Primary culture of mouse adipose and fibrous synovial fibroblasts under normoxic and hypoxic conditions

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ABSTRACT

Synovial fibroblasts have attracted considerable attention in studies of joint diseases. Although mice are useful and powerful in in vitro and in vivo experiments, primary cultures of mouse synovial fibroblasts are notoriously difficult because the mouse synovial tissues are much smaller and cell cycle arrests can be induced more easily in murine cells than in human cells. Here, we report a precise protocol for the isolation and culture of fibroblasts from mouse adipose and fibrous knee joint synovia. In both adipose and fibrous synovial fibroblasts, proliferation was decreased in addition to a higher rate of cellular senescence under normoxic conditions (20% O₂); however, it was maintained over 20 days with low cellular senescence under hypoxic conditions (3% O₂). The marker gene expression in adipose and fibrous synovial fibroblasts was not markedly altered after a 3-week culture. Both cells displayed similar potencies for chondrogenic, osteogenic, and adipogenic differentiation, and responses to a proinflammatory cytokine. The present method provides a sufficient amount of mouse synovial fibroblasts for in vitro and in vivo experiments in joint biology and the pathophysiology of osteoarthritis and rheumatoid arthritis.

Two leading causes of disability worldwide are osteoarthritis (OA), the most prevalent joint disorder, and rheumatoid arthritis (RA), a common autoimmune disease characterized by persistent synovitis and systemic inflammation. Several studies have been performed to clarify their molecular mechanisms and develop novel therapeutics. Synovial fibroblasts, which are also referred to type B synoviocytes, fibroblast-like synoviocytes and synovium-derived mesenchymal stem cells in previous studies (17), have been the focus of these research fields. In RA, synovial fibroblast invasion causes destruction of cartilage and bone (16, 21). Recent studies have indicated that the synovial fibroblast–chondrocyte interaction plays pivotal roles in joint homeostasis and OA pathology (1, 2, 20, 24). The synovial fibroblast is also one of the potent cell sources for regenerative therapy of joints (18, 26).

In vitro and in vivo experiments using mice are necessary to investigate the physiological and pathological roles of the synovial fibroblasts. However, primary cultures of mouse synovial fibroblasts are notoriously difficult to perform because the mouse synovial tissues are small. Furthermore, cell cycle arrests can be induced more easily in murine cells than in human cells (23). Some studies have used mouse synovial fibroblasts (8, 18, 19); however, this murine cell cycle arrest issue has not been examined to date. A long culture period is necessary to obtain enough mouse synovial fibroblasts, but may also increase their cellular senescence and change their phenotype (33). To address this issue, oxygen concentrations in vitro have been studied intensively.
Previous studies indicate that the oxygen concentration of in vivo joints is hypoxic (7, 22). Moreover, under 3% O₂ concentration, murine cells are resistant to cellular senescence and expressing a human-like senescence-associated secretory phenotype (3, 23). Therefore, in the present study, we employed physiological hypoxic culture, approximately 3% O₂ (7, 22), to expand synovial fibroblasts from small mouse synovial tissues and examined the effects of oxygen concentrations in cell proliferation and cellular senescence.

There are two kinds of synovia: i.e., adipose and fibrous synovial tissues. The adipose synovium, such as infrapatellar fat pads, consists of fibroblasts in the lining layer and abundant adipocytes in the sublining zone, while the fibrous synovium lacks adipose tissues. Because adipose and fibrous synovial tissues are histologically different, fibroblasts derived from adipose synovium (adipose synovial fibroblasts; ASF) and those derived from fibrous synovium (fibrous synovial fibroblasts; FSF) may have different characteristics. A few studies have reported the difference between ASF and FSF in human and canine (11, 27). However, this difference has not been investigated deeply and no reports have compared mouse ASF and FSF.

Here, we report the isolation of fibroblasts from mouse adipose and fibrous knee joint synovia, primary cultures, and characterization. We compared the cell proliferation and cellular senescence of ASF and FSF under normoxic and hypoxic conditions. We also investigated marker gene expression, multipotency in both cells by in vitro experiments, and responses to a proinflammatory cytokine.

MATERIALS AND METHODS

Preparation of primary cells. Adipose and fibrous synovia were resected from knee joints of 8-week-old male C57BL/6 mice (Fig. 1). Those synovia were digested in 2 mg/mL collagenase in high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin–streptomycin (Sigma-Aldrich) at 37°C for 1 h, and then seeded onto 12-well plates. Primary cells from adipose and fibrous synovia were defined as adipose synovial fibroblasts (ASF) and fibrous synovial fibroblasts (FSF). Additionally, primary cells were isolated from inguinal fat, muscle, patellar tendon, and meniscus by collagenase digestion. Primary cells from tendon were defined as tendon-derived cells, including tenocytes and tenon-derived stem cells (15), and those from meniscus were defined as meniscus-derived cells, including fibroblast-like cells and sporadic round cells (31). Bone marrow-derived mesenchymal stem cells were collected by flushing the femur. These cells were cultured with high-glucose DMEM with 10% FBS and 1% penicillin–streptomycin. ASF and FSF were cultured under normoxic (20% O₂, 5% CO₂) or hypoxic (3% O₂, 5% CO₂) conditions using nitrogen gas. The medium was 2-mm deep and changed every 2 or 3 days. Before reaching approximately 60–70% confluence, these cells were detached with 0.25% trypsin-ethylenediaminetetraacetic acid, and seeded at the density of 5.0×10⁴ cells/cm². The number of cells was counted using Countess (Thermo Fisher Scientific, Waltham, MA, USA).

Senescence-associated β-galactosidase (SA-β-gal) staining. SA-β-gal staining was performed with a cellular senescence detection kit (Cell Biosabs, San Diego, CA, USA), following the manufacturer’s instructions. In brief, ASF and FSF at passage 1 (day 7) and day 21 were washed with phosphate-buffered saline (PBS), fixed for 5 min at room temperature in fixing solution, washed with PBS again, and incubated for 6 h at 37°C with a freshly prepared SA-β-gal staining solution.

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis. Total RNA was extracted from cells with TRI reagent (Molecular Research Center, Cincinnati, OH, USA) and RNeasy Mini Kit (Qiagen, Hilden, Germany), and was reverse transcribed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). qRT-PCR analyses were performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO). The amount of target genes was normalized with an endogenous control, β-actin (Actb).

Chondrogenic differentiation. Briefly, 2×10⁵ cells at 14 days under hypoxic differentiation were centrifuged at 400 ×g for 5 min in 96-deep-well polypropylene plates (Evergreen Scientific, Vernon, CA, USA). The pellets were cultured for 21 days under hypoxic conditions in high-glucose DMEM supplemented with 10 ng/mL transforming growth factor β, 10 nM dexamethasone, ascorbate–phosphate, proline, insulin-transferrin–sodium selenite, and 1% penicillin-streptomycin. The medium was changed every 3 days. The pellets were then embedded in paraffin, cut into sections, and stained with Safranin O staining.
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RESULTS

Cultures under hypoxic conditions enhanced cell proliferation of synovial fibroblasts and suppressed cellular senescence

We first performed primary cultures of ASF and FSF under normoxic (20% O₂) and hypoxic conditions (3% O₂). Under the normoxic condition, both cells became larger and flattened at 14 days (Fig. 2A). The proliferation of ASF was arrested at 15–20 days, and the number of FSF barely increased tenfold during 20 days (Fig. 2B). In contrast, the cells maintained a spindle-like shape and high proliferation potency under hypoxic conditions in both cells (Fig. 2C).

Osteogenic differentiation. Briefly, 1.5×10⁵ cells at 14 days under hypoxic differentiation were seeded onto a 12-well plate and cultured for 21 days under normoxic conditions in high-glucose DMEM supplemented with 1 nM dexamethasone, 20 mM glycerol phosphate, and 50 mg/mL ascorbate-2-phosphate. The medium was changed every 3–4 days. The cells were then stained with 0.5% alizarin red solution.

Adipogenic differentiation. Briefly, 5.0×10⁴ cells at 14 days under hypoxic differentiation were seeded onto a 12-well plate and cultured for 7 days under hypoxic conditions in an adipogenesis differentiation kit (Thermo Fisher Scientific). The cells were then stained with oil red-O solution.

IL-1β treatment. Two-step interleukin-1β (IL-1β) treatment was performed as described previously (5). Briefly, 1.0×10⁵ cells at 14 days under hypoxic conditions were seeded onto a 12-well plate. On the following day, the cells were treated with or without 10 ng/mL mouse IL-1β (PROSPEC, Rehobot, Israel) for 24 h, cultured without IL-1β for 24 h, and treated with or without 10 ng/mL mouse IL-1β for an additional 24 h.

Statistical analysis. The statistical significance between groups was determined with one-way analyses of variance using SPSS software (IBM SPSS, Armonk, NY, USA). P-values below 0.05 were considered to be significant.

ALTERATION OF MARKER GENE EXPRESSION

We next examined marker gene expression in ASF and FSF under normoxic and hypoxic conditions. Prg4, coding lubricin responsible for joint lubrication, is a representative marker gene for synovial

Fig. 1 Identification of mouse fibrous and adipose knee joint synovia. The synovia at the suprapatellar pouch were identified as fibrous synovia and that at the infrapatellar fat pads as adipose synovia. Scale bars, 200 μm.
Thy1, also known as CD90, is one of the mesenchymal stem cell markers. mRNA levels of Thy1 were also comparable to that of Prg4, lining layer cells. mRNA levels of Prg4 were similar in both cells under both conditions, and much higher than those in adipose-derived stem cells (ASC) (Fig. 3).
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and were higher than that for bone marrow-derived stem cells (BMSC) (Fig. 3). Sometimes it is difficult to histologically distinguish synovium from tendon and meniscus (28). Therefore, we examined expression of Coll1a1, which is highly expressed in tendon-derived cells (TDC), and Col2a1, which is highly expressed in meniscus-derived cells (MDC) (31). Coll1a1 and Col2a1 expressions in ASF and FSF were lower than that in TDC and that in MDC, respectively (Fig. 3). These data indicated that ASF and FSF have different characteristics from ASC, BMSC, TDC and MDC.

Cell potency
To investigate the multipotency of ASF and FSF cultured under hypoxic conditions, we performed in vitro differentiation experiments using standard protocols; chondrogenic, osteogenic, and adipogenic potential were estimated by safranin O staining, alizarin red staining, and oil red-O staining, respectively. When we induced chondrogenic differentiation in pellet culture, ASF and FSF formed larger pellets compared with adipose-derived stem cells (ASC) and bone marrow-derived stem cells (BMSC) (Fig. 4A). Some parts of ASF and FSF pellets were safranin O-positive, while BMSC pellets were stained uniformly and strongly (Fig. 4A). In osteogenic differentiation, alizarin red staining was most intensive in BMSC, moderate in FSF, slightly weak in ASF, and weakest in ASC (Fig. 4B). In adipogenic differentiation, oil red-O-positive cells were most abundantly observed in ASF, while much lower cells in FSF, muscle-derived stem cells (MuSC), and BMSC (Fig. 4C).

Responses to proinflammatory cytokine
We finally examined responses to proinflammatory cytokines. When synovial fibroblasts are exposed to
DISCUSSION

The present study showed a precise protocol for isolation and culture of fibroblasts from mouse adipose and fibrous knee joint synovia. Under hypoxic conditions, proliferation could be maintained over 20 days and cellular senescence was markedly suppressed. Meanwhile, expression levels of marker genes were not markedly changed in both ASF and FSF at 21 days.

Mouse synovial fibroblasts were used in previous studies (8, 18, 19). Futami et al. precisely reported the identification of infrapatellar fat pads (8), which are identical to adipose synovial tissues in the current study. They further optimized collagenase reac-

a two-step inflammatory stimuli, they show augmented responses to the second stimuli (5). Therefore, we performed a two-step treatment with IL-1β (Fig. 5A; NT, no treatment). We examined expression of Mmp3 and Mmp13, because their expression patterns are different in arthritis and cellular senescence (3, 14). In both cells cultured under hypoxic conditions, mRNA levels of Mmp3 and Mmp13 were increased immediately after the single IL-1β treatment (T1), and decreased to the baseline 48 h later (T2) (Fig. 5B). Immediately after the two-step treatment (T3), Mmp3 expression was enhanced markedly compared with the single treatment in ASF, meanwhile it was similar to that immediately after the single treatment in FSF (Fig. 5B). Mmp13 expression was also significantly increased by the two-step treatment compared with the single treatment (Fig. 5B).

**Fig. 4** Chondrogenic, osteogenic, and adipogenic differentiation of ASF and FSF. (A) Chondrogenic differentiation of ASF, FSF, ASC, and BMSC by pellet culture. Upper and lower panels indicate gross appearance and safranin O staining of pellets, respectively. Scale bars, 1 mm. (B) Osteogenic differentiation of ASF, FSF, ASC, and BMSC determined by alizarin red staining. (C) Adipogenic differentiation of ASF, FSF, muscle-derived stem cells (MuSC), and BMSC determined by oil red-O staining. Scale bars, 100 μm.
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 cellular senescence was markedly suppressed throughout the culture. Mak et al. examined chondrogenic, osteogenic, and adipogenic differentiation of synovial cells isolated from MRL and C57BL/6 mice (18). They employed a hypoxic culture, but did not describe the method of identification of synovial tissues in detail, and did not compare the normoxic and hypoxic conditions (18).

Previous studies experimentally showed that the partial pressure of O2 (PO2) in human synovia is 22.5 mmHg (range 3.2–54.1 mmHg) (22) and the PO2 in mouse synovia is about 29 mmHg (7), which is approximately equivalent to 3.0 to 3.8% O2 concentration. Taken together, these data may suggest that hypoxic conditions, which are similar to physiological O2 concentrations of in vivo synovia, is suitable for culture expansion of synovial fibroblasts. Meanwhile, it is technically difficult to determine accurate PO2 in in vivo synovial tissues, which would change in response to various factors such as the pathological conditions of articular joints (6, 12). Moreover, in vitro pericellular PO2 can be altered according to cell densities, atmosphere, temperature, medium thickness, and time course (25). Consider-

Fig. 5 Responses to proinflammatory cytokine. (A) Scheme showing two-step IL-1β treatment (10 ng/mL). Cells were divided to 4 groups and treated as follows; T0, no treatment (NT) from 0 to 72 h; T1, NT from 0 to 48 h, and IL-1β treatment from 48 to 72 h; T2, IL-1β treatment from 0 to 24 h, and NT from 24 to 72 h; T3, IL-1β treatment from 0 to 24 h, NT from 12 to 24 h, and IL-1β treatment from 48 to 72 h. (B) mRNA levels of Mmp3 and Mmp13 in ASF and FSF after the two-step IL-1β treatment under hypoxic conditions. Bars indicate means. *P < 0.05.
ing these issues, the methods and conditions of cell culture and oxygen concentrations should be determined carefully according to the objectives of each experiment.

In the present study, we harvested primary fibroblasts from mouse adipose and fibrous synovia, and compared their cell proliferation, marker gene expression, multipotency, and responses to IL-1β. Katagiri et al. reported a primary culture of cells isolated from human fibrous synovia and found that the colony number from fibrous synovial tissues was significantly greater than that from adipose ones (11). Sasaki et al. harvested mesenchymal stem cells (MSC) from fibrous synovia, infrapatellar fat pads, inguinal adipose, and bone marrow tissues from six canines and compared their characteristics (27). The MSC from fibrous synovia and infrapatellar fat pads, which are probably equivalent to FSF and ASF in the current study, respectively, had similar potency in colony formation and cell proliferation (27). In chondrogenic differentiation, the MSC from fibrous synovia formed larger pellets than that from infrapatellar fat pads, while no significant differences were observed for the glycosaminoglycan/DNA ratio or the staining intensity for toluidine blue and collagen type II (27). Notably, the MSC from infrapatellar fat pads showed much higher potency for adipogenic differentiation (27). The current data for mouse ASF and FSF are consistent with the previous data from canine cells.

Mouse adipose and fibrous synovia contain various kinds of cells, including fibroblasts, adipocytes, macrophages, and other immune-related cells. The recent single-cell technology indicates unknown subsets for each cell (4, 29, 32). Because the heterogeneity of synovial tissues was not considered in the present protocol, the primary ASF and FSF cells should be characterized in further studies to investigate the specific roles of these cells. Meanwhile, mouse primary synovial fibroblasts can be applied in transplantation experiments using mice. In combination with various kinds of genetically-modified mice, the present protocol for primary cultures of ASF and FSF may be useful in studying the physiological roles of fibrous and adipose synovial tissues.

In conclusion, enough nonsenescent synovial fibroblasts could be obtained from one wild-type normal mouse by primary culture under hypoxic conditions. The primary ASF and FSF may contribute to the elucidation of the roles of mouse fibrous and adipose synovia, and further studies of joint biology and the pathophysiology of OA and RA.

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