Synovial fluid-derived mesenchymal stem cells increase after intra-articular ligament injury in humans

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Objective. The existence of mesenchymal stem cells (MSCs) in SF was previously reported. However, the behaviour and properties of MSCs derived from SF have not been fully elucidated.

Methods. Human SFs were obtained from 19 knee joints with anterior cruciate ligament injury around the time of reconstruction surgery, and from three healthy volunteers. SF was plated, cultured and examined for colony-forming number, in vitro differentiation, surface epitopes and gene profiles. Also, rabbit synovium-MSCs were injected into the knee joint in a rabbit partial anterior cruciate ligament defect model, and the injected cells were traced.

Results. SF-MSCs from IA ligament injury patients were 100 times more in number than those from healthy volunteers. Total colony number was positively correlated with post-injury period. No significant differences were observed among the cells derived from SF around the time of the surgery in relation to surface epitopes and differentiation potentials. Cluster analysis of gene profiles demonstrated that SF-MSCs were more similar to synovium MSCs than bone marrow MSCs. In rabbit experiments, the MSCs injected into the knee in which IA ligament was partially defective were observed more on the defected area than on the intact area of the ligament at 24 h.

Conclusion. We demonstrated that SF-MSCs, similar to synovium MSCs, increased in number after IA ligament injury and surgery without partially defective were observed more on the defected area than on the intact area of the ligament at 24 h.

Key words: Synovial fluid, Synovial fluid-mesenchymal stem cells, Synovium mesenchymal stem cells, Bone marrow mesenchymal stem cells, Intra-articular ligament injury, Kinetics.

Introduction

Mesenchymal stem cells (MSCs) can be defined as being derived from mesenchymal tissue and by their functional capacity both to self-renew and to generate a number of differentiated progeny [1]. An increasing number of reports describe various mesenchymal tissue-derived stem cells [2, 3]. Jones et al. [4] reported the existence of MSCs in SF of swollen knee joint suffering from OA and RA. They showed that SF-MSCs existed more in OA than in other arthropathies and suggested their possible role in the pathophysiology of arthritis. However, knowledge of SF-MSCs is still limited.

The anterior cruciate ligament is located in the centre of the knee joint and functions to provide stability to the knee and to minimize stress across the knee joint. A tear to the anterior cruciate ligament results from overstretching of the ligament within the knee. After the initial injury, the knee swells from IA bleeding but the symptoms decrease unless another dislocation occurs. Often, but not always, depending on a person’s activity level, a torn ligament needs to be fixed, and currently reconstruction surgery is the standard treatment [5, 6]. A damaged anterior cruciate ligament can restore function spontaneously in limited cases; however, injured ligament alters its appearance [7] and constitution [8] years after surgery.

Determination of the putative mechanisms of the transition of injured and reconstructed IA ligament is intriguing, and it is possible that the existence of MSCs in the knee joint can explain these mechanisms. In this study, we tried to clarify the kinetics and characteristics of SF-MSCs and to speculate on their role in patients with anterior cruciate ligament injury before and after reconstructive surgery.

Materials and methods

Collection of SFs

The study was approved by an institutional review board, and informed consents were obtained from all study subjects. SFs were obtained from the knee joints of 19 patients with anterior cruciate ligament injury of the knees and from 3 healthy volunteers as a control group. These subjects did not have their knees aspirated nor had they received any drugs within 1 month prior to the aspiration for the analyses. Patients with severe cartilage defect and OA were eliminated for the study. In the injury group, average age was 26 yrs (range 12–45 yrs) and average disease duration was 37 weeks (range 2–200 weeks). In the control group, average age was 33 yrs (range 29–40 yrs). SFs were also collected 1 and 6 days after operation of the anterior cruciate ligament reconstruction [6]. For healthy volunteers and patients before the operation, 15 ml of isotonic saline solution was injected into the knee joint, the knee was moved several times and SF with saline solution was collected. The original volume of SF was recorded without 15 ml of saline solution.

Cultures of colony-forming cells in SFs

SFs were diluted with 4 volumes of PBS, filtered through a 70 µm nylon filter (Becton Dickinson, Franklin Lakes, NJ, USA) to remove debris, and resuspended after centrifugation. One-fourth of the volume of SF solution was used to count nucleated cell numbers, and 4 ml Ficoll–Paque PLUS (Amersham Biosciences, Uppsala, Sweden) was layered beneath 8 ml of SF solution with Hanks balanced salt solution (Invitrogen, Carlsbad, CA, USA) and centrifuged at 400 g for 10 min at room temperature. The mononuclear cell layer was collected from the interface and the number was counted. An additional three-fourths of the volume of SF solution was used to harvest colony-forming cells in SF.
They were plated in six culture dishes of 60 cm² (Nalge Nunc International, Rochester, NY, USA) in complete culture medium: α-modified essential medium (α-MEM; Invitrogen) containing 10% fetal bovine serum (Invitrogen; lot selected for rapid growth of human bone marrow MSCs), 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 CO₂. After 24h, the adherent cells were washed with phosphate buffered saline (PBS), and complete culture medium was changed every 3 or 4 days. Fourteen days after initial plating, the cells in three dishes were harvested with 0.25% trypsin and 1 mM EDTA (Invitrogen) (Passage 0), replated at 500 cells/cm² in a 145-cm² culture dish (Nalge Nunc International) and cultured for 14 days for further analyses. The other three dishes were stained with 0.5% Crystal Violet (Wako, Osaka, Japan) in methanol and 4% paraformaldehyde for 5 min. The dishes were washed twice with distilled water and the number of colonies was counted. Colonies < 2 mm in diameter and faintly stained colonies were ignored.

Isolation and culture of synovium and bone marrow MSCs

Bone marrow was aspirated from the tibia with an 18-gauge needle during the operation. For isolation of bone marrow-MSCs, nucleated cells were separated with a Ficoll density gradient [2, 9]. Synovium was harvested from the inner side of the medial joint capsule with a pituitary rongeur under arthroscopic observation. Collected synovium was digested in a 3 mg/ml collagenase D solution (Roche Diagnostics, Mannheim, Germany) in α-MEM at 37°C for 3 h, and digested cells were filtered through a 70 μm nylon filter. Nucleated cells from bone marrow and synovium were plated in a 60-cm² dish and cultured in a similar method as described earlier.

Epitope profile

One million cells were resuspended in 200 μl PBS containing 20 μg/ml antibody. After incubation for 30 min at 4°C, the cells were washed with PBS and resuspended in 1 ml PBS for fluorescent-activated cell sorter analysis. (FITC-) or phycoerythrin (PE)-coupled antibodies against CD34, CD45, CD 90, CD147 and anti-nerve growth factor receptor (NGFR) antibody were from Becton Dickinson; CD31, CD44, CD54, CD106 (vascular cell adhesion molecule: VCAM-1) and CD117 were from eBioscience (San Diego, CA, USA); CD105 and CD166 (activated leukocyte cell adhesion molecule: ALCAM) were from Ancell (Bayport, MN, USA); Flk-1 (VEGFR2) was from Genzyme-Technne (Minneapolis, MN, USA); CD10 was from DakoCytomation (Copenhagen, Denmark) and anti-iCXC4 was from R&D Systems (Minneapolis, MN, USA). For STRO-1 staining, the cells were incubated for 30 min with an antibody against STRO-1 (mouse IgM; Genzyme-Technne Minneapolis, MN, USA). The cells were then incubated with a secondary antibody (fluorescein-conjugated goat anti-mouse IgM; Vector Laboratories, Burlingame, CA, USA) for 30 min. For D7-FIB staining, the cells were incubated for 30 min with an antibody against D7-FIB (mouse IgG; Serotec). The cells were then incubated with a secondary antibody (fluorescein-conjugated rabbit anti-mouse IgG; Serotec) for 30 min. For recombinitant human receptor (BMPR-1A) staining, the cells were incubated for 30 min with an antibody against BMPR-1A (Genzyme-Technne). The cells were then incubated with a secondary antibody (fluorescein-conjugated rabbit anti-goat IgG; Southern Biotech, Birmingham, AL, USA) for 30 min. For isotype control, FITC- or PE-coupled non-specific mouse immunoglobulin G (IgG; Becton Dickinson) was substituted for the primary antibody. Cell fluorescence was evaluated by flow cytometry using a FACSCalibur instrument (Becton Dickinson). The data were analysed using CellQuest software (Becton Dickinson).

In vitro chondrogenesis

Two hundred thousand cells were placed in a 15-ml polypropylene tube (Becton Dickinson) and centrifuged at 450 g for 10 min. The pellets were cultured at 37°C with 5% CO₂ in 400 μl chondrogenic media that contained 500 ng/ml BMP-2 (Astellas Pharma, Tokyo, Japan) in high-glucose DMEM (Invitrogen) supplemented with 10 ng/ml TGF-β3 (R&D Systems, Minneapolis, MN, USA), 100 nM dexamethasone, 50 μg/ml ascorbate-2-phosphate, 40 μg/ml proline, 100 μg/ml pyruvate (Sigma-Aldrich, St Louis, MO, USA) and 50 μg/ml ITS +Premix. The medium was replaced every 3–4 days for 21 days. For microscopy, the pellets were embedded in paraffin, cut into 5 μm sections and stained with toluidine blue [10–12]. For type II collagen detection, sections were pre-digested with proteinase K enzyme for 15 min. Then, sections were incubated with primary antibodies: mouse monoclonal antibody to human type II collagen (Daichi Fine Chemical, Toyama, Japan) for 1 h at room temperature. Sections were incubated for 30 min with biotinylated secondary antibodies. Immunostaining was detected by Vector ABC kit horse anti-mouse IgG (H+L) (Vector Laboratories). Colour was developed with 3,3'-diaminobenzidine tetrahydrochloride. Counterstaining was performed with Mayer’s haematoxylin.

Adipogenesis in a colony-forming assay

One hundred cells were plated in 60-cm² dishes and cultured in complete medium for 14 days. The medium was then switched to adipogenic medium that consisted of complete medium supplemented with 100 nM dexamethasone, 0.5 mM isobutylmethylxanthine (Sigma-Aldrich) and 50 μM indomethacin (Wako) for an additional 21 days. The adipogenic cultures were fixed in 4% paraformaldehyde and stained with fresh Oil Red-O (Sigma-Aldrich) solution, and the number of Oil Red-O-positive colonies was counted [13]. Colonies < 2 mm in diameter or faint colonies were ignored. The same adipogenic cultures were subsequently stained with Crystal Violet, and the total number of cell colonies was counted.

Calcification in a colony-forming assay

One hundred cells were plated in 60-cm² dishes and cultured in complete media 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 for over 5 min and stained with 40 μM alizarin red solution (pH 4.1; Sigma-Aldrich) for 2 min, and the number of alizarin red-positive colonies was counted [9]. Colonies < 2 mm in diameter or faint colonies were ignored. The same calcification cultures were subsequently stained with Crystal Violet, and the total number of cell colonies was counted.

GeneChip expression analysis

To clarify which SF-MSCs were closer to bone marrow MSCs or synovium MSCs, gene profiles were analysed by gene chips. SF, synovium and bone marrow were collected during the operation. SF was also aspirated 6 days after the operation. After 14 days of initial plating, colony-forming cells (Passage 0) were replated at 500 cells/cm² and cultured for 14 days (Passage 1). Total RNA was prepared from colony-forming cells (Passage 1) using the RNeaquate Kit (Ambion, Austin, TX, USA), according to the manufacturer’s instructions. Human genome-wide gene expression was examined on a total 23 000 genes with the Human Genome U133 Plus 2.0 Array (GeneChip; Affymetrix, Santa Clara, CA, USA). Double-stranded cDNA was synthesized from total RNA, and the cDNA was subjected to in vitro transcription
in the presence of biotinylated nucleoside triphosphates, according to the manufacturer’s protocol (One-Cycle Target Labeling and Control Reagent package). The fluorescence intensity of each probe was quantified by using the GeneChip Analysis Suite 5.0 computer program (Affymetrix). The level of gene expression was determined as the average difference (AD). Specific AD levels were then calculated as percentages of the mean AD level of probe sets for housekeeping genes (actin and GAPDH genes). Further data analysis was performed with GeneSpring GX 7.3.1 (Agilent Technologies, Inc., Santa Clara, CA, USA). Hierarchical clustering analysis was performed with a Pearson correlation as a similarity measurement using an average linkage as a clustering algorithm.

**RT–PCR**

Total RNA was prepared by RNAqueous Kit (Ambion) according to the manufacturer’s instructions. RNA was converted to cDNA and amplified by the Titan One Tube RT–PCR System (Roche Diagnostics, Basel, Switzerland). RT was performed by a 30-min incubation at 50°C, followed by a 2-min incubation at 94°C to inactivate the RT. PCR amplification of the resulting cDNAs was performed under the following conditions: 35 cycles of 94°C for 30 s, 58°C for 45 s, and 68°C for 45 s, in which the 68°C step was increased by 5 s every cycle after 10 cycles. The reaction products were resolved by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide. PCR primers were as follows:

**CHI3L1** (forward) 5'-TGTGAAGCCGTCTCAACAG-3'

**CHI3L1** (reverse) 5'-AAATTCGGCCTTATTCC-3'

(478 bp)

**β-Actin** (forward) 5'-CCAAGGCAACCCGGAAGAT

GAC-3'

**β-Actin** (reverse) 5'-AGGGTACATGTTGTGCAGCCGAC

AC-3' (587 bp).

**Injection of synovium-MSCs into a rabbit knee**

Skeletal mature Japanese white rabbits weighing about 3.1 kg (range 2.9–3.3 kg) were used. Animal care was in strict accordance with the guidelines of the Animal Committee of Tokyo Medical and Dental University. Under anaesthesia, synovium was harvested from the inner side of the medial joint capsule and synovium-MSCs were prepared [14].

The anteromedial side of the anterior cruciate ligament was partially defected. The size of the defect was 2 mm long, 3 mm wide and 1 mm deep. After the joint capsule was closed, 2 x 10⁷ autologous synovium-MSCs labelled with DiI (Molecular Probes, Eugene, OR, USA) in 200 μl PBS were injected into the knee joint. After 24 h, the ligaments with bones were removed and observed by fluorescence [14].

To examine whether MSCs tend to adhere to rough surface of ligament obtained through partial removal of the ligament or smooth surface of ligament untouched, rough surface area, smooth surface area and DiI-positive area were defined. Then, DiI-positive area in rough surface area, rough surface area, DiI-positive area in smooth surface area and smooth surface area were image analysed.

For histological examination, sagittal sections of a 5 μm thickness were obtained from defected ligament and stained with haematoxylin–eosin. For visualization of DiI-labelled cells, nuclei were counterstained by 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI).

**Statistical analysis**

The StatView 5.0 program (SAS Institute, Cary, NC, USA) was used for statistical analyses and P-values < 0.05 were considered to be statistically significant. For surface epitopes and differentiation assays, Wilcoxon signed rank test was used.

**Results**

**Analyses of colony-forming cells in SF**

Cell colonies were observed 14 days after plating SFs (Fig. 1A: upper and middle panels). Most of them were spindle-shaped (Fig. 1A: lower panel). Total colony number in SF from ligament injury patients was 100 times more than those of healthy volunteers (Fig. 1B). Colony-forming ratio of nucleated cells in SF from ligament injury patients was 2.1 ± 7.0%.

In ligament injury patients, duration between the injury and aspiration ranged from 2 to 200 weeks. Total colony number was positively correlated with post-injury period (r = 0.693, P = 0.0008) (Fig. 1C).

SFs were transparent without contamination of blood before operation, bloody at post-operative day 1 possibly due to bleeding by drilling to the bone and became clearer at post-operative day 6 (Fig. 1D). The nucleated cell number in SFs increased at post-operative day 1 and was maintained at post-operative day 6 though contamination of reduced blood (Fig. 1D and E). Surprisingly, there were fewer total colony numbers in SFs at post-operative day 1 when SF was potently affected by drilling to the bone at the operation. Total colony numbers in SFs increased at day 6 though SFs looked clearer (Fig. 1D and F). These indicate that colony-forming cells in SF increase after ligament injury and ligament surgery and that the number of colony-forming cells is not reflected by IA bleeding from bone tunnels after ligament surgery.

**Epitope profiles of colony-forming cells in SF**

In two populations of the cells derived from SFs aspirated before and after the operation, positive rates were <5% for CD31, CD34, CD45, CD106 (VCAM-1), CD117 (c-kit), Flk-1 (VEGF receptor 2), CXCR4, BMPR-1A and NGFR; between 10% and 30% for CD10 and CD166 (ALCAM) and >40% for CD44, CD54, CD90 (Thy-1), CD105 (SH2), CD147, D7-FIB and STRO-1 (Fig. 2A). There were no significant differences in each epitope between two populations. Epitope profiles of the colony-forming cells in SFs were similar to those of MSCs derived from bone marrow [9] and synovium [2, 15].

**Multipotency of colony-forming cells in SF**

Differentiation potentials of the colony-forming cells in SFs aspirated before and after the operation were compared. After chondrogenic induction, both types of cell pellets had a similar spherical and glistening transparent appearance (Fig. 2B). The development of proteoglycans (Fig. 2B) and type II collagen (Fig. 2B) looked similar, and there was no significant difference of pellet weight in both populations (Fig. 2C). After adipocyte induction, the ratio of Oil Red-O-positive colonies was similar in both populations (Fig. 2D and E). After osteogenic induction, the ratio of alizarin red-positive colonies was also similar in both populations (Fig. 2F and G). Colony-forming cells in SFs contained multipotentiality as MSCs, and the ligament surgery did not affect their differentiation potentials.

**Gene profiles of SF-MSCs**

To elucidate the character of SF-MSCs, we compared gene profiles of MSCs derived from bone marrow, synovium and SFs before and 6 days after the operation. Hierarchical cluster analysis demonstrated that gene profiles in both SF-MSCs were more similar to those in synovium MSCs than in bone marrow MSCs (Fig. 3A). Gene profile analysis demonstrated that the expression levels of chitinase 3-like 1 (CHI3L1), aggrecan 1, WNT 1-inducible signalling pathway protein 2 (WISP2), fibrin 1 and S100 calcium-binding protein were extremely low in bone marrow MSCs, and high in synovium- and SF-MSCs. RT–PCR demonstrated that synovium-, SF (pre)- and SF (PO6)-MSCs expressed...
FIG. 1. Analyses of colony-forming cells in SF. SFs were collected from healthy volunteers and patients with anterior cruciate ligament injury of the knees at pre-operation (Pre), post-operative day 1 (PO1d) and post-operative day 6 (PO6d). The SFs were plated, cultured for 14 days, and stained with Crystal Violet. (A) Culture dishes indicated with 1 cm bar. Cell morphology indicated with 50 μm bar. (B) Total colony number per volume (millilitres) of SFs from healthy volunteers and patients with ligament injuries. Data are shown as average ±S.D. (n = 3 in control, n = 19 in injury, P < 0.05 by Mann–Whitney U-test). (C) Correlation coefficient between post-injury period (weeks) and total colony number per volume (millilitres) of SF from patients with ligament injuries (n = 19, r = 0.693, P < 0.001). (D) Appearances of SFs. For only pre-operation, 15 ml saline was injected into the knee joint and collected together. (E) Nucleated cell number per volume (millilitres) of SF (n = 13, *P < 0.05, **P < 0.001 by one-way analysis of variance). (F) Total colony number per volume (millilitres) (n = 13, *P < 0.05 by paired t-test).

CH13L1 mRNA but bone marrow MSCs did not in the other three donors (Fig. 3B). These results indicate that SF-MSCs are more similar to synovium MSCs than to bone marrow MSCs.

**Adhesion of synovium MSCs to injured ligament in a rabbit model**

We speculated that the increased number of SF-MSCs adhered to injured ligament. To examine this hypothesis, DiI-labelled MSCs were injected into the knee joint in which anterior cruciate ligament was partially defective in a rabbit model (Fig. 4A). Since obtaining a sufficient amount of SF-MSCs was difficult in rabbits, we used synovium MSCs [14] in which gene profile was similar to that of SF-MSCs as shown earlier. Macroscopic analysis showed that DiI-labelled MSCs tended to adhere to the rough surface of ligament obtained through partial removal of the ligament (Fig. 4B). Magnified histologies showed that DiI-positive cells existed in the ligament (Fig. 4C and D). Quantitative analysis demonstrated that the ratio of the DiI-positive area to the total area of the rough surface was higher than that of the smooth surface (Fig. 4E). This indicates the possibility that increasing SF-MSCs tend to adhere to injured ligament in humans (Fig. 5).

**Discussion**

We demonstrated that SF contained cells that adhered to the culture dish, formed cell colonies and differentiated into chondrocytes, osteoblasts and adipocytes in vitro. We herein define MSCs as being derived from mesenchymal tissue and by their functional capacity both to self-renew and to generate a number of differentiated progeny [1]. Since the earliest work by Friedenstein [16], the standard assay used to identify MSCs is the colony-forming unit-fibroblast assay that identifies adherent, spindle-shaped cells that proliferate to form colonies. SF-derived cells expressed CD44, CD90, CD105, CD166 and showed low expression levels of CD34 or CD45, which were also identical to the definition of MSC [17]. Therefore, all of the cells studied in this article were called MSCs.

Interestingly, the number of SF-MSCs from IA ligament injury patients was 100 times more than that from healthy donors. Furthermore, the SF-MSC number increased along with post-injury period. Why did IA ligament injury cause MSCs to increase in SF?

IA bleeding can trigger an increase of SF-MSCs. When the ligament tears, vessels in and around the ligament rupture and bleed, and consequently, the SF contains blood for several days. Vessel injuries and bleedings promote expression of cytokines, chemokines and consequently recruit MSCs. Generally, SFs within a few days after IA ligament injury are bloody, and then become transparent thereafter. In this study, we aspirated SFs on average 37 weeks after the ligament injuries, and most SFs appeared transparent, indicating that IA bleeding can trigger an increase of SF-MSCs in the early phase.

Inflammation may affect SF-MSCs. Jones et al. [4] examined SF-MSCs derived from swollen knee joint and described their possible role in the pathophysiology of arthritis. In our study, the amount of SFs from pre-operative patients was ~1 ml or less in most cases, and SF appeared not to be cloudy, indicating a low number of inflammatory cells. Also, blood tests for CRP and ESR were negative at that time (data not shown). Inflammation may increase the number of SF-MSCs in the early phase but would not maintain the increase of SF-MSCs.

The environment of the torn ligaments in the joint may influence the increase of SF-MSCs. Torn anterior cruciate ligaments rarely regain their original strength in the natural course of complete tear [18, 19], therefore reconstruction surgery is required for athletes. However, biological response of injured ligaments will continue for several years after anterior cruciate ligament injuries.
FIG. 3. Gene expression profiles of colony-forming cells derived from SF at pre-operation (Pre) and post-operative day 6 (PO6d), synovium and bone marrow. (A) Hierarchical clustering of colony-forming cells. (B) CHI3L1 expression by RT–PCR.

ligament injuries. By arthroscopical observations, torn ligaments often attach to the mismatched location, and injured ligaments are covered with synovial tissue [7, 20]. On the other hand, the volume of torn ligaments decreases and finally disappears in some cases [7]. Sequential morphological alteration of injured ligament is probably due to cells constituting ligament. Another possibility is that SF-MSCs may interact with injured ligament.

What is the origin of SF-MSCs? We had previously anticipated that SF-MSCs originated from bone marrow. We collected SFs 1 and 6 days after ligament reconstructed surgery. For the surgery, bone tunnels were created into the femur and tibia bones, and bleeding from the bone, which would contain bone marrow cells, flowed into the joints. According to our results, SFs at post-operative day 1 showed more blood and contained more nucleated cells than those at day 6; however, the numbers of SF-MSCs at day 6 were much higher than those at day 1. If the origin of SF-MSCs were from blood [21, 22], then we would have obtained many colonies at post-operative day 1. These suggest that the origin of SF-MSCs is neither from bone marrow nor from circulating MSCs.

We assume that SF-MSCs were derived from synovium, because the morphology and colony size of SF-MSCs seemed to be more similar to synovium MSCs than to bone marrow MSCs. Furthermore, reconstructed ligaments are recovered with synovial tissue in the natural course of healing [23]. Analyses of gene expression profiles demonstrated that SF-MSCs obtained before and after the operations were more closely related to synovium MSCs.
of the ligament, and the ratio of DiI-positive area to the total area of smooth surface of ligament untouched.

FIG. 4. Adhesion of synovium MSCs to the defect of anterior cruciate ligament in a rabbit model. The ligament was partially defected and $2 \times 10^7$ MSCs in 200 μl PBS were injected into the knee joint. The ligament was observed at day 1. (A) Macropscopic feature of the ligament with 1 mm bar. The defect of the ligament is shown by arrow. (B) Injected MSCs labelled with DiI. (C) Magnified feature of injured ligament stained with haematoxylin–eosin with 50 μm bar. (D) DiI positive cells in the injured ligament. The nuclei were counterstained by DAPI with 50 μm bar. (E) The ratio of DiI-positive area to the total area of rough surface of ligament obtained through partial removal of the ligament, and the ratio of DiI-positive area to the total area of smooth surface of ligament untouched.

However, similar gene expression patterns between SF-MSCs and synovium MSCs may well be due to the environmental cues; both tissues are within the knee joint, the environment of which is quite different from that of bone marrow, and are constantly exposed to the same cytokines and growth factors in the knee joint, thus resulting in similar gene expression pattern. It is likely that MSCs that are recruited from bone marrow through systemic circulation will change in gene expression pattern after they are sent to SF in response to the environmental cues in the SF.

Another possibility for the origin of SF-MSCs may be cartilage. The inner space of the knee joint mostly consists of articular cartilage in addition to synovium. There are some papers which describe articular cartilage containing MSCs [24, 25]; however, it is still debatable [26], especially in subjects over 40 yrs [26].

As other possibilities, MSCs may be mobilized from other tissues including ligaments in the joint due to liberation of molecules associated with repair. Further experiments are required to identify the origin of SF-MSCs.

We had previously expected that SF-MSCs might be activated after the operation. Contrary to our expectation, 18 surface epitopes, in vitro chondrogenic, adipogenic and osteogenic differentiation potentials were not altered. Six days may be too short a time to determine the phenotypic alterations of SF-MSCs after the operation.

In order to examine the function of SF-MSCs to the ligament injury, we attempted injection of MSCs into the knee joint in a partial ligament injury model of rabbit. Higher number of injected MSCs was observed in the injured area than in the uninjured area of the ligament, indicating the possibility that increased SF-MSCs adhered to injured ligament in humans. Kanaya et al. [27] injected bone marrow MSCs into the knee in which anterior cruciate ligament was partially resected in rats and demonstrated adhesion of the cells to the injured site and their contribution to the repair. These suggest that SF-MSCs have a possibility to contribute to the repair of torn ligament and to promote healing of operated ligament.

Why are MSCs mobilized to the defected area of the ligament? The injured ligament is able to produce some cytokines/chemokines such as stromal cell-derived factor-1 (SDF-1)/CXCR4 or CX3CL1/CX3CR1 [28, 29] or VEGF [30]. These are shown to induce stem cell homing to injured sites [31–34]. In our case, similar mechanisms are supposed.

This report demonstrated the kinetics, characterizations and possible roles of SF-MSCs of patients with IA ligament injury (Fig. 5). This study will shed light on the elucidation of pathological mechanisms and the advancement of treatments for damaged tissues in joints from the standpoint of MSCs derived from SF.

**Rheumatology key messages**

- MSCs in SF increase after IA ligament is injured.
- MSCs in SF efficiently adhere to the injured ligament.

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