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なし

H. 知的財産権の出願・登録状況

なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表（研究代表者：関矢一郎）

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Ⅳ. 研究成果の刊行物・別刷

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BASIC RESEARCH

Mesenchymal Stem Cells in Synovial Fluid Increase After Meniscus Injury

Yu Matsukura MD, Takeshi Muneta MD, PhD, Kunikazu Tsuji PhD, Hideyuki Koga MD, PhD, Ichiro Sekiya MD, PhD

Received: 19 September 2013 / Accepted: 27 November 2013

Abstract

Background Although relatively uncommon, spontaneous healing from a meniscus injury has been observed even within the avascular area. This may be the result of the existence of mesenchymal stem cells in synovial fluid.

Questions/purposes The purpose of this study was to investigate whether mesenchymal stem cells existed in the synovial fluid of the knee after meniscus injury.

Methods Synovial fluid was obtained from the knees of 22 patients with meniscus injury just before meniscus surgery and from 8 volunteers who had no history of knee injury. The cellular fraction of the synovial fluid was cultured for 14 days followed by analysis for multilineage potential and presentation of surface antigens characteristic of mesenchymal stem cells. Colony-forming efficiency and proliferation potential were also compared between the two groups.

Results Cells with characteristics of mesenchymal stem cells were observed in the synovial fluid of injured knees to a much greater degree than in uninjured knees. The colony-forming cells derived from the synovial fluid of the knee with meniscus injury had multipotentiality and surface epitopes identical to mesenchymal stem cells. The average number of colony formation, obtained from 1 mL of synovial fluid, in meniscus-injured knees was 250, higher than that from healthy volunteers, which was 0.5 ($p < 0.001$). Total colony number per synovial fluid volume was positively correlated with the postinjury period ($r = 0.77$, $p < 0.001$).

Conclusions Mesenchymal stem cells were found to exist in synovial fluid from knees after meniscus injury. Mesenchymal stem cells were present in higher numbers in synovial fluid with meniscus injury than in normal knees. Total colony number per synovial fluid volume was positively correlated with the postinjury period.

Clinical Relevance Our current human study and previous animal studies suggest the possibility that mesenchymal stem cells in synovial fluid increase after meniscus injury contributing to spontaneous meniscus healing.

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Each author certifies that his institution approved the human protocol for this investigation that all investigations were conducted in conformity with ethical principles of research, and that informed consent for participation in the study was obtained.

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Introduction

The meniscus plays an important role in knee function and mechanics [24]. Meniscal injuries are a common and important source of knee dysfunction [13]. Meniscal repair is usually considered for the outer third of the meniscus because a rich network of arborizing vessels within the

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peripheral capsular and synovial attachments supplies vascularization to the menisci [7]. The remaining two-thirds of the meniscus have a poor vascular supply and thus a limited ability to heal spontaneously. However, spontaneous healing can be observed at the avascular area even in clinical situations (although relatively uncommon) [25] and in animal studies [3, 4, 16]. One of the possible mechanisms to account for this may be ascribed to the existence of mesenchymal stem cells in synovial fluid.

Mesenchymal stem cells are defined as being derived from mesenchymal tissue and having the functional capacity to self-renew and generate a number of differentiated progeny [2]. These cells participate in tissue homoeostasis, remodeling, and repair by ensuring replacement of mature cells that are lost during the course of physiological turnover, senescence, injury, or disease [1]. There are increasing reports that mesenchymal stem cells can be isolated from various adult mesenchymal tissues including intraarticular components [14, 19, 20, 26]. We previously reported that the number of mesenchymal stem cells in synovial fluid from knees with anterior cruciate ligament (ACL) injury and osteoarthritis was greater than that from healthy knees [17, 22]. Furthermore, the gene profiles of mesenchymal stem cells from synovial fluid were much closer to that of synovium than to that of bone marrow [17, 22].

According to our studies concerning meniscus regeneration in rat and rabbit models, synovium-derived mesenchymal stem cells injected into the knee adhered to the lesion, differentiated into meniscal cells directly or produced trophic support factors, and enhanced meniscus healing and regeneration [8, 10]. In a clinical situation, meniscus injuries have the potential to heal spontaneously, although it depends on the type and location of the lesion [25], raising the possibility that when the meniscus is injured, mesenchymal stem cells mobilize into synovial fluid, increase in number, and function to promote meniscal healing. However, the degree to which these cells may or may not be present in the human knee after meniscus injury has not been determined.

In this study, we investigated whether mesenchymal stem cells existed in synovial fluid of knees with meniscus injury and whether the number of mesenchymal stem cells in synovial fluid increased after meniscal injury in vivo in the human knee.

Materials and Methods

Collection of Synovial Fluid

This study was approved by an institutional review board, and informed consent was obtained from all study subjects. Synovial fluid was obtained from the knees of 22 patients with meniscus injury after induction of anesthesia for arthroscopic procedures for suture or partial resection of the injured meniscus in the operating room. These procedures took place at a mean of 12 weeks after injury (range, 2–39 weeks). Synovial fluid was also obtained from 8 healthy volunteers as a control group. Patients with anterior cruciate injury, a severe cartilage defect (International Knee Documentation Committee Grade 2–4), and osteoarthritis (Kellgren-Lawrence Grade 2–4) were eliminated from the study. In the injury group, mean age was 29 (SD, ± 17) years and mean postinjury period was 3.0 (SD, ± 2.3) months. In the control group, mean age was 34 (SD, ± 6) years.

Definition of Mesenchymal Stem Cells

For purposes of our analysis, we defined a mesenchymal stem cell as one that has three characteristics [4]. First, mesenchymal stem cells must be adhered to plastic culture dishes and colony-formed when maintained in standard culture conditions. Second, the cells must express CD73, CD90, and CD105 at high rates and not express CD34 and CD45. Finally, the cells must differentiate into chondrocytes, adipocytes, and calcified tissue using standard in vitro conditions.

Cultures of Colony-forming Cells in Synovial Fluid

Synovial fluid was diluted with three volumes of phosphate-buffered saline (PBS), filtered through a 70-μm nylon filter (Becton Dickinson, Franklin Lakes, NJ, USA) to remove debris, and plated in six culture dishes of 60-cm^2 (Nalge Nunc International, Rochester, NY, USA) in complete culture medium: α-modified essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (Invitrogen). The dishes were incubated at 37° C with 5% humidified CO_2. After 24 hours, the nonadherent cells were washed out with PBS. Fourteen days after initial plating, three dishes were stained with 0.5% crystal violet (Wako, Osaka, Japan) in 4% paraformaldehyde for 10 minutes and the number of colonies was counted. Colonies less than 2 mm in diameter and faintly stained colonies were ignored. The other three dishes were harvested with 0.25% trypsin and 1 mM EDTA (Invitrogen) (Passage 0), and the number of isolated cells was counted. Then the cells were replated at 500 cells/cm^2 in a 145-cm^2 culture dish (Nalge Nunc International) and cultured for 14 days for further analyses (Fig. 1).

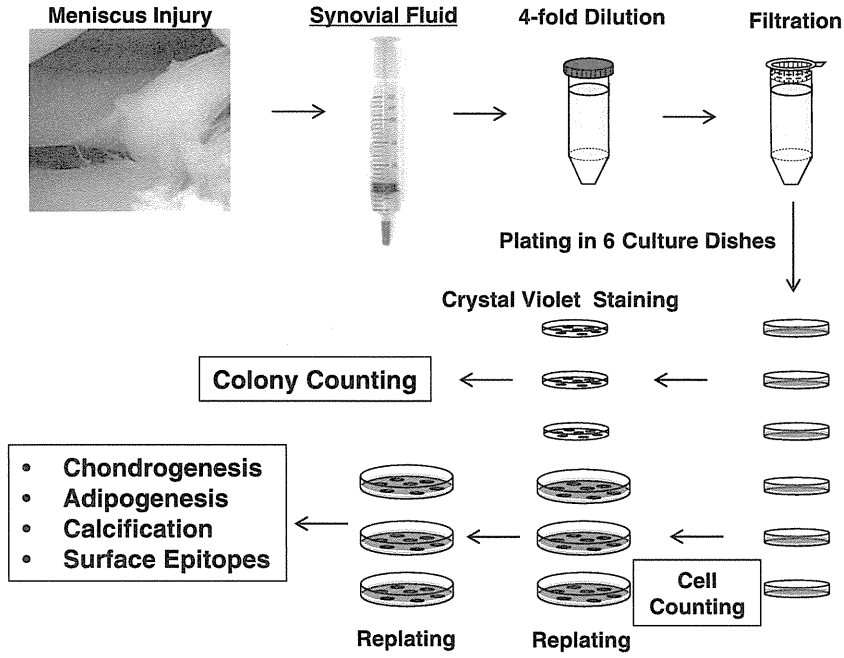

Fig. 1 The study scheme is shown. Synovial fluid was obtained from knees with meniscus injury before surgery. Synovial fluid was diluted, filtered, and plated in six culture dishes. Fourteen days after initial plating, three dishes were stained with crystal violet and the number of colonies was counted. The remaining three dishes were harvested, number of cells counted, then replated and cultured for 14 days for further analyses.

Chondrogenesis

Two hundred thousand cells were placed in a 15-mL polypropylene tube (Becton Dickinson) and centrifuged at 450 x g for 10 minutes. The pellets were cultured at 37° C with 5% CO_2 in 400 µL chondrogenesis medium that contained 1000 ng/mL BMP-7 (Stryker Biotech, Hopkinton, MA, USA) in high-glucose DMEM (Invitrogen) supplemented with 10 ng/mL transforming growth factor-β3 (R&D Systems, Minneapolis, MN, USA), 100 nM dexamethasone, 50 ng/mL ascorbate-2-phosphate, 40 µg/mL proline, 100 µg/mL pyruvate (Sigma-Aldrich, St Louis, MO, USA), and 50 mg/mL ITS + Premix (Becton Dickinson). The medium was replaced every 3 to 4 days for 21 days. For microscopy, the pellets were embedded in paraffin, cut into 5-µm sections, and stained with toluidine blue [23].

Adipogenesis

One hundred cells were plated in 60-cm^2 dishes and cultured in complete medium for 14 days. The medium was then switched to adipogenesis medium that consisted of complete medium supplemented with 100 nM dexamethasone, 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), and 50 nM indomethacin (Wako) for an additional 21 days. The adipogenic cultures were fixed in 10% formalin and stained with fresh Oil Red-O (Sigma-Aldrich) solution [21].

Calcification

One hundred cells were plated in 60-cm^2 dishes and cultured in complete medium for 14 days. The medium was switched to calcification medium that consisted of complete medium supplemented with 1 nM dexamethasone (Sigma-Aldrich), 20 mM β-glycerol phosphate (Wako), and 50 µg/mL ascorbate-2-phosphate for an additional 21 days. These dishes were fixed in 10% formalin in PBS and stained with 40 mM alizarin red solution (pH 4.1; Sigma-Aldrich) [18].

Epitope Profile

One million cells at Passage 2 were suspended in 500 µL PBS containing 20 µg/mL antibody. After incubation for 60 minutes at 4° C, the cells were washed with PBS and resuspended in 1 mL PBS for flow cytometric analysis. Fluorescein isothiocyanate (FITC), phycoerythrin-Cy7 (PE-Cy7), peridinin chlorophyll protein-Cy5.5 (PerCP-Cy5.5), or Allophycocyanin-H7 (APC-H7)-coupled antibodies against CD34, CD44, CD45, CD73, CD90, and CD105 (Becton Dickinson) were used. For isotype controls, FITC-, PE-Cy7-, PerCP-Cy5.5-, or APC-H7-coupled nonspecific mouse immunoglobulin G (IgG; Becton Dickinson) was substituted for the primary antibody. Cell fluorescence was evaluated by flow cytometry using a

FACSVerse instrument (Becton Dickinson). The data were analyzed using FACSuite software (Becton Dickinson).

Statistical Analysis

The Mann-Whitney U test was used for comparison of mesenchymal stem cell number and colony number between the control group and injury group. Probability values < 0.05 were considered significant. The correlation analysis was used for analyzing the relationship of the postinjury period and total colony number per volume.

Results

Analyses of Colony-forming Cells in Synovial Fluid

The colony-forming cells in synovial fluid obtained from the knees with meniscus injury had characteristics of mesenchymal stem cells. A large number of colonies composed of spindle-shaped cells was observed after 14 days' culture of cell components in the synovial fluid of the knees with meniscus injury (Fig. 2). The colony-forming cells differentiated into chondrocytes, adipocyte, and calcified tissue when cultured in differentiation medium (Fig. 3). Three populations of colony-forming cells expressed CD44, CD73, and CD90 at high rates; CD105 at a moderate rate; and did not express CD34 or CD45 (Fig. 4). The colony-forming cells from uninjured knees could not be analyzed for multidifferentiation ability and surface epitopes because isolated cells were very few in number, as shown in the next section.

Colony Formation of Synovial Fluid Mesenchymal Stem Cells in Meniscus Injury

More colonies with traits of mesenchymal stem cells were observed in the knees with meniscus injuries than in the uninjured knees. The total colony number per synovial fluid was 253 ± 262 (mean $\pm$ SD) mL^{-1} in the meniscus injury group and 0.5 ± 0.9 (mean $\pm$ SD) mL^{-1} in the control group (Fig. 5). The number of mesenchymal stem cells per synovial fluid was $350 \pm 370 \times 10^3$ (mean $\pm$ SD) mL^{-1} in the meniscus injury group and $3.9 \pm 6.7 \times 10^3$ (mean $\pm$ SD) mL^{-1} in the control group. The number of colonies and total mesenchymal stem cells per synovial fluid were higher in the meniscus injury group than in the control group ($p < 0.001$). Colony number per synovial fluid (mL^{-1}) was positively correlated with the postinjury period ($r = 0.773$, $p < 0.001$) (Fig. 6). The longer time passed from the injury, the more colonies of mesenchymal stem cells were observed in synovial fluid.

Discussion

Meniscal injuries are common and important causes of knee dysfunction. The meniscus has a limited ability to heal spontaneously in the avascular area. However, although relatively uncommon, spontaneous healing from a

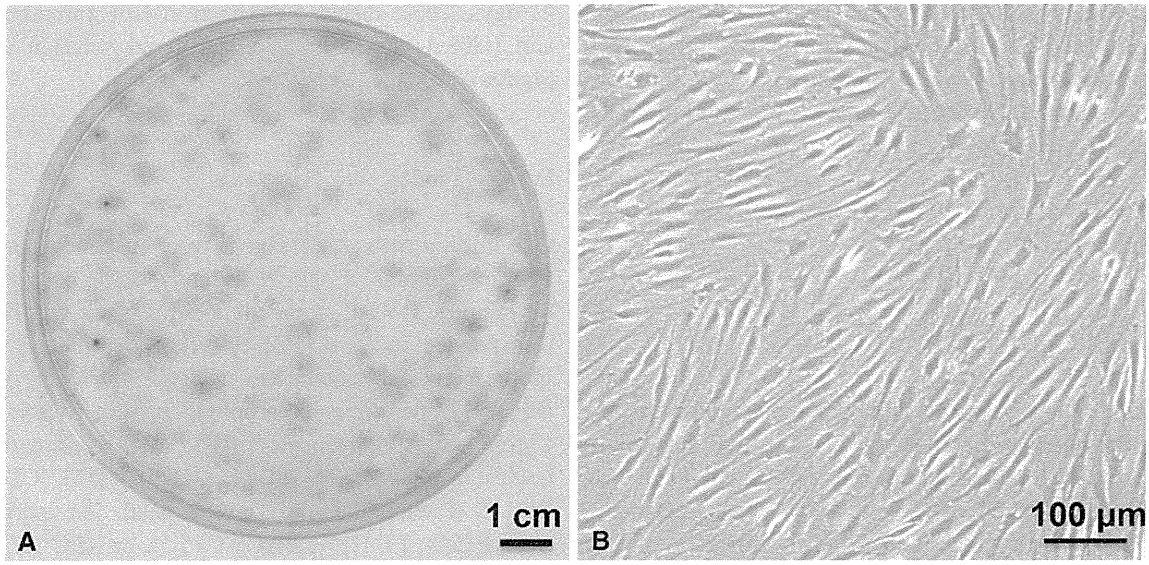

Fig. 2A–B Colony-forming cells in synovial fluid derived from a meniscus injury to the knee are shown. (**A**) Representative cell colonies stained with crystal violet are shown. (**B**) Morphology of colony-forming cells is spindle-shaped (original magnification, ×100).

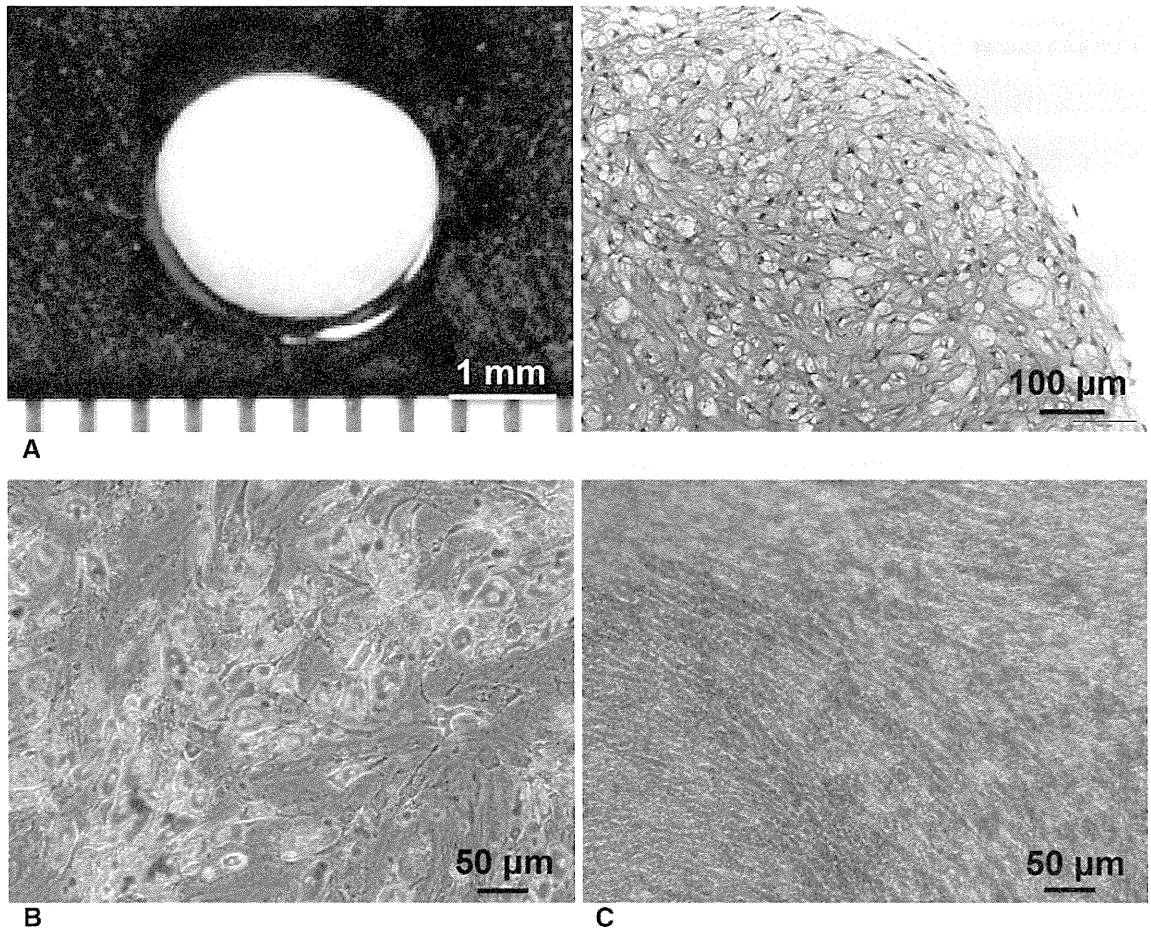

Fig. 3A–C Colony-forming cells in synovial fluids have multipotentiality. (**A**) Cartilage and its histology are shown (Stain, toluidine blue; original magnification, ×100). (**B**) Adipocytes are shown (Stain, oil red-o; original magnification, ×200). (**C**) Calcified tissue is shown (Stain, alizarin red; original magnification, ×200).

meniscus injury has been observed even within the avascular area. This may be the result of the existence of mesenchymal stem cells in synovial fluid. However, the degree to which these cells may or may not be present in the human knee after meniscus injury had not been determined. Therefore, we investigate whether mesenchymal stem cells existed in the synovial fluid of the knee after meniscus injury.

This study had several limitations. The first limitation relates to the small cell number available in synovial fluid from uninjured knees. We could not compare the colony-forming ability in the same density of nucleated cells in the two groups. Initial cell density may affect the colony-forming ratio, but in this study, it was difficult to incubate two populations of the cells at similar densities because there were very few cells in synovial fluid in the uninjured knees. Also, the colony-forming cells from uninjured knees could not be analyzed for multipotentiality and surface epitopes because of the same reason. The second limitation is related to surface markers we examined. The International Society for Cellular Therapy proposed a criteria to define human mesenchymal stem cells, describing them as positive for CD105, CD73, and CD90 and negative for CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR [2]. In this study, we examined all positive markers recommended. However, we examined only two negative markers among five recommended negative markers. To save the effort and expense, we carefully selected two negative markers that we thought to be the most promising. The third limitation is related to heterogeneity. We demonstrated that mesenchymal stem cell number per synovial fluid was higher in the meniscus injury group, and the majority of the cells were identical to the definition of mesenchymal stem cells in colony-forming, surface epitopes and multipotentiality. However, mesenchymal stem cells we termed here might contain other cells including adherent hematopoietic cells and just fibroblasts, although their number would be quite low because they do not form cell colonies like mesenchymal stem cells.

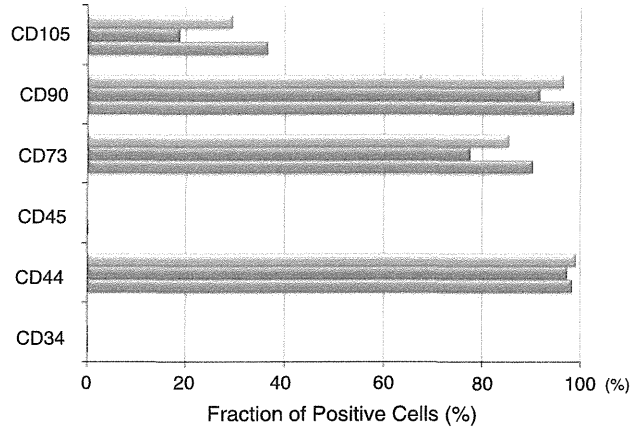

Fig. 4 Surface epitopes of colony-forming cells in synovial fluids obtained from three donors are shown.

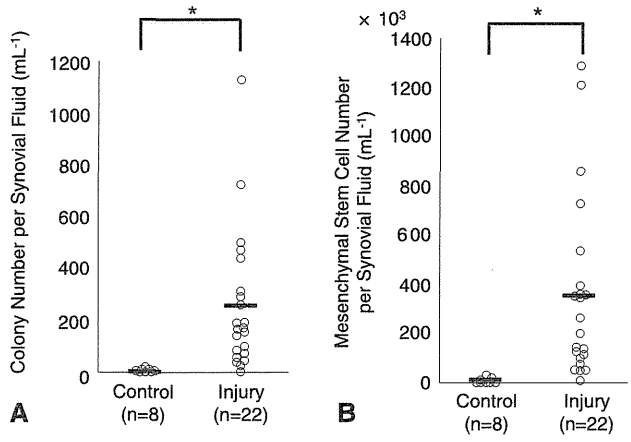

Fig. 5A–B Quantification of mesenchymal stem cells derived from synovial fluid is shown. **(A)** Total colony number per volume (mL^{-1}) of synovial fluid from healthy volunteers (control) and patients with meniscus injuries. Average values are shown as bars (n = 8 in control, n = 22 in injury, *p < 0.001 by Mann-Whitney U test). **(B)** Number of synovial fluid-derived mesenchymal stem cells per volume (mL^{-1}) of synovial fluid (n = 8 in control, n = 22 in injury, *p < 0.001 by Mann-Whitney U test).

In this study, we demonstrated that mesenchymal stem cells existed in the synovial fluid of knees with meniscus injury. The cells studied in this article adhered to the culture dish, were spindle-shaped, formed cell colonies, and differentiated into chondrocytes, adipocytes, and calcified tissue. Also, the cells expressed CD44, CD73, and CD90 at a high rate; CD105 at a moderate rate; and did not express CD34 or CD45. These are consistent with the definition of mesenchymal stem cells [2].

There have been several papers describing mesenchymal stem cells derived from synovial fluid in the knee after ACL injury [17], osteoarthritis [11, 12, 15, 22], and rheumatoid arthritis [12]. Jones et al. [12] observed mesenchymal stem cells in synovial fluid in knees with

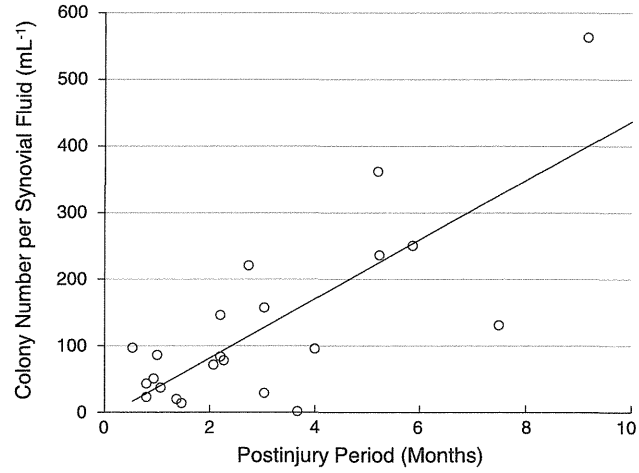

Fig. 6 Colony number per synovial fluid (mL^{-1}) was positively correlated with postinjury period (n = 22, r = 0.773, p < 0.001).

meniscus injury. They compared mesenchymal stem cells in synovial fluid between the osteoarthritis group and the nonosteoarthritis group. This nonosteoarthritis group included meniscal injuries diagnosed by arthroscopy for otherwise unexplained joint pain, although our study is the first report that focused on mesenchymal stem cells in the synovial fluid in knees solely with injury to the meniscus.

The number of mesenchymal stem cells in synovial fluid was higher in the knees with meniscus injury than in healthy knees and increased during the postinjury period. We propose two possible mechanisms, one at the early phase and one at the late phase after injury.

Early on, bleeding in the knee can trigger an increase in mesenchymal stem cells in the knee. When the meniscus is injured, vessels in and around the meniscus rupture at the vascular area and bleed; consequently, the synovial fluid contains blood for several days. Vessel injury and bleeding promote the expression of cytokines and chemokines and consequently recruit mesenchymal stem cells. Generally, within a few days after meniscus injury, synovial fluid is bloody and then becomes transparent thereafter. In this study, the shortest period from the onset of meniscus injury to the time of synovial fluid aspiration was 14 days and the average period was 90 days. Most synovial fluid appeared transparent, indicating that intraarticular bleeding can trigger an increase of mesenchymal stem cells in synovial fluid only at the early postinjury phase. Inflammation may affect mesenchymal stem cells in synovial fluid. In our study, the amount of synovial fluid aspirated was 3.2 mL on average, and the synovial fluid appeared largely transparent, indicating a low number of inflammatory cells. Also, blood tests for C-reactive protein and erythrocyte sedimentation rate were within the normal range in all patients at that time (data not shown). Inflammation may increase the number of mesenchymal stem cells in synovial

fluid at the early phase but would not maintain the increase of mesenchymal stem cells in synovial fluid for a long-term period.

Later, uncertain cartilage degeneration along with meniscus injury may cause an increase of mesenchymal stem cells in synovial fluid. In this study, all injured menisci were indicated for operations as a result of instability and in some cases locking had occurred. Several meta-analyses have demonstrated that an unstable meniscus causes osteoarthritis [5, 6, 9]. In this study, patients with severe cartilage injury or osteoarthritis were excluded; however, cartilage may degenerate after meniscus injury. According to our earlier work, the number of mesenchymal stem cells in synovial fluid was directly correlated to cartilage degeneration, evaluated with arthroscopy in ACL-injured knees, and the number of mesenchymal stem cells in synovial fluid increased along with the radiological grading of osteoarthritis [22]. If synovial fluid could be collected sequentially from immediately after meniscus injury, the number of mesenchymal stem cells in synovial fluid may transiently increase at an early phase because of bleedings in the knee, decrease after withdrawal of bleeding, then continue to increase along with cartilage degeneration resulting from dysfunction of the meniscus.

We postulate that mesenchymal stem cells participate in tissue repair. We previously reported that intraarticular injection of synovium-derived mesenchymal stem cells enhanced meniscus healing and regeneration in rat [10] and rabbit models [8]. Therefore, mesenchymal stem cells appear to promote meniscal healing. However, there may be too few mesenchymal stem cells in synovial fluid for menisci to heal spontaneously in most cases. We speculate that if mesenchymal stem cells in synovial fluid could be increased through an intervention, this may promote meniscus healing. This will call for future study.

In conclusion, we found that mesenchymal stem cells exist in synovial fluid in knees with meniscus injury. More mesenchymal stem cells were present in the synovial fluid of knees with meniscus injury than in normal knees. The number of colonies from synovial fluid was positively correlated with the postinjury period. Mesenchymal stem cells in synovial fluid may have a function to enhance spontaneous meniscal healing. Therefore, in future studies we will seek to clarify a mechanism for increasing mesenchymal stem cells in synovial fluid after injury, and we will try to determine whether this results in increased healing of meniscal tears.

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Transplantation of aggregates of synovial mesenchymal stem cells regenerates meniscus more effectively in a rat massive meniscal defect

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ABSTRACT

Transplantation of mesenchymal stem cells (MSCs) derived from synovium is a possible therapy for meniscus regeneration. We have previously reported that intraarticular injection of 5 million synovial MSCs promoted meniscal regeneration in rat meniscal defects. However, if a similar cell number per body weight were required, preparation of required human MSCs would not be practical in a clinical situation. The use of aggregates of MSCs may be one of the solutions. Here, we investigated whether the use of aggregates of synovial MSCs regenerated meniscus more effectively in a rat meniscectomized model. The total number of synovial MSCs was adjusted to 25,000 cells, and aggregates consisting of MSCs or 25,000 MSCs suspended in PBS were placed on the meniscal defects. Five million MSCs suspended in PBS were also used as another control. For the regenerated menisci, the area was larger and the histological findings were closer to that of the normal meniscus in the aggregate groups than to that in the suspension groups at 4 weeks. The effects of transplantation of aggregates were still observed at 12 weeks. Luminescence intensity remained higher at 3 weeks and thereafter in the aggregate group than in the suspension group when the same number of luciferase expressing MSCs were transplanted. We confirmed that MSCs transplanted as aggregates existed in the regenerated meniscus focally and partially. Transplantation of aggregates of synovial MSCs regenerated meniscus more effectively in a rat massive meniscal defect.

1. Introduction

The meniscus mainly acts like a shock absorber in the knee joint [1]. For meniscus injury, efforts have been made to preserve the meniscus as much as possible to prevent degenerative arthritis. For meniscal defects after meniscectomy or meniscal degeneration, transplantations of meniscal graft or artificial meniscus have been attempted, however, the invasiveness, durability and safety of the transplant remain controversial [2]. A novel strategy is required for meniscus regeneration.

Mesenchymal stem cells (MSCs), especially those derived from synovium, are an attractive cell source for meniscus regeneration, because synovial MSCs have remarkable proliferation and chondrogenic potentials [3]. We previously reported that intraarticular injection of 5 million synovial MSCs in rat models promoted meniscal regeneration [4] and injection of fewer cells did not [5]. Simply, when the body weights of rats and humans are compared, 100 times more synovial MSCs are required in a clinical situation. However, preparation of 500 million human synovial MSCs is not practical. To address this problem, another strategy is needed.

The use of aggregates of synovial MSCs may be one of the solutions because aggregation of synovial MSCs increased chondrogenesis- and antiinflammatory-related gene expressions [6]. The purpose of this study was to investigate whether the use of aggregates of synovial MSCs regenerated meniscus more effectively in comparison with the use of suspension of synovial MSCs when the same number of the cells was used in a rat massive meniscectomized model. The number of synovial MSCs was adjusted to 25,000 and the effects of the "number per aggregate" and the "number of aggregates" were also examined from viewpoints of meniscal regeneration.

2. Materials and methods

2.1. Isolation and culture of rat synovial MSCs

This study was approved by the Animal Experimentation Committee of Tokyo Medical and Dental University. Synovial

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membranes of bilateral knee joints were harvested from wild type male Lewis rats (Charles River Laboratories Japan, Kanagawa, Japan) and transgenic rats expressing luciferase [7], LacZ or GFP at 12–14 weeks. The minced synovial membranes were digested for 3 h at 37 °C with type V collagenase (0.2%; Sigma–Aldrich, St. Louis, MO), and passed through a 45-μm filter (Becton Dickinson, Franklin Lakes, NJ). The digested cells were cultured in a complete culture medium (αMEM; Invitrogen, Carlsbad, CA; 10% FBS; Invitrogen; penicillin, streptomycin and amphotericin B; Invitrogen) in a normoxic condition for 14 days. The cells were then trypsinized, replated and cultured at 70% subconfluency. The characteristics of the MSCs were previously demonstrated [3,4].

For cell tracking, a fluorescent lipophilic tracer DiI (Molecular Probes, Eugene, OR) was used. The cells were suspended at 1×10^6 cells/ml in αMEM without FBS, and DiI was added at a final concentration of 5 μl/ml. After incubation for 20 min at 37 °C, the cells were washed twice.

2.2. Preparation of aggregates of synovial MSCs

500, 5000 or 25,000 synovial MSCs were suspended in 30 μl complete culture medium, and plated on an inverted culture dish lid. Then, the lid was inverted, and placed on a culture dish (Fig. 1A). The cells were cultured at 37 °C with 5% humidified CO_2 for 3 days in hanging drops.

2.3. Meniscectomy

Wild-type male Lewis rats at 12–14 weeks old were used. A straight incision was made on the anterior side of the bilateral knees, and the anterior insertional ligament of the medial meniscus was transected. Then, the anterior half of the medial meniscus was dislocated and resected at the level of the medial collateral ligament.

2.4. Transplantation of aggregates

The total cell number for transplantation per each knee was adjusted to 25,000 cells. "500 cells × 50 aggregates", "5000 cells × 5 aggregates" and "25,000 × 1 aggregate" were placed on the sites of meniscus defects. The suspension of 25,000 cells or 5,000,000 cells was injected into the knee joint in the suspension groups. No cells were transplanted in the untreated group. The rats were allowed to walk freely in each group, and they were sacrificed at 4 weeks ($n = 8$) and 12 weeks ($n = 4$).

2.5. Macroscopic observation

Meniscus was carefully separated from femoral and tibial condyles at 4 and 12 weeks after transplantation. Tibial condyles were stained with India ink to identify location, size and severity of cartilage degeneration. Macroscopic pictures were taken using an Olympus MVX 10 (Olympus, Tokyo, Japan). Quantification for the size of regenerated meniscus was performed using the software Image J (National Institutes of Health, Bethesda, Maryland).

2.6. Histological examination

Meniscus tissue was fixed in 4% paraformaldehyde for 7 days, decalcified in 20% ethylene–diamine–tetra-acetic acid (EDTA) solution for 7 days, respectively and embedded in paraffin wax. The specimens were sectioned in a sagittal plane at 5 μm and stained with safranin-o and fast green. Histological sections were visualized using an Olympus BX 53 microscope (Olympus, Tokyo, Japan). Regenerated meniscus was evaluated using the modified Pauli's score, in which a full score was 18 and a lower score indicated values closer to the normal meniscus [8].

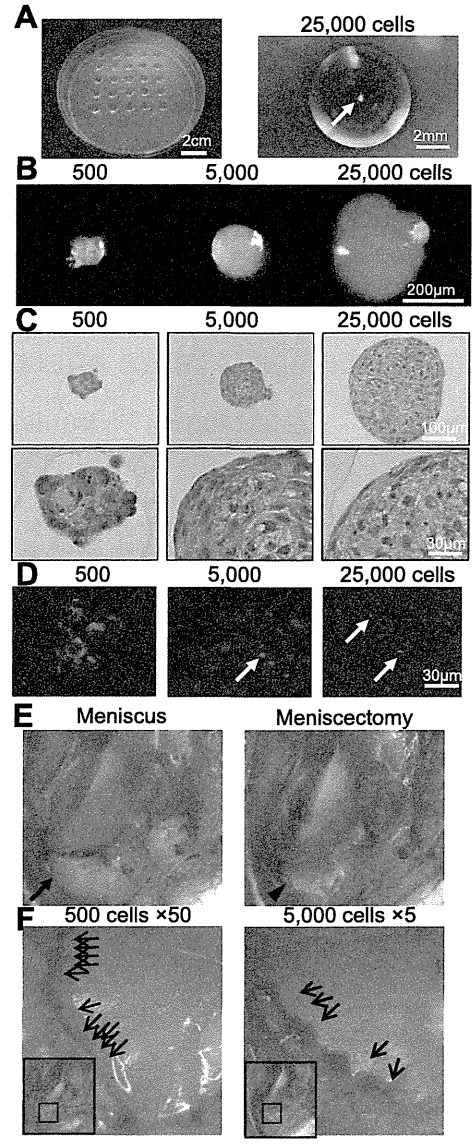

Fig. 1. Preparation, appearance and transplantation of aggregates of rat synovial MSCs. (A) Drops hanging on the cover of dish. Aggregates consisting of 25,000 MSCs are indicated with arrow. (B) Macroscopic images of aggregates consisting of 500, 5000 and 25,000 MSCs 3 days after cultured in hanging drops. (C) Histologies of aggregates stained with hematoxylin and eosin. (D) Aggregates stained with TUNEL (green) and Hoechst (blue). (E) Procedure for removal of meniscus. The medial meniscus (arrow) was exposed, and half of the meniscus was removed (arrow head). (F) Transplantation of aggregates of MSCs on the site of meniscal defects. MSCs were labeled with DiI, and the aggregates are indicated with arrows. (For interpretation of color in this figure legend, the reader is referred to the web version of this article).

2.7. Immunohistochemistry

Paraffin-embedded sections were deparaffinized in xylene and pretreated with 0.4 mg/ml proteinase K (DAKO, Carpinteria, CA) in Tris–HCl buffer for 15 min. Endogenous peroxidases were quenched by using 0.3% hydrogen peroxidase in methanol for 15 min. Nonspecific antigen was blocked by preincubation with PBS containing 10% normal horse serum (Vector Laboratories, Burlingame, CA) or rabbit serum (Vector Laboratories) for 20 min. A primary anti-rat monoclonal antibody against human type II collagen (1:200 in dilution, Daiichi Fine Chemical, Toyama, Japan) or a primary anti-rat polyclonal antibody against goat CXCR4 (1:500