sources of MSCs, synovium is the closest tissue to articular cartilage, has a higher chondrogenic capacity and can be harvested easily when clinicians confirm a diagnosis of articular cartilage damage by routine arthroscopic examination without harming otherwise normal tissues (Sakaguchi et al., 2005; Mochizuki et al., 2006). The ability of spontaneous regeneration of articular cartilage is rather low. For this reason the research of healing of articular surface defects centres on the possibility of better repair of damaged cartilage using transplantation of cells (chondrocytes, mesenchymal stem cells) and tissue engineering (Nečas et al., 2010).

Synovium is the only tissue that can produce hyaline cartilage in benign conditions, such as in synovial chondromatosis and osteochondral spurs in osteoarthritis, which suggests that synovial membrane acts as a cell source for articular cartilage repair (Nagase et al., 2008).

Normal synovial tissue consists of two anatomically distinct layers: the surface layer (intima) and the underlying layer (subintima). The intima is loosely organized, avascular and not supported by a basement membrane. The subintima consists of a meshwork of connective tissue interspersed with cells and blood vessels. In the normal intima and subintima, two cell types predominate: macrophage-like (type A) synoviocytes, and fibroblast-like (type B) synoviocytes (Mor et al., 2005). Synovial fibroblast-like cells are involved in the production of specialized matrix constituents including hyaluronan, collagens and fibronectin for the intimal interstitium and synovial fluid (Jones et al., 2004). The proliferative potential of these fibroblast-like cells is much higher than

that of macrophages (Vandenabeele et al., 2003). Synovial fluid may induce the migration of mesenchymal stem cells from synovial tissue to the synovial fluid in the joints of osteoarthritis patients (Zhang et al., 2008).

In the present study, we characterized MSCs isolated from human synovial membrane (SM) and synovial fluid (SF) by using various known cell-surface markers and evaluated their differentiation potential. We show that SM-derived and SF-derived cells can be expanded *in vitro* and can be induced into multilineage differentiation pathways.

Material and Methods

Harvesting of synovial tissue and synovial fluid and isolation of the cells

Random biopsies of SM (1 × 1 cm) were obtained aseptically from the knee joints of cadaver tissue donors within 12 h of death before the processing. The experiments were performed with the approval of the University's ethical committee. SM specimens were placed into the transport medium containing sterile phosphate-buffered saline solution (PBS) (GIBCO BRL, Life Technologies, Carlsbad, CA) supplemented with 1% (v/v) antibiotic/antimycotic solution (10,000 units/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin B; GIBCO BRL). Fragments were digested with 0.1% (v/v) bacterial collagenase type II (GIBCO BRL) in high-glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL) containing 1% (v/v) antibiotic/antimycotic solution. Following overnight incubation at 37 °C and after removing undigested tissue using a 40 μm nylon cell strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ), cells were collected by centrifugation at 150 *g* for 7 minutes at 4 °C, rinsed twice with DMEM and resuspended in complete culture medium containing Alpha MEM medium (Biochrom AG, Berlin, Germany) supplemented with 20% (v/v) foetal bovine serum (FBS)(GIBCO BRL) and 1% (v/v) antibiotic/antimycotic solution.

Samples of synovial fluid (SF) from patients with initial stage of knee joint osteoarthritis were harvested by syringe aspiration before the instillation of intra-articular drugs. Briefly, the synovial fluid was centrifuged at 150 *g* for 7 minutes at 4 °C, and the cell pellet was resuspended in complete culture medium.

Isolated SM- and SF-derived cells were plated into flasks and allowed to become adherent. Following overnight incubation, non-adherent cells were removed by replacement with fresh culture medium and the attached cells were cultured in complete culture medium at 37 °C in a humidified 5% CO_2 atmosphere to reach confluence. When cells reached 80–90% confluence, they were washed with DMEM and detached by incubation with trypsin-EDTA (GIBCO BRL) for 5 min, washed twice with DMEM and then resuspended in complete culture medium at a density of 2.0×10^5 cells/75 cm^2 dish. Subsequent passages were performed in the same way when cells reached confluence.

Immunohistochemistry

SM- and SF-derived cells obtained after two passages were seeded at a density of 2.0×10^4 cells/cm^2 on glass chamber slides (Lab-Tec®, Nalgene Nunc International, Naperville, IL). After 3-day cultivation, the cells were fixed in 4% (w/v) paraformaldehyde (Sigma–Aldrich, Co., St. Louis, MO) in phosphate-buffered saline (PBS), pH 7.6 and stained with monoclonal antibodies mouse anti-human CD45 (R&D Systems Inc., Minneapolis, MN) and mouse anti-human CD105 (Endoglin) (Dako Cytomation, Carpinteria, CA). Cell and Tissue Staining Kit (R&D Systems Inc.) was used for chromogenic detection of CD45 labelling and Dako EnVision+System-HRP (Dako Cytomation) for detection of CD105 labelling.

Immunomagnetic separation of $CD105^+$ cells

The subpopulation of $CD105^+$ cells was separated by magnetic separation (MACS® separator, Miltenyi Biotec, Bergisch Gladbach, Germany) using an anti human-CD105 antibody (Miltenyi Biotec) after three passages. SM- and SF-derived cells were incubated with colloidal MicroBeads coated with CD105 monoclonal antibody for 15 min at 10 °C. $CD105^+$ cells were separated using the MINI MACS System (Miltenyi Biotec), according to the manufacturer's specifications. Cell counts before and after immunomagnetic separations were determined in Burker's chamber. The subpopulation of $CD105^+$ cells was then used for further analysis.

In vitro differentiation of SM- and SF-derived cells

To evaluate multilineage differentiation of SM- and SF-derived cells *in vitro*, Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, Inc.) was used. Differentiation studies were performed with the subpopulation of $CD105^+$ cells. For that, cells were seeded at a density of 2.0×10^4 cells/cm^2 on glass chamber slides (Lab-Tec®). Cells were allowed to become near-confluent and then cultured for two to three weeks in adipogenic, chondrogenic and osteogenic media as recommended by the manufacturer. Cells cultured in complete culture medium were used as a negative control.

Calcium deposits characteristic for osteogenic differentiation in cultures were visualized by von Kossa staining or Alizarin red staining. Alcian blue staining was used to assess the formation of proteoglycans, which confirmed chondrogenic differentiation. Cytoplasmic inclusions of neutral lipids after adipogenic differentiation were stained with Oil Red O.

Flow cytometry

Immunophenotype characterization of SM- and SF-derived cells after five passages was performed by flow cytometry. After detaching cells from the dishes, they

were washed twice with PBS buffer supplemented with 2% (v/v) FBS. Aliquots of 2.0×10^5 cells were incubated with mouse anti-human CD90/RPE and mouse anti-human CD44/RPE for 30 min in the dark. Monoclonal antibodies for flow cytometry were purchased from BD Pharmigen (San Diego, CA). Negative control was determined using equal aliquots of the cells that were not labelled with monoclonal antibodies. Immunophenotype characterization of separated $CD105^+$ cells was also analysed by flow cytometry. Cells were stained with primary antibodies mouse anti-human CD105/RPE (BioLegend, San Diego, CA) and mouse anti-human CD45/FITC (BD Pharmigen) and prepared as previously. Data were analysed in a Becton Dickinson FACSCalibur using CellQuest software (Becton Dickinson).

Results

Cell isolation

The morphology of SM- and SF-derived cells was compared using light microscopy. Non-adherent or few adherent small round cells were present in the primary culture after isolation from synovial membrane and synovial fluid and were discarded after first medium change. SM- and SF-derived cells changed their morphology from two days after the beginning of cultivation. A heterogeneous population of cells with different morphology was observed after 4-day cultivation. Some of the SM-derived cells had oligo- and polydendritic morphology (Fig. 1a). In the case of SF-derived cells, single cell colonies with fibroblastic spindle-like shape occurred; however, large and flat cells were also observed (Fig. 1b). Microscopic examinations showed that the spindle cells proliferated much more rapidly than the round cells under the same culture conditions. Typical fibroblasts became the predominant subtype in the culture after 10 days of cultivation. The cells with round and polydendritic morphology persisted over time in the culture.

Immunohistochemistry

The immunophenotype of the obtained SM- and SF-derived cells after two passages was analysed by immunohistochemistry. Cells immunostained with anti-human CD105 stained strongly brown. Negative control and cells immunostained with anti-human CD45 showed no staining (data not shown).

Immunomagnetic separation of $CD105^+$ cells

The subpopulation of $CD105^+$ cells was separated by immunomagnetic separation from cells pre-adherent to the flasks after 2–4 weeks of cultivation. The percentage of $CD105^+$ cells in the MSC population from SM and SF before immunomagnetic separation was between 40–50 % and increased to 95 % after the immunomagnetic separation. After cultivation of $CD105^+$ cells, cell morphologies became more uniformly spindle-shaped and a confluent cell layer with fibroblastoid morphology was obtained (Fig. 2 a, b).

Differential potential of SM- and SF-derived cells

The subpopulation of $CD105^+$ cells obtained after immunomagnetic separation of SM- and SF-derived cells was able to differentiate into adipose, bone and cartilage tissue when incubated with adipogenic, osteogenic and chondrogenic medium (Fig. 3 a, b, c, d, e, f).

Flow cytometry

Flow cytometric analysis showed that SM- and SF-derived cells after five passages were positive for CD90 and CD44 antigens. Immunomagnetically separated $CD105^+$ cells were positive for CD105 and negative for CD45 antigen. The expression profile of cell surface antigens of SM-derived adherent cells was similar to that of SF-derived cells (Fig. 4 a, b).

Discussion

We investigated the morphological and immunophenotype characteristics of the cultured SM- and SF-derived cells using light microscopy and flow cytometry. A heterogeneous population of SM- and SF-derived cells with different morphology was evident after isolation and four days of cultivation.

In the last three decades, both long passaging culture method and limited dilution method were usually utilized for MSC purification (Djouad et al., 2005). In re-

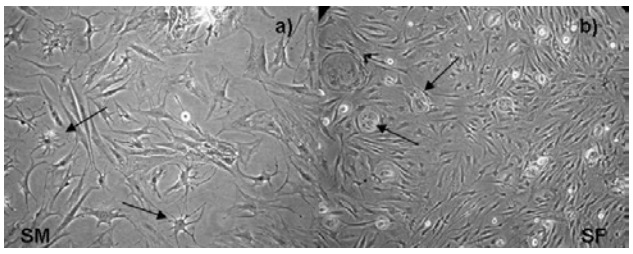

Fig. 1. Light microscopy of a heterogenous population of MSCs isolated from **a**) synovial membrane (SM), 12 days of cultivation, arrows show oligo- and polydendritic morphology of cells (150×); **b**) synovial fluid (SF), 12 days of cultivation, arrows show round morphology of cells (100×).

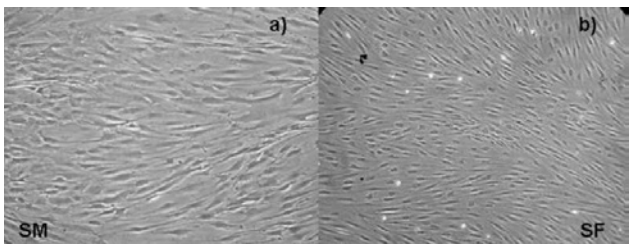

Fig. 2. Light microscopy of immunomagnetically separated $CD105^+$ cells from **a**) synovial membrane (SM) (150×), 16 days of cultivation from separation **b**) synovial fluid (SF) (100×), 16 days of cultivation from separation.

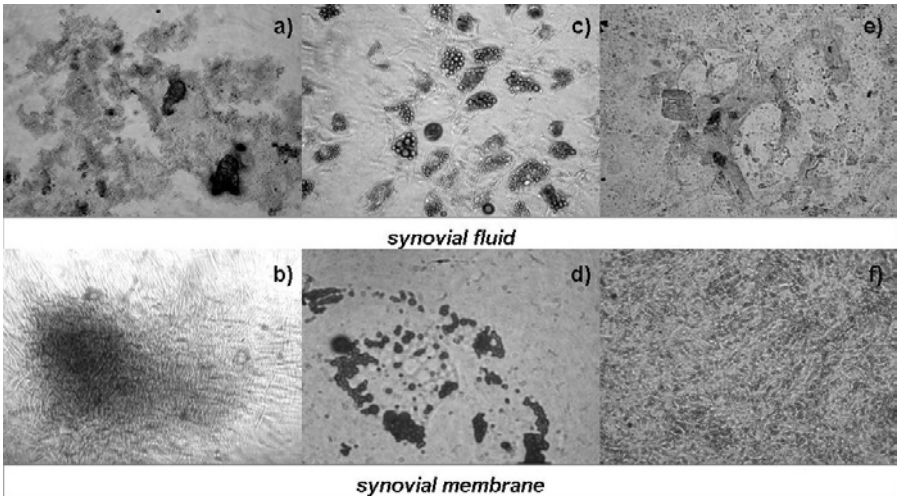

Fig. 3. Differential potential *in vitro* after passage 1 of immunomagneticaly separated CD105^{+} cells from synovial fluid and synovial membrane. Differentiation to osteoblasts was confirmed by **a**) von Kossa staining (×100) and **b**) Alizarin red staining (×100); the presence of adipocytes was demonstrated by **c, d**) Oil red O staining of cytoplasmic inclusions of neutral lipids (×100); chondrocytes were demonstrated by **e, f**) Alcian blue staining (×100).

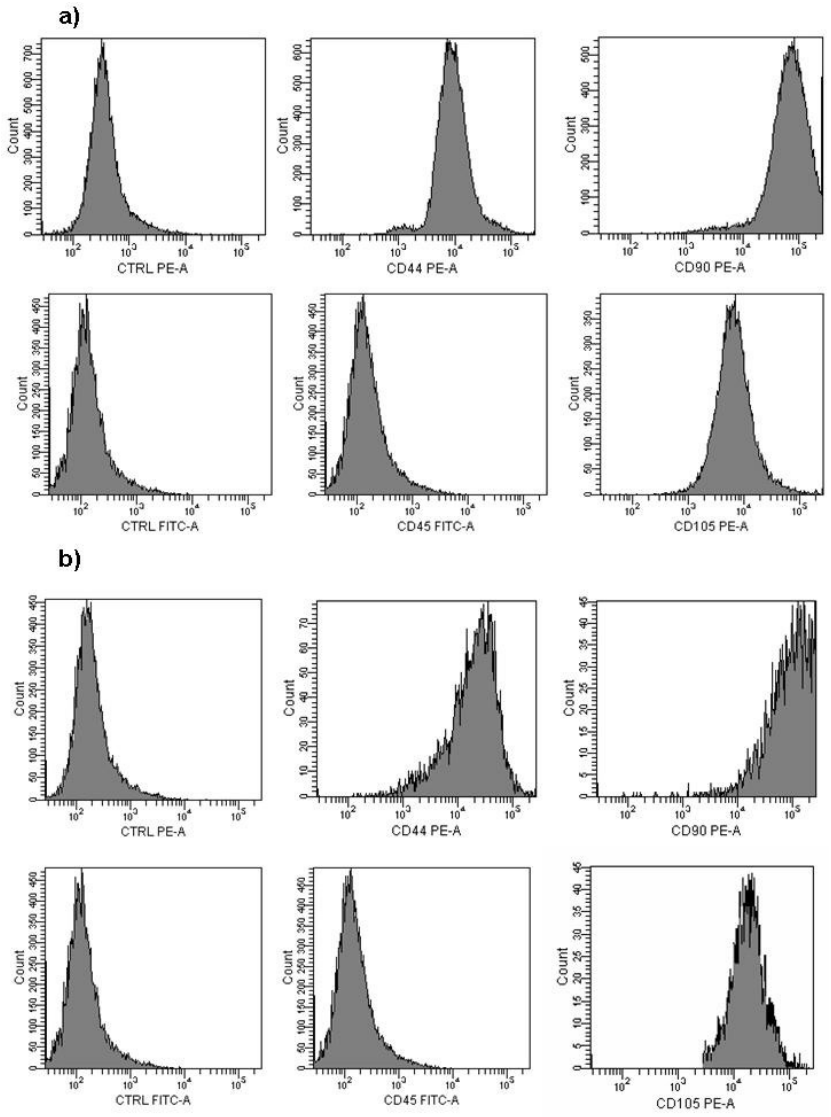

Fig. 4. Flow cytometric analysis of **a**) SM-derived cells after 5th passage **b**) SF-derived cells after 5th passage. The positive cell surface markers were CD44, CD90 and CD105. CD45 was found to be negative.

cent years, novel techniques such as fluorescence-activated cell sorting (FACS) analysis or magnetic-activated cell sorting (MACS®) have been utilized for purifying MSCs based on special cell surface markers (Fickert et al., 2003). It was also demonstrated that positive selection using commercialized anti-endoglin (CD105) antibody makes it possible to obtain homogenous cultures of MSCs without contamination with haematopoietic-derived cells (Goussetis et al., 2005; Arufe et al., 2009). Cell sorting can be performed either from cells pre-adherent to the flasks for a period of time and then subjected to selection or from freshly isolated cells from the synovial tissue.

In our study immunomagnetic separation based on the CD105 marker was used to enrich $CD105^+$ SM- and SF-derived cells. We obtained a homogenous population of $CD105^+$ adherent cells with the typical spindle-shaped fibroblast-like appearance. Immunomagnetic separation represents a suitable method to provide a satisfactory number of cells within a short period of time compared to conventional passaging. Antigen CD105 was shown to bind TGF-β1 and TGF-β3 with high affinity, which may play a role in mediating TGF-β signalling during chondrogenic differentiation of MSCs (Cheifetz et al., 1992; Pei et al., 2008a). In particular, CD105 (SH2, endoglin) and CD90 (Thy-1) are present on MSC surface, but are lost during the developmental progression into differentiated phenotypes (Bruder et al., 1998; Chen et al., 1999). We demonstrated by immunohistochemical analysis that immunomagnetically separated $CD105^+$ SM- and SF-derived cells are capable to differentiate *in vitro* into chondrogenic, osteogenic and adipogenic lineages under specific differentiation culture conditions.

Detection of the expression of CD90 was used to identify the cells as fibroblast-like cells with MSC properties. Our results correspond with many papers which describe that MSCs are negative for haematopoietic marker CD45 and positive for CD44, CD90, CD105 (Dominici et al., 2006; Pei et al., 2008b). We determined that SM- and SF-derived adherent cells had similar cell surface markers and maintained proliferative properties after eight passages. These findings indicate that adherent cells derived from synovial membrane and synovial fluid display typical characteristics of MSCs. There are studies revealing that human MSCs derived from the synovium retain their proliferative ability even after passage 10 (De Bari et al., 2001; Zimmermann et al., 2001).

Our results confirmed that cells isolated from synovial membrane and synovial fluid have characteristics of mesenchymal stem cells and the immunomagnetic separation technique based on CD105 antigen is a suitable method to enrich the subpopulation of $CD105^+$ synovial MSCs. The superiority of synovial tissue as a source of MSCs is that large amounts of cells can be easily obtained from a small part of synovial tissue. Synovial mesenchymal stem cells appear to be a promising cell source for cartilage regeneration.

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