**Local fibroblast proliferation but not influx is responsible for synovial hyperplasia
in a murine model of rheumatoid arthritis**

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Abstract

Synovial fibroblasts play crucial roles in inflammation and joint destruction in rheumatoid arthritis (RA). How they accumulate in the RA joints remains unclear. This study was conducted to discern whether cellular influx from the outside of the joints and local proliferation are responsible for synovial fibroblast accumulation in an animal model of RA. We found that synovial fibroblasts were identified as GFP⁺ cells using collagen type I alpha 2 (Col1a2)-GFP transgenic reporter mice. Then, bone marrow transplantation and parabiosis techniques were utilized to study the cellular influx. Irradiated wild-type mice were transplanted with bone marrow from Col1a2-GFP mice. Col1a2-GFP and wild-type mice were conjoined for parabiosis. The transplanted mice and the parabionts were subjected to collagen antibody-induced arthritis (CAIA). We found no GFP⁺ cells in the hyperplastic synovial tissues from the transplanted mice with CAIA and from the wild-type parabionts with CAIA. Furthermore, normal and CAIA synovial tissues from Col1a2-GFP mice and from fluorescent ubiquitination-based cell cycle indicator (Fucci) transgenic mice, in which cells in S/G₂/M phases of the cell cycle express Azami-Green, were studied for Ki67, a cellular proliferation marker, and vimentin, a fibroblast marker, expression. The percentages of Ki67⁺/GFP⁺ and Azami-Green⁺/vimentin⁺ cells in the CAIA synovial tissues were

higher than those in the untreated synovial tissues (34% vs. 0.40% and 19% vs. 0.26%, respectively). These findings indicate that local fibroblast proliferation but not cellular influx is responsible for the synovial hyperplasia in CAIA. Suppression of proliferation of the local synovial fibroblasts should be a promising treatment for RA.

Keywords:

Rheumatoid arthritis; Collagen antibody-induced arthritis; Synovial fibroblast; Synovial hyperplasia; Cellular proliferation; Cellular influx.

Abbreviations;

RA, rheumatoid arthritis; CAIA, collagen antibody-induced arthritis; CIA, collagen-induced arthritis; Col1a2, collagen type I alpha 2; Col2, anti-type II collagen; MSC, mesenchymal stem cell.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that presents as polyarthritis with structural joint destruction [1]. Accumulation of fibroblasts and inflammatory cells results in formation of the pannus, which characterizes the pathology of the RA. Synovial fibroblasts play crucial roles in inflammation and joint destruction in RA [2,3] and its animal models including collagen antibody-induced arthritis (CAIA) [4] and collagen-induced arthritis (CIA) [4–6]. However, how the synovial fibroblasts accumulate in the arthritic synovial tissues remains unclear.

Possibly, the hyperplastic pannus might result from increased proliferation of resident synovial fibroblasts [2,7]. In support of this notion, cultured RA synovial fibroblasts proliferate more vigorously and are less sensitive to apoptotic stimuli than cultured synovial fibroblasts derived from the osteoarthritis joints [8,9]. However, prevalence of proliferating synovial fibroblasts estimated with the Ki67, proliferating cell nuclear antigen (PCNA) and c-myc staining varied among the studies of the RA synovial tissues [10–14].

Alternatively, the synovial fibroblasts might accumulate via influx of non-resident cells from the outside of the joints. Recent studies have reported that bone-marrow-derived mesenchymal cells, circulating fibrocytes, and synovial

fibroblasts can migrate from the outside of the joints in animal models of RA [15–19].

However, it still remains unclear whether and to what extent the local cellular proliferation and the influx of non-resident cells contribute to the synovial fibroblast accumulation in RA and its animal models.

Ki67, a common cellular proliferation marker, is expressed in G₁, S, G₂, and M phases but not in a quiescent (G₀) phase of the cell cycle. It is expressed not only by proliferating cells but by cells being arrested in the G₁ phase and entering the G₀ phase from the G₁ phase [20,21]. In fluorescent ubiquitination-based cell cycle indicator (Fucci) transgenic reporter mice, every cell emits either green fluorescence (Azami-Green) or red fluorescence (Kusabira-Orange) [22,23]. Azami-Green is positive in the S, G₂, M phases while Kusabira-Orange is positive in the G₁ and G₀ phases. Although Azami-Green⁺ cells in these mice do not include the proliferating cells entering the S phase from the G₁ phase, studies of the fluorescence in the Fucci mice and Ki67 expression should collaborate to demonstrate the cellular proliferation precisely.

The present study was conducted to the contribution of the local cellular proliferation and the cellular influx to the synovial hyperplasia. To study the influx of non-resident cells into the arthritic joints, we utilized bone marrow transplantation and

parabiosis techniques. In our previous reports, these techniques have revealed the presence or absence of the cellular influx in multiple disease models including skin fibrosis, pulmonary fibrosis, and liver fibrosis [24–26]. They should also be of use for studying the origin of the synovial fibroblasts in arthritis models.

Material and methods

Mice

Collagen type I alpha 2 (Col1a2)-GFP transgenic mice on a C57BL/6 background were established previously [26]. FucciG₁-#639/FucciS/G₂/M-#474 double transgenic mice on a C57BL/6 background were gifts from A. Miyawaki (RIKEN BioResource Center, Ibaraki, Japan) [22]. Wild-type C57BL/6 (CD45.2+) and B6.SJL (CD45.1+) mice were purchased from Charles River Japan (Kanagawa, Japan) and the Jackson Laboratory (Bar Harbor, ME) respectively. Experiments were performed on 6- to 10-week-old male and female mice. All procedures in the animal experiments were approved by the Institutional Animal Care and Use Committee of TMDU.

Induction of arthritis

CAIA was induced by the administration of 5 mg of Arthrogen-CIA antibodies (Chondrex, Redmond, WA) and lipopolysaccharide (serotype 0111:B4) according to the manufacturer's instruction.

Bone marrow transplantation

Wild-type C57BL/6 or B6.SJL mice was administered a single 10-Gy dose of

whole body irradiation. After 6 hours, 5 million unfractionated bone marrow cells from Colla2-GFP mice were injected intravenously. Bone marrow reconstitution was confirmed with the chimerism of the peripheral blood from the recipients using either real-time PCR analysis quantifying a GFP transgene normalized to an internal control, glyceraldehyde-3-phosphate dehydrogenase gene as described previously [24] or flow cytometric analysis with PE anti-CD45.1 (clone A20, 12-0453; eBioscience, San Diego, CA) and APC anti-CD45.2 (clone 104, 17-0454; eBioscience) antibodies.

Parabiosis

Pairs of age matched male Colla2-GFP mice and wild-type littermates were surgically joined for parabiosis. Cross-circulation was confirmed with the chimerism using the pairs of peripheral blood as described above.

Immunostaining and histological analysis.

Frozen sections of murine knee joints were studied histologically using a confocal microscope. Five random fields of view were observed for each section. Cell counts were assessed in 1 section per joint (1 joint per mouse). The experiments using bone marrow transplantation and parabiosis techniques were performed on 2 sections

per joint (1 joint per mouse). Detailed protocol and materials used are described in Supplementary materials and methods.

Flow cytometric analysis

The peripheral blood and disaggregated knee joint tissues were harvested from mice with CAIA (1 joint per mouse). Detailed protocol and materials used are described in Supplementary materials and methods.

Statistical analysis.

The number of cells was analyzed statistically using unpaired t-test (two-tailed) for comparisons between 2 groups. One-way analysis of variance was used with Bonferroni correction for comparisons between 3 groups.

Results

Accumulation of synovial fibroblasts as GFP+ cells in Col1a2-GFP mice during CAIA

The fibroblasts in Col1a2-GFP transgenic mice that express GFP in collagen type I-producing cells have been identified in other studies as GFP+ cells in the models of wound healing, liver fibrosis, pulmonary fibrosis, and adipose tissue fibrosis [24–28]. To study if the synovial fibroblasts can be identified as GFP+ cells similarly in the same reporter mice, they were subjected to administration of anti-type II collagen (Col2) antibodies. As was reported earlier [29], after the antibody injection (day 0), arthritis peaked day 5–8, persisted until at least day 10, and gradually decreased until day 20. When synovial thickening was maximal (day 10), the joints were collected for histological studies with confocal microscopy.

The untreated Col1a2-GFP mice had relatively few GFP+ cells in the synovial tissues, while the arthritic Col1a2-GFP mice demonstrated frequent GFP+ cells (Fig. 1A). Next, we examined the GFP+ cells for expression of cell-type specific antigens. All GFP+ cells expressed the common fibroblast marker, vimentin (Fig. 1B). They also expressed another fibroblast marker, Hsp47 [30] (Fig. 1C). Thus, the GFP+ cells in the reporter mice were all synovial fibroblasts. In addition, the number of GFP+ cells

reached a maximum and was as abundant as that of leukocytes, CD45+ cells, when the arthritis peaked (day 10) (Fig. 1D). Thus, the synovial fibroblasts as well as the leukocytes accumulated in the hyperplastic synovial tissues in CAIA.

Synovial fibroblast accumulation without influx of bone marrow-derived cells in CAIA

To discern whether bone-marrow-derived cells, including mesenchymal cells and fibrocytes, are responsible for the accumulation of the synovial fibroblasts in the arthritic joints, Col1a2-GFP bone marrow was transplanted into wild-type mice. Two months after the transplantation, successful engraftment was confirmed with chimerism (> 87%) in the peripheral blood from all bone-marrow-transplanted mice ($n = 7$). They were then subjected to the anti-Col2 antibody administration. When the arthritis persisted for more than three days after the arthritis peak, the joints were assessed. GFP+ cells were absent in the hyperplastic synovial tissues from the bone-marrow-transplanted mice based on histological analyses (Fig. 2A), and were also absent in their joint tissues based on quantitative flow cytometric analyses (Fig. 2B, 2C). Thus, the synovial fibroblasts did not derive from the cells in the bone marrow.

Synovial fibroblast accumulation without influx via circulation in CAIA

While bone marrow transplantation is a standard approach to tracing bone-marrow-derived cells, the pretreatment with whole body irradiation might alter the synovial microenvironment. In addition, the bone marrow transplantation procedures might not replace all mesenchymal progenitor cells with the donor-derived cells.

To overcome the above limitations, we conjoined wild-type mice and Col1a2-GFP mice for parabiosis. Since the parabionts shared common blood circulation, the migration of the synovial fibroblasts from one joint to another can also be assessed. Two months after the surgical joining, cross-circulation between the parabionts was confirmed with chimerism (41–59%) in the peripheral blood from wild-type parabionts ($n = 6$). As is in conventional CAIA, the parabionts developed arthritis 3 to 5 days after the anti-Col2 antibody administration. The joints were assessed when synovial thickening was maximal. GFP⁺ cells were absent in the hyperplastic synovial tissues from the wild-type parabionts while GFP⁺ cells accumulated in those from the Col1a2-GFP parabionts with histological and flow cytometric analyses (Fig. 3A, 3B, 3C). Thus, the synovial fibroblasts did not accumulate in the synovial tissues via circulation in CAIA.

Local proliferation of synovial fibroblasts in CAIA

The above results indicated that the accumulated synovial fibroblasts in the arthritic joints arise from resident cells. To investigate the proliferation of the synovial fibroblasts in arthritis, the inflamed Col1a2-GFP synovial tissues were examined immunohistochemically for the Ki67 expression. The percentage of Ki67+ cells out of GFP+ cells in the synovial tissues from the Col1a2-GFP mice with CAIA was over 80-fold higher than that from the untreated Col1a2-GFP mice in the arthritis peak (34% vs. 0.40%) (Fig. 4A, 4B). The proliferation of the synovial fibroblasts was also evaluated by Fucci transgenic mice. The percentage of Azami-Green+ cells out of vimentin+ cells in the synovial tissues from the Fucci mice with CAIA was over 70-fold higher than that from the untreated Fucci mice in the arthritis peak (19% vs. 0.26%) (Fig. 4C, 4D). These results are consistent with the hypothesis that the local cellular proliferation is responsible for the synovial fibroblast accumulation in CAIA.

Since Ki67 and Azami-Green are expressed in the different phases of the cell cycle as was described earlier, the number of proliferating cells should be smaller than that of Ki67+ cells and larger than that of Azami-Green+ cells. Our results using the two markers demonstrated that the percentage of the proliferating synovial fibroblasts is between 19% and 34%.

Discussion

In the present study, we demonstrated that the local cellular proliferation accounts for most of the accumulation of synovial fibroblasts in an animal model of RA. The influx of non-resident cells did not contribute to the accumulation in this system.

It was reported that cultured bone-marrow-derived mesenchymal cells and isolated circulating fibrocytes migrated into the inflamed synovial tissues when they were transferred with systemic injection [15,16]. When cultured RA synovial fibroblasts were implanted subcutaneously with the human cartilage into severe combined immunodeficient (SCID) mice, they migrated into the synovial tissues of the distant osteoarthritic joints [17]. In sharp contrast to our findings, these observations suggested that the circulating mesenchymal cells should contribute to the accumulation of synovial fibroblasts in the arthritic joints. Possibly, the isolation and the *in vitro* culture of the cells might alter the expression of molecules associated with cell adhesion and motility, making the isolated and cultured cells artificially prone to the migration.

The inflamed synovial tissues from mice with their bone marrow transplanted from mice expressing GFP ubiquitously were studied in a different report [18]. It revealed GFP+ cells in the *ex vivo* cultured cells isolated from the synovial tissues. In

this regard, we studied the synovial tissues directly to eliminate the possibilities of biased expansion of contaminating bone marrow cells and/or rare cells in the joints.

In murine models of kidney fibrosis and skin fibrosis, the influx of bone-marrow-derived cells contributed to the accumulation of fibroblasts [24,31]. In these models, the cells have to be mobilized from the bone marrow, recruited and lodged to the target tissues, and differentiate to the fibroblasts. During these processes, cytokines, adhesion molecules, and growth factors in the traffic pathway should play crucial roles. One or more of these factors might be missing in the arthritis.

The CAIA model was employed in this study since it could be induced readily in mice on the C57BL/6 background. To confirm that our findings hold true in a chronic arthritis model, we induced collagen-induced arthritis (CIA) in mice with the Colla2-GFP bone marrow transplantation (n = 4) and parabiosis (n = 4), and studied their synovial tissues. We found no GFP⁺ cells in the arthritic synovial tissues from the bone-marrow-transplanted mice with CIA (Supplementary Fig. S1A) or from the wild-type parabionts with CIA (Supplementary Fig. S1B).

In human diseases, the presence of bone-marrow-derived fibroblasts carrying Y chromosomes in the gastric cancer tissue were revealed in female patients who had undergone allogeneic stem cell transplantation from male donors [32]. Similarly, RA

synovial tissues from female patients with male bone marrow transplants would serve to clarify the presence or absence of the bone-marrow-derived synovial fibroblasts in RA.

We found that increased proliferation should be responsible for the accumulation of synovial fibroblasts in the murine models of RA. Previous immunohistochemical analyses revealed that the number of synovial fibroblasts expressing proliferation markers in RA was also larger than those in normal controls or osteoarthritis [11–14,33]. It should be likely that the local proliferation of the synovial fibroblasts in the joints accounts for the synovial fibroblast accumulation in RA.

Recent studies reported that mesenchymal stem cells (MSCs) in the *ex vivo* cultured cells were isolated from both the RA and the osteoarthritis synovial tissues [34,35]. The differentiation from MSCs to synovial fibroblasts during arthritis and their subsequent proliferation could also be involved in the accumulation of synovial fibroblasts.

Current therapeutic agents that target inflammatory cytokines and leukocytes are effective in treating RA. However, they share similar limitations in both efficacy and adverse effects including serious infections. Suppression of proliferation of the local synovial fibroblasts should be a promising treatment for RA. Elucidation of the

proliferative processes will facilitate the development of anti-rheumatic drugs that would concert with current immunosuppressive treatments.

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Figure legends

Fig. 1. Evaluation of GFP+ cells in the synovial tissues from the Col1a2-GFP mice. The synovial tissues of the knee joints from the untreated Col1a2-GFP mice (no CAIA) and the Col1a2-GFP mice with CAIA (CAIA) 10 days after the anti-Col2 antibody administration were stained with hematoxylin and eosin (H&E) and DAPI (A). The boxed regions in the H&E panels correspond to the magnified images in the right panels. Scale bars = 200 μm . The synovial tissues from the Col1a2-GFP mice with CAIA were stained with the antibody against vimentin (B) or Hsp47 (C). The images of GFP and vimentin (B) or Hsp47 (C) were merged. Scale bars = 10 μm . Representative images from 7 mice are shown (A-C). Quantification of the numbers of GFP+ cells and CD45+ cells in the synovial tissues from the untreated mice (no CAIA), and the arthritic mice (CAIA) 10 and 20 days (day 10, day 20) after the anti-Col2 antibody administration (4 mice each) (D). Bo: bone (femur or tibia), JS: joint space, M: meniscus, Sy: synovial tissue. Data represent means $\pm$ SEM. NS = not significant. * = $P < 0.05$.

Fig. 2. Evaluation of the influx of bone-marrow-derived cells into the synovial tissues in CAIA. The synovial tissues of the knee joints from the bone-marrow-transplanted mice with CAIA (BMT) and the Col1a2-GFP mice with CAIA (Control) 10 days after the antibody administration were stained with H&E and DAPI (A). The images of DAPI and GFP were merged. The boxed regions in the H&E panels correspond to the magnified images in the right panels (DAPI, GFP, Merge). Representative images from 7 transplanted mice are shown. Scale bars = 100 μ m. The disaggregated arthritic knee joint tissues from the bone-marrow-transplanted mice (BMT) and the Col1a2-GFP mice (Control) were examined for GFP expression with flow cytometric analyses (B, C). Representative data (B) and quantitative analyses (C) from 4 mice in each group are shown. Bo: bone (femur or tibia), M: meniscus, Sy: synovial tissue, SSC: side scatter. Data represent means $\pm$ SEM.

Fig. 3. Evaluation of the influx via circulation into the synovial tissues in CAIA. The synovial tissues of the knee joints from the wild-type parabionts with CAIA (Wild-type parabiont) and the Colla2-GFP parabionts with CAIA (Colla2-GFP parabiont) 10 days after the antibody administration were stained with H&E and DAPI (A). The images of DAPI and GFP were merged. The boxed regions in the H&E panels correspond to the magnified images in the right panels (DAPI, GFP, Merge). Representative images from 6 pairs of parabionts are shown. Scale bars = 100 μ m. The disaggregated arthritic knee joint tissues from the wild-type parabionts with CAIA (Wild-type parabiont) and the Colla2-GFP parabionts with CAIA (Colla2-GFP parabiont) were assessed for GFP expression with flow cytometric analyses (B, C). Representative data (B) and quantitative analyses (C) from 4 mice in each group are shown. Bo: bone (femur or

tibia), Li: ligament, M: meniscus, Sy: synovial tissue, SSC: side scatter. Data represent means $\pm$ SEM.

Fig. 4. Evaluation of local proliferation of the synovial fibroblasts. The synovial tissues of the knee joints from the untreated Coll1a2-GFP mice (no CAIA) and the Coll1a2-GFP mice with CAIA (CAIA) 10 days after the antibody administration were stained with DAPI and the antibodies against Ki67 (A). The images of GFP, DAPI, and Ki67 were merged. The percentage of Ki67+ cells out of GFP+ cells in the synovial tissues from the untreated Coll1a2-GFP mice and the Coll1a2-GFP mice with CAIA was calculated (B). Representative images from 7 mice in each group are shown. The synovial tissues of the knee joints from the untreated Fucci mice (no CAIA) and the Fucci mice with CAIA (CAIA) 7–11 days after the treatment were stained with DAPI and the antibodies against vimentin (C). The images of vimentin, DAPI, Azami-Green, and Kusabira-Orange were merged. The percentage of Azami-Green+ cells out of vimentin+

cells in the synovial tissues from the untreated Fucci mice and the Fucci mice with

CAIA was calculated (D). Representative images from 3 mice in each group are shown.

Scale bars = 30 μm . JS: joint space, Sy: synovial tissue. Data represent means $\pm$ SEM; *

= $P < 0.05$.