Characterisation of fibroblast-like cells in pannus lesions of patients with rheumatoid arthritis sharing properties of fibroblasts and chondrocytes

Chengsen Xue, Masayoshi Takahashi, Tomoko Hasunuma, Hiroyuki Aono, Kazuhiko Yamamoto, Shinichi Yoshino, Takayuki Sumida, Kusuki Nishioka

Abstract

***Objective*—To better understand the characteristics of synoviocytes located in the rheumatoid arthritis (RA) pannus.**

***Methods*—One cell line, termed PSC, was cloned from RA pannus lesions. Phenotypic analysis was done by contrast microscopy, indirect immunostaining, and safranin O staining. Transcription of several protooncogenes and matrix degrading enzymes was evaluated. The expression of mRNA for collagen II was detected by in situ hybridisation. The ability of anchorage independent growth was assessed by soft agarose culture.**

***Results*—PSCs showed a high transcription of protooncogenes c-fos, c-myc and c-jun. They also expressed mRNA for matrix degrading enzymes, such as collagenase, cathepsin B, and cathepsin L. Anchorage independent growth assay demonstrated that PSCs formed colonies in soft agar culture. Phenotypic analysis showed that this fibroblast-like PSC was stained intensely with anti-vimentin and anti-fibroblast antibody. In situ reverse transcriptase assay showed that the cell line expressed type II collagen mRNA.**

***Conclusion*—Alternative fibroblast-like cells were identified in the pannus lesion of RA sharing properties of fibroblasts and chondrocytes. These findings suggest that this fibroblast-like cell derived from pannus lesions may contribute to the destruction of the cartilage in RA.**

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Department of Rheumatology and Immunology, Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan
C Xue
M Takahashi
T Hasunuma
H Aono
K Yamamoto
T Sumida
K Nishioka

Division of Rheumatology, Nippon Medical College, Tokyo, Japan
S Yoshino

Correspondence to:
Dr K Nishioka, Department of Rheumatology and Immunology, Institute of Medical Science, St Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216, Japan.

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Pannus formation is a prominent feature of rheumatoid arthritis (RA). Several reports have suggested that pannus expresses 'aggressive, invasive and tumour-like' properties.[1–4] Moreover, pannus synoviocytes have been suggested to represent a T cell independent pathway responsible for joint destruction in RA.[5] Such synoviocytes express excessive amounts of vascular cell adhesion molecules, but a large part of phenotypic and functional features of pannus synoviocytes remains obscure.

Although the origin of pannus is still under debate,[6–9] observations based on detailed morphological studies have provided sufficient evidence indicating that the area of cartilage-pannus junction contains chondrocyte derived cells,[10] that react with monoclonal anti-type II collagen antibodies.[10 11] The articular cartilage has been considered to contribute, at least in part, to the formation of pannus.[9 10] Others have suggested that the immature mesenchymoid cells serve as a source for initiating pannus formation.[12] Because of the limitation of conventional histochemical stains, isolation techniques, and culture conditions used, a putative distinct cell population, which may be different from the well characterised synovial lining cells, has not been isolated and well characterised.[13] In this study, we isolated a cell line, termed PSC, from biopsy specimens of RA pannus. The phenotypic and functional features of the cell line were characterised.

Methods

CELL PREPARATION

Synovial tissue samples were obtained during arthroplasty from the knee joints of two female patients (aged 69 and 72 years) with clinical and laboratory features consistent with RA according to the criteria of American College of Rheumatology. During the operation, pannus samples were carefully selected from granulation tissue invading the articular cartilage of the femur and tibia. At the same time, synovial tissue samples from the hypertrophic villi surrounding the joint capsule were obtained, which served as non-pannus samples (SC). As a control, synovial tissues from two female patients with osteoarthritis (OA) and one female patient with traumatic joint injury were also obtained during knee surgery. The control sample was taken from tissues far from the cartilage area. The experimental protocol was approved by the Ethics Review Committee for Human Experimentation at our institution and a signed consent form was obtained from all subjects.

Cloning of synovial cells was performed according to the method described previously by our laboratory.[14] Briefly, following removal of adipose tissue from the biopsy material, the specimen was minced into small pieces and digested with 1.0 mg/ml of collagenase (Sigma Chemical Co, St Louis, MO) in Dulbecco's modified Eagle's medium (DMEM, GIBCO Laboratories, Grand Island, NY) at 37°C for two hours. After digestion, the dissociated cells were collected by centrifugation at 500 *g* for five minutes, resuspended in Ham F-12 medium (GIBCO Laboratories) supplemented with 10% fetal calf serum (FCS, Bioserum, Melbourne, VIC, Australia), 100 U/ml penicillin, 100 mg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol (2-ME). The cell suspen-

sion was then poured into a 90 mm diameter dish (Sumitomo Medical Co, Tokyo). The adherent synoviocytes became confluent in three to five days and served as the primary culture. A limited dilution method was established for developing colonies. Briefly, the cells in culture were detached using 0.25% trypsin (GIBCO Laboratories), collected by centrifugation at 500 *g* for three minutes, and resuspended in Ham F-12. After counting, the cells were seeded into 96 well flat bottom plates (Corning Glass Works, Corning, NY) at a density of 0.5 cells per well. Single cells were picked up and expanded. In addition, cells forming colonies in soft agar culture (see below) were also picked up and expanded in 90 mm dishes. These cells were termed PSC.

INDIRECT IMMUNOFLUORESCENCE

Phenotypic analysis was performed by indirect immunofluorescence test using fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies (MAbs). All MAbs used in the study were purchased from commercial sources, and included anti-vimentin and 5B5 (DAKO A/S, Glostrup, Denmark), MY4, a monocyte/macrophage marker (CD14, Coulter Immunology, Hialeah, FL), and several antibodies including S100, LCA, DRC-1 and CD35 (DAKO A/S), S100A (DAKO Co, CA) and Leu6 (Becton-Dickinson, Mountain View, CA). Mouse IgG1 (Coulter Immunology) was used as a control. Briefly, cells were seeded into slide chambers (Nunc Inc, Naperville, IL) at a density of 3 × 103 cells per chamber. After a culture period of five days, the slides were fixed with cold acetone and subjected to indirect immunostaining as described previously.[15] The results were evaluated using a fluorescence microscope.

STAINING WITH SAFRANIN O

Staining with safranin O was performed according to the method described by Ishikawa *et al*.[16] The cells studied were grown in slide chambers. The chambers were incubated at 37°C and 5% CO_2 in air. The slides were fixed five days later with acetone, and then immersed into 0.2% solution of safranin O (Schmid GmbH, Kongen, Germany). The staining procedure lasted for two hours.

Table 1 Oligonucleotide sequences used in PCR amplification

Gene	*Primer*	*Position*	*Sequence*	*GenBank Accession No*
c-fos	Fos5L	2490-2511	AGGAGAATCCGAAGGGAAAGGA	
	Fos6R	3000-2979	AGTCATCAAAGGGCTCGGTCTT	K00650
c-myc	Myc1L	1374-1395	TCCTGGCAAAAGGTCAGAGTCT	
	Myc2R	1624-1602	TCTTGACATTCTCCTCGGTGTC	V00568
c-jun	c-jun1L	1275-1296	GGAAACGACCTTCTATGACGAT	
	c-jun2R	1590-1571	GAACCCCTCCTGCTCATCTG	J04111
Cathepsin L	CAT1L	232-253	AGTTTAGAGGCACAGTGGACCA	
	CATL2R	444-423	GCCATTCATCACCTGCCTGAAT	M20496
Cathepsin B	CTB1L	974-995	GCTCTACAAGTCAGGAGTGTAC	
	CTB2R	1182-1161	GAATTCCAGCCACCACTTCTGA	M14221
Collagenase	Coll1L	55-75	ATGCACAGCTTTCCTCCACTG	
	Coll2R	220-240	TTCAACCACTGGGCCACTATT	M15996
β actin	BAC1L	1561-1581	AAGGCCAACCGCGAGAAGATG	
	BAC4R	2596-2575	AAGGTAGTTTCGTGGATGCAAC	M10277

All primers are shown in 5' to 3' direction. Sense strand oligonucleotides are indicated with an L and antisense oligonucleotides with an R.

IN SITU REVERSE TRANSCRIPTASE ASSAY

In situ reverse transcription was performed using the Digoxigenin Detection System Kit (Kreatech Biotechnology BV, Amsterdam).[17] Briefly, cells were seeded into slide chambers. After culture for three days, they were fixed with 10% paraformaldehyde for one hour. The cells were treated with 0.5% NP-40 for one hour to ensure permeability. Ten µl of hybridisation mixture containing 5.0 pM type II collagen primer (5'-GAGGCAGTCTTTCACGTCTTCA-3'), 10 mM DTT, 1.0 mM dNTP, 10 U/ml reverse transcriptase, 2 U/ml RNase inhibitor, and 0.1 nM digoxigenin-11-dUTP was added. Each slide was covered with a coverslide and incubated at 42°C for one hour. The coverslides were removed at a later stage by washing with 2 × standard saline citrate buffer (SSC, 150 mM NaCl, 15 mM sodium citrate, pH 7.0). They were incubated with 100 µl of rhodamine-conjugated sheep anti-digoxigenin-Fab fragments at room temperature for one hour, and then rinsed in phosphate buffered saline. The results were evaluated under a confocal microscope. Reaction with type II collagen sense primer was used to monitor non-specific binding.

RNA PREPARATION AND SEMI-QUANTITATIVE REVERSE TRANSCRIPTION PCR (RT-PCR)

After 5×10^5 cells were cultured in near confluent status, RNA was extracted from the cells using the AGPC method[18] and analysed semi-quantitatively by RT-PCR with Mo-MuLV reverse transcriptase (GIBCO Laboratories).[19] Table 1 summarises the primers used in the present experiment for determining the individual expression. The sequence numbers correspond with those of the GenBank database. Amplification of β actin mRNA served as a loading control. PCR was performed on a thermocycler (Hybaid Limited, Middlesex, UK) in a 50 µl reaction volume, including cDNA sample, 2.0 U Taq DNA polymerase (GIBCO Laboratories), 50 pmol of each primer, 200 mmol of each deoxynucleotide triphosphate and 5 mCi (α-32P)-dCTP (NEN, Wilmington, DE). The annealing temperature was 64°C for one minute. PCR products were separated by 10% polyacrylamide gel electrophoresis. The gel was exposed to an *x* ray film, and the bands on the film were measured under a densitometer with an image analyser (TIAS Image PRO, ACI Japan Co, Kanagawa). The ratio of the individual density of the protooncogene/β actin was calculated.

ANCHORAGE INDEPENDENT GROWTH ASSAY

An anchorage independent growth assay similar to that described by Lafyatis *et al*[20] was performed in six well plates (Corning Inc) with minor modifications. A gel mixture of 0.55% SeaPlaque agarose (FMC BioProducts, Rockland, ME) was prepared and used to precoat each well. Eighty per cent of confluent synoviocytes were washed twice in phosphate buffered saline, trypsinised, centrifuged, and resuspended in Ham F-12 medium supple-

mented with 10% fetal calf serum. In the next step, 2.0 ml of the soft agar solution (0.33% agarose in Ham), consisting of 10 000 synoviocytes, was spread over the base layer. The plates were incubated at 37°C-5% CO_2. All wells were examined daily for colony growth. Colonies formed in 10-14 days after seeding. Three weeks later, the number of colonies was counted under a contrast microscope. The cells in colonies were picked up and expanded in 90 mm dishes.

Results

MORPHOLOGICAL CHARACTERISTICS OF PSCS

Morphological studies were performed to determine the characteristics of PSC. According to our previous classification,[14] RA synoviocytes are heteromorphous, consisting of three types, including dendritic cells, macrophage cells, and fibroblast-like cells. PSCs were homogeneous (fig 1A) and phenotypically resembled fibroblasts. However, they expressed more filopodia and spherical phase-dense cytoplasmic granules (fig 1B) than that of SCs. The cytoplasm of PSCs was oval or rhombic in shape. When the cells became confluent on culture dishes, they resembled fibroblasts. In addition, morphologically, there are no hyaline cartilage cells in the culturing PSCs under contrast microscopy.

IMMUNOFLUORESCENCE STAINING

To further characterise the phenotype of PSC, the cells were stained with several MAbs. PSCs were intensely stained with MAb to vimentin. The intermediate filament vimentin exists on fibroblast cells and epithelial cells as well as the mononuclear phagocyte system.[21] Vimentin surrounded the nucleus and extended out to the cell periphery (fig 2). The synoviocytes were also immunostained positively with 5B5 antibody, but reacted weakly with antibody MY4 of the monocytes/macrophage surface marker. The cells failed to stain with antibodies such as S100, S100A, LCA, DRC-1, and CD35.

STAINING WITH SAFRANIN O

Safranin O is known to react with proteoglycan present in the cartilage and has been used as a marker for chondrocyte-like cells. Safranin O was positive in PSCs (fig 3) but not in SCs.

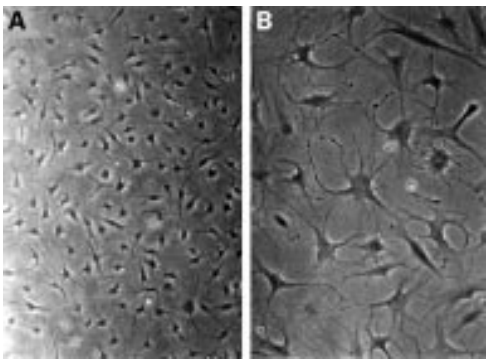

Figure 1 Contrast microscopy of cultured pannus synoviocytes. PSCs were cultured in a 90 mm dish. They were a homogeneous group of cells (A) and had many filopodia (B) (original magnification × 200).

Figure 2 Immunofluorescence micrograph of vimentin filaments in cultured PSCs. Under contrast microscope, almost all PSCs were stained with anti-vimentin antibody labelled with FITC. The network of intermediate filament vimentin was stained with antibody to vimentin protein. An extensive network of vimentin filament surrounds the nucleus and extends out to the cell periphery (fluorescence microscopy, original magnification × 400).

More than half of cloned PSCs picked up from soft agarose showed positive results. The percentage of positive cells in those with several filopodia was higher compared with spindle cells. Both the cytoplasm and nuclei of PSCs were stained, but the latter showed a less dense staining. In addition, as the passage of cells was increased, the positive staining gradually decreased. Almost all cells were negative after the eighth passage.

IN SITU REVERSE TRANSCRIPTASE ASSAY FOR TYPE II COLLAGEN MRNA

To confirm the chondrocyte nature of PSC, we evaluated the expression of mRNA for type II collagen by in situ reverse transcriptase assay. Confocal microscopy demonstrated detectable expression of type II collagen mRNA transcripts in PSCs from both rheumatoid

Figure 3 Safranin O staining of synoviocytes. Cloned cells were seeded into the slide chamber, and fixed three days later with acetone, then subjected to the staining procedure described in the text. PSCs stained with red colour represent positive staining (original magnification × 200).

patients. In contrast, SCs from the same patient, and patients with OA and traumatic joint injury were negative for the gene. Quantitative evaluation by in situ reverse transcriptase assay demonstrated that less than 5% of the cells in primary bulk culture of pannus specimens showed mRNA expression of type II collagen, suggesting that these cells might represent the PSC phenotype. All cells that were picked up from the soft agar were positive (fig 4A). These cells also expressed several filopodia (fig 4B). Characteristically, type II collagen mRNA in cultured PSCs showed a scattered pattern, and were distributed over or adjacent to the nuclei.

EXPRESSION OF PROTOONCOGENES AND PROTEASE

Semi-quantitative RT-PCR assay was used to examine the relative expression level of different protooncogenes, including c-myc, c-fos and c-jun in synoviocytes. To accurately control PCR amplification of all genes for the plateau phenomenon, the PCR conditions were modified in a cycle dependent manner. The most suitable cycle numbers were 16 cycles for β actin, 28 cycles for c-myc, and 30 cycles for c-fos and c-jun. These protooncogenes were expressed in synoviocytes obtained from RA pannus as well as non-pannus synoviocytes. However, PSCs expressed larger amount of protooncogenes compared with SCs (table 2).

Synoviocytes derived from RA are well known to secrete several matrix degrading enzymes, such as collagenase, cathepsin B, and cathepsin L. The expression of such genes in PSCs was examined by RT-PCR. Both PSCs and SCs were found to express similar values of the genes (data not shown).

ANCHORAGE INDEPENDENT GROWTH OF PSCS

To examine the ability of PSC for anchorage independent growth, we used the soft agarose culture method. After two weeks of culture, PSCs in primary culture developed colonies in soft agar culture (fig 5A). The maximum cell number in one colony was more than 55 cells when culture continued for four weeks. To examine for differences in colony growth, PSCs and SCs of the same passage were seeded into soft agar at the same time. These experiments demonstrated a stronger tendency for colony formation by PSCs (147 colonies/104 cells at primary culture) compared with SCs (6 colonies/104 cells at primary culture). The colony number of PSCs diminished with increasing passages. The number of colonies after the eighth passage was as low as 12/104 cells.

Two phenomena characterised the anchorage independent growth of PSCs. Firstly, under the phase contrast microscope, one third of the colonies has a ring formed in the extracellular matrix (fig 5B). Secondly, the morphology of the colonies changed with time, for example, culture for approximately five weeks reduced the density of colonies, so that the originally compact colony became scattered gradually.

Table 2 Expression of protooncogenes in PSCs and SCs

	PSCs		*SCs*	
	*Case 1**	*Case 2*	*Case 1*	*Case 2*
c-fos/β actin	1.72†	1.34	0.12	0.93
c-jun/β actin	1.44	ND	0.11	0.29
c-myc/β actin	2.85	2.17	0.48	0.21

* PSCs and SCs were obtained from two patients: case 1 and case 2. † The calculated ratio between band density measured by densitometry in protooncogenes and β actin. ND = not detected.

Discussion

In this study, we isolated PSC from rheumatoid pannus lesions. Several tests were performed to identify and characterise these synoviocytes including morphological examination and detection of transcription of several protooncogenes and matrix degrading enzymes. Our results demonstrated clearly that the PSC is a fibroblast-like cell with properties common for both fibroblasts and chondrocytes. The cell did not only have specific phenotypic features, based on the observation of morphological studies, immunostaining

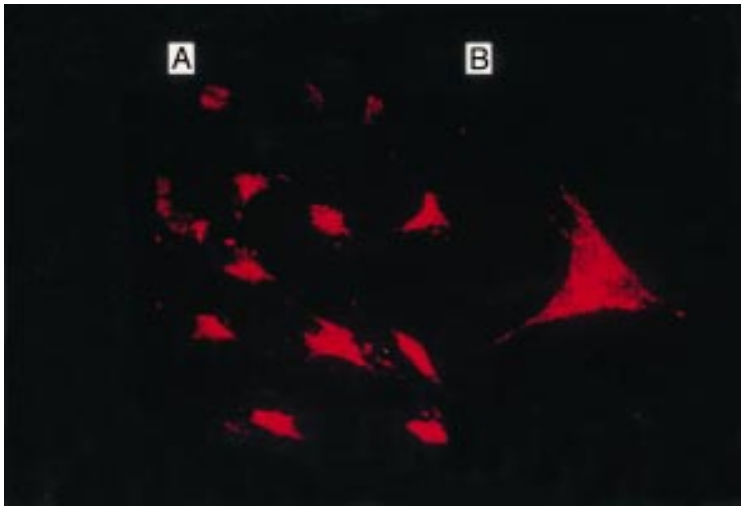

Figure 4 Type II collagen in situ reverse transcription. In situ reverse transcriptase assay was performed on the slide chamber with cultured PSCs to evaluate the expression of type II collagen mRNA. PCR amplification was performed with digoxigenin labelled oligonucleotide probe and sequential immunostaining with sheep anti-digoxigenin conjugated rhodamine. Cells picked up from the soft agar highly expressed type II collagen mRNA (A). High power view shows one PSC with several filopodia. Type II collagen mRNA shows a scattered pattern (B) (confocal microscopy, (A) original magnification × 200, (B) × 400).

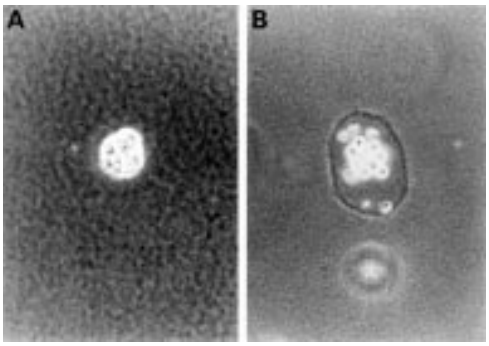

Figure 5 Colony growth in anchorage independent growth assay. After culture for four weeks, PSCs formed colonies in soft agarose (A). Under the phase contrast microscope, a number of the colonies have a ring formed in the extracellular matrix (B) (contrast microscopy, (A) and (B) original magnification × 200).

with anti-vimentin and anti-fibroblast antibodies, safranin O staining, and type II collagen transcription, but it also showed some functional features, including formation of colonies in soft agar culture and high nuclear transcription. Furthermore, like activated macrophages, the PSCs expressed protease mRNAs, such as collagenase, cathepsin B, and cathepsin L. Therefore, we suggest that this fibroblast-like synoviocyte, even though forming a low percentage of the total synoviocytes, less then 5% of the cells isolated from pannus, may constitute an active component of the rheumatoid pannus lesion. In addition, the PSCs could be clearly differed from control synovial cells with positive staining of vimentin and safranin O as well as expression of type II collagen mRNA.

A peculiar property of fibroblast-like PSCs uncovered in this study was that of colony formation in soft agar. It has been well documented that few fibroblast populations are potentially capable of colony formation, as detected in anchorage independent growth assay. For example, in idiopathic pulmonary fibrosis, fibroblast lines derived from the fibrotic tissue form colonies at a frequency almost similar to that seen in cells derived from neonatal lung tissue.[22] Early passage synoviocytes obtained from RA and streptococcal cell wall induced arthritis in rats also form colonies in soft agar.[20]

The nature of anchorage independent cell growth is also a marker of cell transformation.[23] The fibroblasts in RA seem to be functionally 'transformed' mesenchymal cells.[24] Previous studies considered the rheumatoid pannus to behave in a tumour-like fashion.[2] The fact that the PSC is cable of anchorage independent growth may indicate its transformation.

The higher expression of protooncogenes in PSCs may explain partially this transformation. Higher activities of protooncogene c-fos, c-jun, and c-myc revealed abnormal nuclear transcripts in PSCs. Overexpression of protooncogene c-fos in transgenic mouse promotes mesenchymal cell proliferation in the pannus area and joint destruction without inflammatory infiltration.[25] Transcription of protooncogenes also regulates matrix degrading enzymes. It is well known that c-fos binds to the jun/AP-1 protein to regulate the expression of genes, such as collagenase.[26] Protooncogenes also regulate the activity of cathepsin enzymes.[3] In this regard, there is ample evidence confirming the role of cathepsin B and L in degrading cartilage collagen type II, IX, XI and cartilage proteoglycan.[27 28] Using immunostaining analysis, Trabandt *et al*[29] demonstrated a position distribution of cathepsin B along the synovial-cartilage junction. In this study, PSCs expressed the matrix degrading enzyme cathepsin B and L mRNA at a level similar to that of SCs. Furthermore, despite increased mRNA for c-fos and c-jun, these cells made no more collagenase than SCs. The reason that PSC does not make more protease is not clear at present. One possible explanation is the presence of unknown factors responsible for its function in pannus.

As PSC had a number of cartilage features, we examined the expression of S100, which is a marker of macrophage and dendritic cells, and it is also expressed on the cartilage cells. Our result clearly demonstrated that PSC did not express S100, which suggests that this cell population is quite distinct from macrophage or cartilage cells.

It is also possible that PSC may have abnormal differentiation induced by the process of inflammation in RA. There is a substantial evidence that fibroblasts also undergo a differentiation process either in vivo or in vitro. Scleral fibroblasts of the chick embryo differentiate into chondrocytes in soft agarose cultures.[30] Such process of differentiation also occurs in well differentiated tissues, for example, during the repair of full thickness defects of articular cartilage.[31] This phenomenon indicates that connective tissue fibroblasts exhibit extensive interconversion properties.[32 33] In the presence of various signals in the extracellular matrix, fibroblasts can transform into osteoblasts, chondrocytes, adipocytes or even smooth muscle cells. This adaptability of fibroblasts is a key feature and represents a response to tissue damage and repair. PSC in the rheumatoid pannus lesion may also respond by transforming into other types of cells in response to the chronic inflammatory process. The tissue samples studied in this experiment were obtained from joints at a comparatively late stage in the disease process, it is also quite possible that the granulation tissue present adjacent to cartilage and bone in these joints is largely related to the formation of osteophytes, and the cells are able to sythesise components of articular cartilage. The expression of protooncogenes may contribute, at least in part, by promoting cellular interconversion. In this regard, PSC may be the source of the cells responsible for the initiation of pannus.

The underlying mechanisms for the difference in ability of PSC and SCs to form colonies were not investigated in this study. We have already cloned three types of synoviocytes from RA synovial tissues, including dendritic cells, macrophage cells and fibroblast-like cells.[14] Although cultured PSCs had more filopodia compared with fibroblasts, they stained negatively with anti-dendritic cell antibodies. The PSC demonstrated an intermediate type between type A and type B synoviocytes. This finding was also supported by morphological findings, the cells in the rheumatoid pannus tissue were mesenchymal in appearance.[13] Moreover, PSCs had several phenotypic and functional characteristics that were not present in SCs as mentioned above. Therefore, it is reasonable to consider PSC as an alternative synoviocyte compared with SCs.

Further research investigating the proliferation and differentiation of PSC would perhaps permit the blockage of the process leading to perpetuation of joint destruction in RA.

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