American Journal of Pathology, Vol. 150, No. 3, March 1997

Pannocytes: Distinctive Cells Found in Rheumatoid Arthritis Articular Cartilage Erosions

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A distinctive cell was identified from sites of rheumatoid arthritis cartilage injury. Similar cells are not found in lesions of osteoarthritis cartilage. We have designated them as pannocytes (PCs). Their rhomboid morphology differs from the bipolar shape of fibroblast-like synoviocytes or the spherical configuration of primary human articular chondrocytes. Chondrocytes are short-lived, whereas the original PC line grew for 25 passages before becoming senescent. Features in common with cultured primary chondrocytes include maximal proliferation in response to transforming growth factor-β a catabolic response to interleukin-1β, collagenase production, and mRNA for the induced lymphocyte antigen and inducible nitric oxide synthase. Despite the presence of the inducible nitric oxide synthase message, PCs do not produce NO either constitutively or when cytokine stimulated. Each of the mesenchymal cells, fibroblast-like synoviocytes, primary chondrocytes, and PCs have the gene for type I collagen, but the type II collagen gene is detected only in primary chondrocytes. PCs can be distinguished from fibroblast-like synoviocytes and primary chondrocytes by their morphology, bright VCAM-1 staining, and growth response to cytokines and growth factors. Their prolonged life span* in vitro *suggests that PCs might represent an earlier stage of mesenchymal cell differentiation, and they could have a heretofore unrecognized role in rheumatoid arthritis joint destruction. (Am J Pathol 1997, 150:1125–1138)

Joint destruction brought about by disruption of ligaments, tendons, articular cartilage, and bone is an almost invariable consequence of chronic rheumatoid arthritis (RA).[1,2] The responsible cells are believed to have their origin in the synovial lining. These, under the influence of locally produced cytokines and growth factors, proliferate and express a broad array of proteinases, such as collagenase and stromelysin, that are thought to be directly responsible for the tissue destruction.[3–6] Simultaneously, chondrocytes digest the matrix in which they are normally embedded and osteoclasts destroy bone. *In vivo* studies of the erosive, destructive granulation tissue in RA (called pannus) have usually been limited to materials obtained at the time of joint replacement surgery and thus reflect long-standing, established lesions. Indeed, it is not absolutely certain that the pannus seen in conventional light and electron microscopy represents the cause of the observed tissue destruction or reflects a process of healing, because the lesions have many attributes of a scar.[7]

To characterize the cellular elements in the pannus, numerous studies have been performed using enzymatically dispersed synovial membranes. Lymphocytes and macrophages constitute approximately one-third each of the isolated populations,[8] but a major interest has focused on what has loosely been called synovial lining cells. A portion of these have the appearance of macrophages, are phagocytic, and display surface HLA-DR antigens (type A synoviocytes), IgG Fc receptors, and other markers associated with cells of the monocyte lineage.[9–11] The remainder of the adherent cells are nonphagocytic, fibroblast-like cells. In time, the monocytes, the macrophages, and the T lymphocytes die out with *in vitro* culture, and only the fibroblast-like synoviocytes (FLSs) are perpetuated. These, also known as type B

Supported in part by funds from National Institutes of Health grants AR40525, AG07996, and AR39799 and a grant to N. J. Zvaifler from Immulogic Corporation.

Accepted for publication November 4, 1996.

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synoviocytes, form the basis of many investigations on synoviocyte proliferation, proteinase production, and HLA-DR expression, both at rest or under the influence of cytokines and growth factors (reviewed in Ref. 2). Indeed, many investigators consider the cultured FLSs derived from digested synovial tissues as *in vitro* surrogates for pannus. However, we have recently isolated cells directly from erosions in RA cartilage at a distance from the synovial membrane or beneath the leading edge of the hyperplastic synovium where it burrows into cartilage and adjacent bone. These are a homogeneous population with morphological, phenotypic, genotypic, and functional characteristics that differ from chondrocytes and type B FLSs. A description of their distinctive features forms the basis of this report and suggests that these cells, which we have called pannocytes (PCs), may have relevance to the tissue destruction observed in RA.

Materials and Methods

Reagents

Human recombinant interleukin (IL)-1β (specific activity) (sp. act.), 5×10^7 U/mg; purity, >95%; lipopolysaccharide (LPS) content of the concentrate stock, <0.015 ng/mg) was purchased from Amgen Biologicals (Thousand Oaks, CA). Human recombinant tumor necrosis factor (TNF)-α (sp. act., 5×10^7 U/mg; purity, >95%; <0.008 ng/mg) was provided by Genentech (South San Francisco, CA). Human recombinant interferon (IFN)-γ (sp. act., 2×10^7 U/mg; purity >98%; LPS, <0.048 ng/mg) was a gift from Amgen Biologicals. Human recombinant IL-6 (sp. act., 5×10^9 U/mg; purity, >95%; LPS, <0.006 ng/mg) and human recombinant transforming growth factor (TGF)-β1 (purity, >97%; LPS, <0.02 ng/mg) were purchased from R&D Systems (Minneapolis, MN). In each case, the final concentration of LPS in culture after dilution of recombinant cytokines was <0.005 ng/ml. Platelet-derived growth factor (PDGF)-α and basic fibroblast growth factor (bFGF) were obtained from R&D Systems. Actinomycin D, cycloheximide, and polymyxin B (7500 U/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). Culture medium and supplements were from GIBCO BRL Laboratories (Grand Island, NY).

Patient Selection and Preparation of Tissues

Synovial tissues and cartilage are obtained routinely in the Rheumatology Research Laboratory at the University of California, San Diego, Medical Center from patients undergoing joint replacement surgery. The majority are patients with osteoarthritis, but samples are also obtained from patients with definite (American College of Rheumatology criteria) chronic destructive RA. Samples from hip surgery include the femoral head and neck with attached synovium. These are placed in petri dishes and washed three times with phosphate-buffered saline (PBS) to remove blood. The pinkish-brown synovial tissue is separated by scissors and scalpel and cut into small pieces (1 cm^3). Some are snap-frozen in 2-methyl butane and liquid nitrogen and stored at −70°C until used for routine staining or immunohistology. The remaining pieces are minced and digested as described below.

PCs were obtained from intact articular cartilage from hips and knees. The cartilages from smaller joints came in bits and pieces. The cells were obtained by scraping erosive lesions in the cartilage at a distance from invading synovium into PBS in a sterile petri dish. These isolated erosions were observed only in intact femoral heads. An alternative site for PC isolation was from the cartilage-pannus junction. The synovial tissue overlying erosive lesions was removed with a scalpel blade down to the underlying cartilage or bone. The eroded area was scraped with a scalpel blade and the tissues obtained were combined with those from isolated erosions when available. The tissues were placed in a 50-ml conical tube, spun, washed with PBS, and resuspended in a digestion solution of collagenase (type VIII, Sigma) at 0.5 mg/ml in RPMI. The digesting material was stirred at 37°C for 90 minutes and filtered through a 70-μm cell strainer (Falcon, Franklin Lakes, NJ). The filtered cell mixture was collected into a 50-ml conical tube, centrifuged, washed once with 10% fetal calf serum (FCS), and plated at 100,000 cells/ml in 10% FCS in complete Dulbecco's minimal essential medium (DMEM) in 24-well cell culture cluster plates (Costar, Cambridge, MA). The next day the nonadherent cells are removed; the adherent cells were washed and then cultured in complete DMEM/10% FCS until confluent. Thereafter, PCs and FLSs were handled identically. Six PC lines were established from RA samples, three from femoral head samples, two from knees, and one from a metatarsal head, removed at arthroplasty. In two instances, simultaneous cultures of PCs and FLSs were established from the cartilage lesions and synovial membrane of the same operative samples. These are designated lines 2A and 2C, and RA FLS line 227 and PC line 1 (see Figures 10, 5, and 8, respectively).

Cell Culture

Synovial cells (FLSs) were isolated by enzymatic dispersion of synovial tissues obtained from patients with RA undergoing joint surgery as described.[5] Briefly, the tissues were minced and incubated with 0.5 mg/ml collagenase (Sigma), in serum-free DMEM for 2 hours at 37°C, filtered through a nylon mesh, extensively washed, and cultured in DMEM supplemented with 10% FCS (GIBCO BRL; endotoxin content, <0.006 ng ml), penicillin, streptomycin, and L-glutamine (hereafter referred to as complete DMEM) in a humidified 5% CO_2 atmosphere. After overnight culture, nonadherent cells were removed and adherent cells were cultivated in complete DMEM/10% FCS. At confluence, cells were trypsinized, split at a 1:3 ratio, and recultured in medium. Synoviocytes were used from passages 3 through 9 in these experiments, during which time they were a homogeneous population of FLS (<1% CD11b, <1% phagocytic, and <1% Fc-γ RII receptor positive).

Chondrocytes were obtained at the time of autopsy from donors without a history of joint disease. Cartilage slices removed from the femoral condyles and tibial plateau are washed in complete DMEM, minced with a scalpel, transferred into a digestion buffer containing DMEM/5% FCS and 2 mg/ml clostridial collagenase type IV (Sigma), and incubated on a gyratory shaker at 37°C until the fragments were digested (typically overnight). Residual multicellular aggregates were removed by sedimentation ($1 \times g$) and the cells washed three times in complete DMEM/5% FCS before use in experiments. Unless specifically stated, only primary cultures were studied.

Electron Microscopy

PCs and synoviocytes were dislodged from culture plates with 1 mmol/L EDTA in cold Ca^{2+}/Mg^{2+}-free PBS. The cells were washed twice with RPMI at room temperature and fixed in 2% glutaraldehyde (Electron Microscopy Science, Fort Washington, PA), RRMI with 20 mmol/L HEPES (GIBCO BRL), pH 7.4, for 1 hour in single-cell suspensions. The cells were washed three times with PBS and postfixed in a pellet form with 1% osmium tetroxide for 1 hour on ice. The cell pellets were embedded in Spur for transmission electron microscopy. An EM 300 (Philips Electronic Instruments, Mahwah, NJ) was used for transmission electron microscopy analysis.

Immunoperoxidase Staining

Cells (PCs, FLSs, or primary chondrocytes) were removed from culture plates by treatment with 0.5% trypsin and 0.53 mmol/L EDTA, and washed in DMEM/5% FCS, followed by a wash of 0.1% bovine serum albumin (BSA)/PBS and cytocentrifuged (300 rpm) onto poly-L-lysine-coated glass slides. The cells were then fixed with 3.7% formaldehyde (10 minutes), rinsed in PBS, and treated with methanol at −20°C (5 minutes) and acetone at −20°C (3 minutes). After three washes with PBS, the slides were stored at −70°C and used within 1 week. Before staining, the slides were left at room temperature to equilibrate and then blocked sequentially with 0.1% BSA/PBS (5 minutes), 1% horse serum/PBS (5 minutes), and 2% human AB serum/PBS (5 minutes). The fixed and blocked cells were incubated with a 1 to 5 μg/ml dilution of the appropriate monoclonal antibody for 60 minutes at room temperature. Controls were performed with an isotype-matched mouse Ig at identical concentrations. After washing the slides three times with PBS, biotinylated horse anti-mouse second antibody (Vector Laboratories, Burlingame, CA) was added for 30 minutes at 4°C. Slides were washed three times with PBS, and endogenous peroxidase was depleted with 0.3% hydrogen peroxide in PBS for 20 minutes. Cells were then incubated with ABC horseradish peroxidase complex (Vector) for 30 minutes at 4°C. The peroxidase-catalyzed reaction was developed with 0.5 mg/ml diaminobenzidine and 0.02% hydrogen peroxide for 3 to 5 minutes at room temperature. Cells were considered positive when the specific staining was equal to or exceeded the controls.

FACS Analysis

FLSs or PCs (1×10^5 to 2×10^5) cultured in six-well plastic dishes (Costar) in complete DMEM/10% FCS were harvested with 1 mmol/L EDTA Ca^{2+}/Mg^{2+}-free PBS, washed once with cold DMEM at 4°C, and stained with the corresponding monoclonal antibody (MAb) as described.[12] Phycoerythrin-conjugated Fab_2 anti-mouse IgG (Tago, Burlingame, CA) was used as a secondary antibody instead of a fluorescein isothiocyanate conjugate to avoid problems with the green autofluorescence exhibited by synoviocytes. Pooled mouse IgG (Cappel Laboratories, Malvern, PA) or an isotype-matched MAb was used as negative control. A total of 5000 cells were analyzed from each sample adjusting the fluorescence gain so that the mean fluorescence channel of cells stained with the negative control was 40 to 50. The

cutoff for the positive cells was arbitrarily established in the channel that would include 98% of control cells. The results are displayed in a logarithmic scale of increasing fluorescence and presented as linear mean fluorescence.

Cell Growth

PCs and FLSs were removed from culture and resuspended at 1×10^4/ml in complete DMEM/10% FCS and incubated overnight in 24-well Falcon plates at 1 ml/well. The supernatants of these cultures were then removed and the cells exposed to medium without FCS for 24 hours. Thereafter the cells were cultured for 5 days in complete DMEM/10% FCS in the presence or absence of 5 ng/ml IL-1β, 100 ng/ml TNF-α, or both. Each condition was performed in triplicate. On day 5, the cells were removed from each well with cold trypsin/EDTA, washed once, resuspended in 1 ml of DMEM, and counted with a Coulter counter. Each sample was counted twice. In a few experiments cells were enumerated with both a hemocytometer and Coulter counter with good concordance.

Proliferation

Cells (FLSs, PCs, or chondrocytes) were distributed into 96-well plates (1×10^4/well) in a total volume of 200 μl of complete DMEM/10% FCS. The cells were allowed to adhere for 24 to 48 hours and then washed twice with DMEM. After the final wash, 200 μl of complete DMEM was left in the well for a 24-hour starvation period. At this point, the medium was replaced with 200 μl of cytokines or growth factors in complete DMEM/1% FCS and cultured for an additional 72 hours, and 16 hours before harvesting, [^{3}H]thymidine ([^{3}H]TdR; 1 μCi/well) was added and the cells harvested on a Cambridge Technologies cell harvester. Total radioactivity was determined by liquid scintillation counting. All experiments were performed in triplicate.

Jurkat Binding

Confluent FLS or PC monolayers were grown in 96-well plates. The T cell lymphoblastoid line Jurkat was labeled with ^{51}Cr (50 μCi/10^8 cells) for 2 hours and then washed sequentially with RPMI 1640 plus 10% FCS, PBS plus 1 mmol/L EDTA, and RPMI 1640 plus 1% BSA. ^{51}Cr-Labeled Jurkat cells were resuspended in RPMI plus 1% BSA and 5×10^4 cells/well added to FLS or PC monolayers that had been washed with RPMI 1640 plus 1% BSA. For inhibition experiments, either labeled Jurkat cells or adherent FLSs or PCs were preincubated for 20 minutes at 4°C with MAbs to adhesion molecules: LB3, 1 (control anti-MHC class II) or P4H9 (anti-β_2), obtained from T. Springer, Harvard Medical School (Boston, MA) or P3H12 (anti-VCAM-1). Jurkat cell adhesion to FLSs or PCs was allowed to proceed for 15 minutes at 37°C followed by the removal of unbound cells by washing three times with RPMI plus 1% BSA. Bound Jurkat cells were lysed with 0.1% sodium dodecyl sulfate, and radioactivity in cell lysates was analyzed in a gamma counter.

Collagenase Production

PCs were cultured to near confluence in 6-well plates with 1 ml of 1% FCS medium with or without IL-1β (1 ng/ml) or TNF-α (100 ng/ml) and incubated at 37°C in a humidified 5% CO_2 atmosphere. Supernatants were harvested after 72 hours and assayed by a solid phase enzyme-linked immunosorbent assay (ELISA) for collagenase employing antibodies provided by Dr. David Taylor. The ELISA consists of a rabbit capture antibody, sheep detection antibody, and signal amplification by biotin-avidin complex formation. The assay can detect as little as 1 ng/ml and was performed as described.[13] Briefly, a 96-well plate was coated overnight at 4°C with 200 μl of rabbit anti-proMMP-1 IgG in PBS followed by overnight blocking at 4°C with 0 to 5% (w/v) BSA in PBS/0.05% Tween 20 (BSA/PBS/Tween). After the wells were emptied and washed in PBS/Tween, either 100 μl of pro matrix metalloproteinase-1 (MMP-1) standard (0.4 to 25 ng/ml) in a 1:21 dilution of horse serum in BSA/PBS/Tween or sample diluted by 1:21 with BSA/PBS/Tween was added, followed by 100 μl of biotinylated anti-human proMMP-1 IgG in a 1:25 dilution of normal sheep serum in 300 mmol/l NaCl, 5 mmol/l $CaCl_2$, 20 mmol/l Tris, pH 7.5. After overnight incubation at 4°C, each well was washed three times in PBS/Tween and once in Tris-buffered saline, and then 200 μl of avidin-biotin complex (Dako) in Tris-buffered saline was added for 30 minutes at room temperature. After three additional washings in Tris-buffered saline and one in 0.5 mmol/l $MgCl_2$ and 0.1 mol/l diethanolamine, pH 9.6, 200 μl of 1 mg/ml *p*-nitrophenyl phosphate (Sigma) was added to each well and, after color development, absorbance was read at 405 nm at approximately 1 hour.

Nitric Oxide (NO) Production

For studies on NO production, chondrocytes (primary culture), PCs, or FLSs are plated in 96-well plates at 50,000 cells per well. The cells were cultured in serum-free DMEM supplemented with L-glutamine and antibiotics for 24 hours in the presence or absence of cytokines (IL-1β and TNF-α) or LPS. The medium was replaced, and 48 hours later, the concentration of nitrites, the stable end products of cellular NO breakdown, in the conditioned media was determined by the Griess reaction using $NaNO_2$ as standard.[14] Briefly, 50 μl of conditioned medium was mixed with 50 μl of Griess reagent (50 ml of 1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylene-diamine dihydrochloride in 25% H_3PO_4) and incubated at room temperature for 5 minutes, and absorbance was read at 550 nm using a Molecular Devices ELISA plate reader. Each pooled sample was assayed in triplicate. All results are expressed as nmoles of nitrates per 10^5 cells.

RNA Isolation

RNA isolation was performed as described.[15] Briefly, total RNA was isolated by a single-step guanidinium thiocyanate-phenol-chloroform method from 500,000 cells. Cells were lysed directly in the flasks using RNA Stat-60 (Tel-Test B, Friendswood, TX) and the samples were processed following the manufacturer's protocol. Poly A+ RNA was obtained with the PolyATract mRNA isolation system (Promega, Madison, WI).

Reverse Transcription

Total RNA (up to 5 μg) was reverse transcribed in a 20-μl volume containing 4 μl of 5X reverse transcription) buffer (GIBCO BRL), 10 mmol/L dithiothreitol, 500 mmol/L dNTPs, 1 μl of random hexanucleotides, 200 U of mouse Moloney leukemia virus reverse transcriptase (GIBCO BRL) and 20 U of RNasin (Promega) for 30 to 120 min at 37°C.

Polymerase Chain Reaction (PCR) for ILA

PCR was performed with 2 μl of the reverse transcription reaction product in a 25- or 50-μl volume with 1 U of *Taq* DNA polymerase (Perkin Elmer Cetus, Emeryville, CA), 140 mmol/L dNTPs, and 10 pmol/L each primer in 10X incubation buffer (100 mmol/L Tris/HCl, 15 mmol/L MgCl, 500 mmol/L KCl, 1 mg/ml gelatin, pH 8.3, at 20°C), 5 U/μl on an Ericomp (San Diego, CA) or a Hybaid-Omnigene thermocycler. On the Ericomp cycler, after a 5-minute denaturation step at 94°C, the reaction proceeded in 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 75 seconds at 72°C, followed finally by 1 cycle of 7 minutes at 72°C. On the Omnigene cycler, a 1-minute denaturation step at 94°C was followed by 35 cycles of 5 seconds at 94°C, 20 seconds at 55°C, and 50 seconds at 72°C with one final cycle of 7 minutes at 72°C.

The following PCR primers were used: included lymphocyte antigen (ILA; 486-bp product) sense, CAT TCC CGG GTC CTT GTA GTA AC (nucleotides 69 to 91), and antisense, CGG TGA TCA TCC TGG CTC TCT CGC AGG GGC (nucleotides 55 to 527); GAPDH (190-bp product) sense, TGG TAT CGT GGA AGG ACT CAT GAC, and antisense, ATG CCA GTG AGC TTC CCG TTC AGC.

PCR for GAPDH was performed on the Omnigene thermocycler using the following program: 1 cycle of 2 minutes at 92°C and 1 minute at 60°C, 25 cycles of 10 seconds at 72°C, 5 seconds at 92°C, and 10 seconds at 60°C, and 1 cycle of 5 minutes at 72°C.

iNOS PCR

iNOS PCR was performed as described.[16] A 2-μl volume of cDNA from the total of 20 μl of reverse transcription product in 20 μl of PCR reaction was mixed with 1 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN), 4 mmol/L dNTPs, 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, and 20 pmol/L each primer. The following amplification protocol was used: 25 cycles for 30 seconds at 94°C, 30 seconds at 60°C, and 60 seconds at 72°C. The primer sequence of the sense strand was nucleotides 2215 to 2147, ACA TTG ATG AGA AGC TGT CCC AC, and that of the antisense was nucleotides 2360 to 2340, CAA AGG CTG TGA GTC CTG CAC. These primers resulted in the amplification of a 236-bp PCR product. Primers for iNOS and ILA span an intron. Genomic DNA thus does not contribute to the PCR signals obtained from cDNA. For the three genes examined here, 25 cycles were used for GAPDH, 25 to 30 cycles for iNOS, and 35 cycles for ILA. These cycle numbers were determined in experiments in which chondrocyte cDNAs were amplified for varying cycle numbers. This calibration showed a linear range of amplification for the indicated cycle numbers.

Collagen PCR

Poly A+-enriched RNA was prepared from cultured cells (at least 10^6 cells) according to a modified protocol of Chomczynski et al.[17] A 2-μg amount of

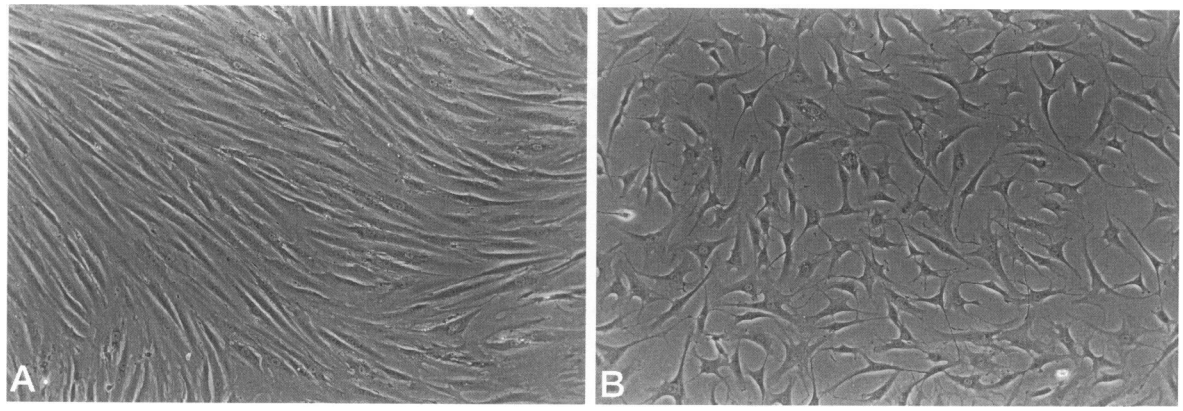

Figure 1. *Phase-contrast microscopic appearance of fibroblast-like synoviocytes and PCs in subconfluent culture.* **A:** *RA synovial fibroblasts (passage 5) have a thin bipolar configuration with occasional long processes, as compared with* **B. B:** *PC line 1 (passage 4) shows more homogeneous, rhomboid-appearing cells. The nucleus is small and hypochromic with prominent nucleoli, often two to three in number. Magnification, ×350.*

RNA was used in the production of cDNA and oligo-dT was used to initiate the synthesis. Superscript reverse transcriptase (400 U; Promega) was used because this enzyme has been engineered to eliminate RNAse H activity, thus resulting in greater full-length cDNA synthesis. A 1/10 volume (2 μl) of the cDNA product and 1 U of *Taq* DNA polymerase (Boehringer Mannheim) were used for the PCR. Thirty PCR cycles (1 minute at 95°C, 1 minute at 60°C, and 1 minute at 72°C) were run followed by extension for 10 minutes, and the amplification products were visualized after electrophoresis on 1.4% agarose gel under ultraviolet illumination in the presence of ethidium bromide.

The following primers were provided by Daniel Cohn (University of California, Los Angeles) (UCLA): collagen 1 primers, sense primer, CGAGAAAGGATCCCCTGGTGCTGAT; antisense primer, TTTACCGGTCTCACCACGGTGA; PCR product size, 327 bp; collagen 2 primers, sense primer, GATGGACAGCCTGGGGCCAA; antisense primer, GGACCTGGTGGACCTTCGGC; PCR product size, 395 bp.

Positive control PCR primers were those that amplify a 450-bp segment of the glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene.

Statistics

Analysis was performed using the Student's *t*-test. Data are presented as mean ± SEM.

Results

Morphology of Pannocytes and Fibroblast-Like Synoviocytes

RA synovial tissues and cartilage were obtained at the time of joint replacement surgery. The synovial tissues were digested with collagenase, filtered, extensively washed, and then cultured on sterile plastic in medium supplemented with 10% FCS. Nonadhering cells were removed and the adherent cells continuously cultivated as FLSs. Tissue obtained from either the cartilage pannus junction or cells removed directly from a cartilage erosion at a distance from the synovium were treated in an identical manner to synovial tissue. Cells obtained from these sites hereafter are referred to as PCs. Six RA cartilage specimens were examined for PCs: three hips, two knees, and one metatarsal head. All yielded similar appearing cells. Despite extensive scraping of eroded areas of five femoral heads removed for advanced osteoarthritis, similar cells were not obtained.

Six PC lines were established and maintained for various periods of time in long-term culture. One, PC-1, was grown continuously through 25 passages before it became senescent; the others were stored after various passages in liquid nitrogen for subsequent study. In contrast to FLSs, which display a thin bipolar configuration with occasional long processes (Figure 1A), PCs have a more homogeneous rhomboid appearance with hypochromic nuclei and two or three prominent nucleoli (Figure 1B). Both PCs and FLSs exhibit contact inhibition and retain their morphology in confluent cultures. Cytospun Giemsa-stained confluent FLSs were heterogeneous in size and larger (12.9 ± 4.2 μm) than the more homogeneous PCs (8.9 ± 0.3 μm; n = 10 fields; $P < 0.006$). Both the appearance and surface phenotype (see below) of PCs were retained through multiple (7 to 15) passages.

Electron Microscopic Appearance

Cells from a multipassaged FLS and the PC-1 line were harvested, washed, pelleted by centrifugation,

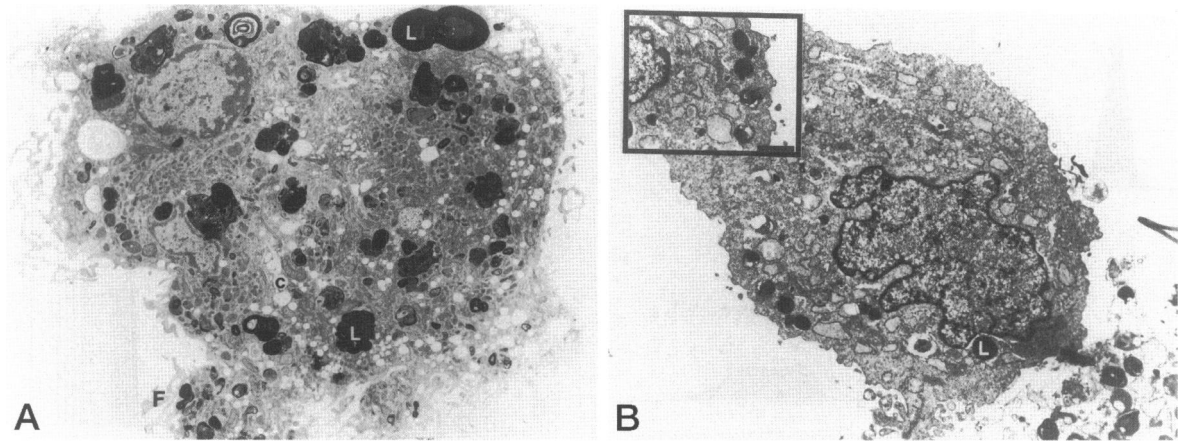

Figure 2. A: *Low magnification transmission electron micrograph of a fibroblast-like synoviocyte cultured from RA synovium. The membrane is folded into multiple well developed filopodia (F). The cytoplasm contains numerous organelles. There is a marked increase in rough endoplasmic reticulum with dilated cisternae (C). Lysosomal bodies (L) containing whorled membranes and electron-dense particles are a regular feature. Magnification, ×7500.* B: *Low magnification transmission electron microscopic appearance of a PC. PC line 1 (passage 4) has a smooth surface membrane without filopodia and does not show the numerous cytoplasmic organelles found in FLSs. The cytoplasm contains many lysosomes and residual bodies (see* ***inset****; magnification ×20,000). Magnification, ×7500.*

and fixed for transmission electron microscopy. The ultrastructure of FLS prepared as single cell suspensions looks similar to earlier descriptions of cultured synovial fibroblasts and type B-fibroblast-like synoviocytes in tissue. [7,18] The FLSs had an irregular surface with multiple, well developed filopodia (Figure 2A). Their cytoplasm contains numerous organelles and a marked increase in endoplasmic reticulum, and their cisternae are invariably dilated (not shown). The ultrastructural appearance of PCs was different from FLSs. They had smooth surface membranes, lacked the numerous cytoplasmic organelles found in FLSs, and showed a lower nucleus to cytoplasm ratio. The nucleus was often irregular and easily distinguished. The cytoplasm of PCs is filled with residual bodies (Figure 2B). Both cell types have lysosomal bodies that contain whorled membranes and electron-dense particles.

Surface Phenotype of PCs

The constitutive expression of surface antigens on PCs was determined on cytospun samples by immunohistology. PCs did not show surface antigens of hematopoietic stem cells (CD34 and CD45), monocytes (CD11b and CD14), T lymphocytes (CD3), or activated endothelial cells (E-selectin). PCs and FLSs were examined by FACS. They did not stain with monoclonal antibodies to von Willebrand factor (factor VIII) or CD31 (PECAM); both were IgG_1 antibodies and were compared with an isotype-specific mouse Ig (data not shown). Isolated PCs did not consitutively express HLA-DR when analyzed by FACS, but class II antigens could be induced with a 72-hour exposure to 100 U of IFN-γ, similar to that observed with FLSs (data not shown).[5] Granulocyte/macrophage colony-stimulating factor treatment had no effect on HLA-DR expression by PCs. Neither FLSs nor PCs showed the distinctive actin array that has been described in myofibroblasts, a late-stage fibroblast thought to be responsible for wound contracture and identified by large amounts of intracellular actin.[19,20]

PCs constitutively express both ICAM-1 (CD54) and VCAM-1. The latter is particularly conspicuous on PCs and present in significantly greater amounts than seen on FLSs. Four separate lines of PCs were 64.75% to 80.3% VCAM-1 positive with linear mean fluorescence ranging from 16.9 to 27.9 (21.5 ± 2.43). For comparison, thirteen different FLSs were 23.53 ± 4.2% VCAM-1 positive, as previously reported by us,[21] with a linear mean fluorescence of 7.85 ± 1.40 (P = 0.011 compared with PCs). A typical experiment comparing two PC lines to a companion culture of FLSs (each fourth passage) is shown in Figure 3. A minority of FLSs display modest amounts of VCAM-1, whereas the majority of PCs routinely showed large amounts of VCAM-1. The expression of this surface molecule on PCs remains unchanged through multiple passages (data not shown).

The functional state of the VCAM-1 on PCs was evaluated by determining the binding of radiolabeled Jurkat cells to tissue culture plates containing a monolayer of PCs. Jurkat cells display large amounts of surface $\alpha 4\beta 1$ integrin, a major ligand for both VCAM-1 and CS-1 fibronectin.[22] As previously shown, although Jurkat binding to resting FLSs is

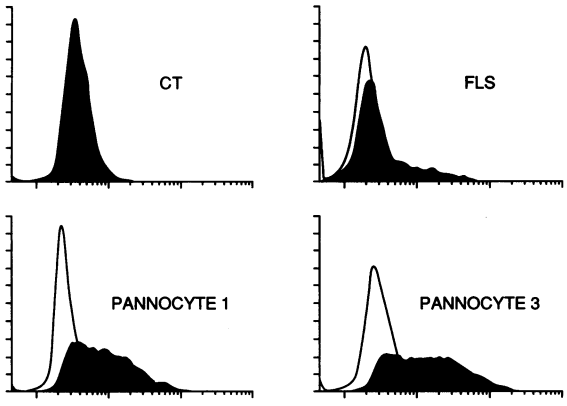

Figure 3. *FACS analysis. A comparison of VCAM-1 surface expression on FLSs and PCs. The outlined histograms show resting unstimulated cells; the shaded areas are stained with either an irrelevant, isotype-matched IgG control* (upper left) *or the anti-VCAM-1 antibody (P3H12) on FLSs* (upper right) *and PC line 1 and PC line 3* (lower left: *and* right, *respectively). Cell numbers are displayed on the vertical axis and fluorescence intensity on a logarithmic scale on the horizontal axis.*

mediated by α4 integrin, VCAM-1 plays only a minor role (presumably binding is mediated through CS-1). In contrast, the VCAM-1 on resting PCs is responsible for approximately one-half of the Jurkat cell binding (see Figure 4).

Cell Growth

Chondrocytes from middle-aged adults seldom propagate beyond 6 to 8 passages in tissue culture. By this time they have assumed the appearance of fibroblasts and enter senescence.[23,24] FLSs can be retained in culture through multiple passages, but after 10 to 12 passages, even when supplemented with growth factors, their doubling time gets progressively longer; they develop signs of senescence and ultimately die. In contrast, the original PC line (line 1) was maintained through 25 passages with little change in morphology or growth rate. (None of the other PC lines were carried for more than 8 passages before being frozen).

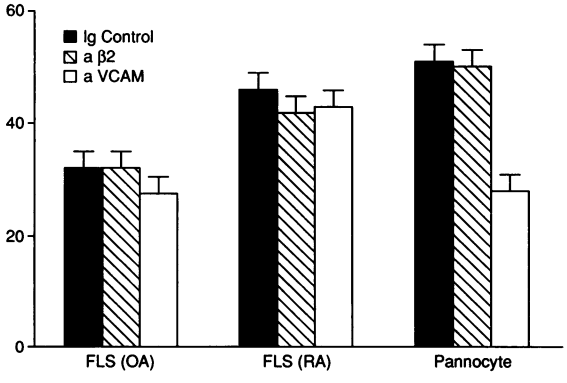

Figure 4. *A representative experiment showing the effect of an anti-VCAM-1 monoclonal antibody (P3H12) on adhesion of Jurkat cells to monolayers of FLSs from osteoarthritis (OA), (RA), or PC line 2 (passage 5). Antibodies were used to block adherence, as described in Materials and Methods. The percentage of cells bound are shown on the* x *axis. The control includes medium and an unrelated monoclonal IgG antibody to MHC class II (LB3.1) or an anti-β2 integrin (P4H9). $P < 0.05$ compared with IgG control.*

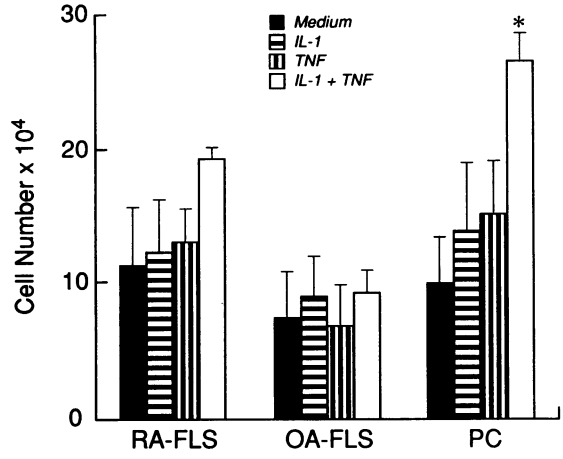

Figure 5. *Growth of synoviocytes and PCs. Subconfluent cultures of either RA-FLS, OA-FLS, or PC line 1 were cultured for 5 days in complete medium in the presence of the indicated factors* (5 ng/ml IL-1β and 100 ng/ml TNF-α). *At 5 days, the cells were harvested and enumerated. The data represent the mean ± SEM for experiments with five different FLS (RA) lines and three separate FLS (OA) lines. PC line 1 was studied five times, including one experiment with the simultaneously obtained FLS (RA) line 227. Growth rates were similar, except when the combination of cytokines caused a significant increase in growth of PC as compared with the FLS lines ($P = 0.015$).*

Subconfluent cultures of FLSs from either RA or osteoarthritis tissues and PCs were established in 24-well culture dishes and maintained in complete DMEM containing 10% FCS. After 5 days, the cells were harvested and enumerated (Figure 5). The cells from the various sources increased in number at approximately the same rates from an initial 1×10^4 to $8.1 \pm 1.1 \times 10^4$ for RA FLSs (5 lines) to $4.0 \pm 0.8 \times 10^4$ for osteoarthritis (3 lines) FLSs and to $7.1 \pm 0.9 \times 10^4$ for PC line 1 ($P > 0.05$). The addition of IL-1β or TNF-α alone did not significantly influence the growth rate in any population, but the combination of the two cytokines caused a synergistic effect on PC growth ($16 \pm 1.3 \times 10^4$; $P < 0.015$; n = 5) as compared with FLSs from either source (Figure 5).

Cell Proliferation

Mesenchymal cells have distinctive proliferation patterns when cultured in the presence of various growth factors. The profile of responses to FGF, TGF-β, PDGF, and IL-1β alone or in combination were examined in chondrocytes, FLSs, and PCs. TGF-β is the primary *in vitro* mitogen for chondro-

Table 1. *Comparison of the Mitogenic Effects of Growth Factors on Chondrocytes and Pannocytes*

Factor (ng/ml)	[^{3}H]TdR cpm	
	PCs	Chondrocytes
Medium	782 ± 31	199 ± 26
TGF-β (10)	1990 ± 18	1635 ± 22
TGF-β (1)	2352 ± 14	2111 ± 24
PDGF (50)	2538 ± 12	459 ± 33
PDGF (10)	3331 ± 52	182 ± 86
PDGF (1)	629 ± 29	254 ± 18

Results are means of triplicate determinations. A total of 3 × 10^5 cells were cultured for 72 hours in medium plus 1% FCS in the presence or absence of various concentrations of growth factors TGF-β and PDGF.

cytes, whereas PCs respond well to both PDGF and TGF-β (Table 1).

Exposure of PCs and FLSs to growth factors results in a similar pattern of proliferation, although the [^{3}H]TdR uptake by PCs is generally greater. However, the effect of IL-1β on these two cell types is different. A summary of one series of experiments is shown in Figure 6. Treatment of FLSs with IL-1β alone caused a modest increase in thymidine incorporation and an additive effect in the presence of TGF-β. By contrast, PCs exposed to IL-1β alone did not proliferate, and when added to TGF-β, the IL-1β significantly reduced PC proliferation (PCs *versus* FLSs; $P < 0.01$; average of five experiments, each with three different PC lines). Similar antiproliferative effects of IL-1β are seen with primary chondrocytes (data not presented).

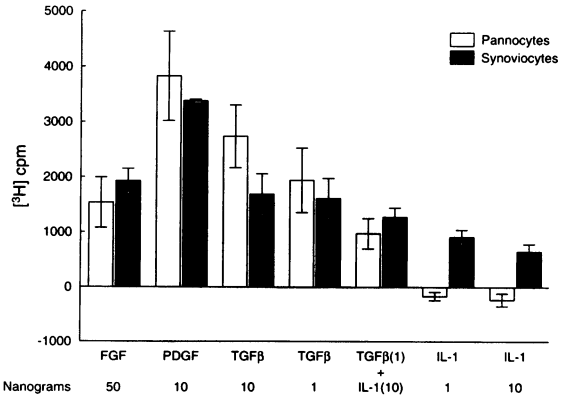

Figure 6. *A comparison of growth factor responses of synoviocytes (■) and pannocytes (□) cultured under identical conditions with the same concentrations of growth factors (ng/ml) for 72 hours. Proliferation was measured by [$_3$H] thymidine uptake during the final 18 hours of culture. Results presented are means ± SEM for five experiments done in triplicate on six different RA-FLS lines (each less than passage 8). Results for PC line 5 (passage 5) are the means ± SEM for triplicate determinations of five separate experiments. Medium controls were 564 ± 37 cpm for PCs and 721 ± 84 cpm for RA-FLSs.*

Table 2. *Pannocytes Produce Collagenase*

Cell Line	Collagenase (ng/ml)		
	Medium	IL-1	TNF-α
PC-1	39	71	51
PC-3	30	51	53
PC-5	39	119	51

Results are averages of duplicate determinations.

Collagenase Production

To determine whether PCs produce collagenase, cells were cultured in the presence or absence of IL-1β (1 ng/ml) or TNF-α (10 ng/ml) for 72 hours and the supernatants assayed using an ELISA specific for human MMP-1. As shown in Table 2, PCs, like FLSs, constitutively produced small amounts of collagenase. Production of collagenase was increased by both IL-1β and TNF-α.

Genotype of PCs

Collagen

The collagen genes expressed by primary chondrocytes, FLSs, and PCs was addressed with reverse transcriptase PCR. As seen in Figure 7, second-passage chondrocytes contained the mRNA for both type II and type I collagen, whereas fourth-passage PCs and FLSs (RA) expressed only the type I collagen gene.

ILA

ILA, a member of the human nerve growth factor/TNF/apo-1/fas receptor family, and the homologue of the murine 4-1BB in mesenchymal cells, was origi-

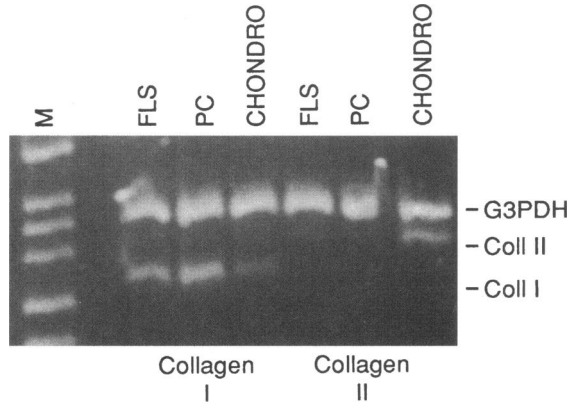

Figure 7. *Collagen gene expression. RT-PCR was performed as described in Materials and Methods using primers for G3PDH, type I collagen, type II collagen. Note that the PC line expresses only type I collagen, whereas the chondrocytes (CHONDRO) express both forms.*

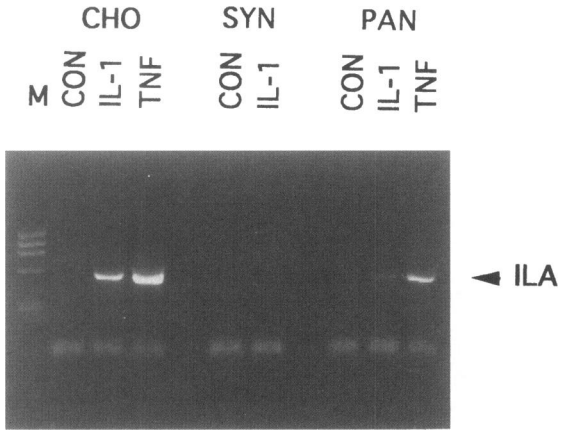

Figure 8. *Regulation of ILA mRNA expression in resting (CON) and stimulated (IL-1, 5 ng/ml; TNF-α, 5 ng/ml), articular cartilage chondrocytes (CHO), synovial fibroblasts (SYN), and PCs (PAN). Molecular weight markers (M) are as indicated.*

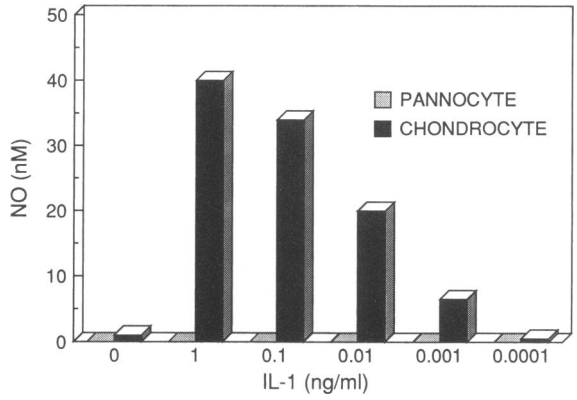

Figure 9. *PCs do not produce NO constitutively or when stimulated with IL-1β. A representative experiment showing NO production (measured by Griess reaction) by first-passage chondrocytes and PC line 5 (passage 4) under the stimulus of varying concentrations of IL-1β is shown.*

nally identified as a mRNA present in activated but not in resting T lymphocytes.[15] Subsequently, ILA expression was found in cytokine-stimulated chondrocytes, but the mRNA could not be identified in resting or induced skin or synovial fibroblasts.[25] Four PC lines were examined for constitutive and cytokine-induced ILA mRNA expression. As seen in Figure 8, chondrocytes (first passage) and PCs each expressed the gene, whereas the one companion FLS line (227) could not be stimulated to produce detectable ILA mRNA. A separate study with synovial fibroblasts demonstrated that the lack of ILA expression was not due to mRNA degradation. Furthermore, the cells responded to IL-1 stimulation with increased expression of IL-6 mRNA (data not shown). The influence of duration of cultures on ILA mRNA expression was determined in several different passages of PC lines 1 and 5. It was observed that ILA mRNA was constitutively expressed only in an early passage of line 1; thereafter, the message could be induced by IL-1β or TNF-α. In still later passages, ILA superinduction was seen with the combination of IL-1β plus cycloheximide (data not shown).

Nitric Oxide

Chondrocytes, unlike other mesenchymal cells, make small amounts of NO constitutively, and their NO production increases dramatically after IL-1β treatment.[26] In five separate experiments, performed under identical conditions with four PC lines, neither constitutive nor IL-1β-stimulated NO production was detected. Figure 9 shows a representative experiment. NO is not made by either resting or stimulated FLSs.[26]

Inducible Nitric Oxide Synthase

Human articular chondrocytes make NO and express the iNOS gene when stimulated by cytokines,[16] but neither resting nor stimulated dermal fibroblasts or FLSs release NO or express the iNOS message.[26] PCs are unusual because, despite their consistent failure to release detectable NO, each of the five PC lines tested could be induced to express the iNOS gene by exposure to IL-1β (5 ng/ml) or IL-1β plus cycloheximide (50 μg/ml. Figure 10).

Discussion

The aim of this study was to characterize a novel cell isolated from destructive lesions in the rheumatoid joint and compare it with the two major mesenchymal cells in the joint, namely, type B synovial fibroblasts

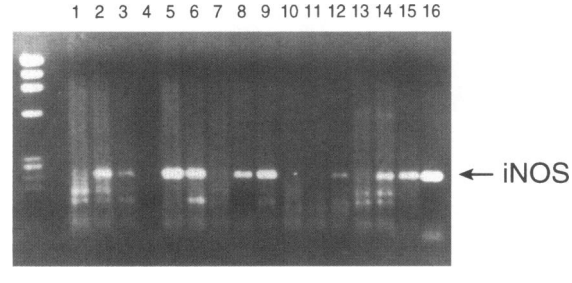

Figure 10. *Regulation of iNOS mRNA expression in resting and stimulated PC lines.* **Lane 1**, *PC line 3 (passage 4);* **lane 4**, *PC line 6 (passage 7);* **lane 7**, *PC line 5 (passage 3);* **lane 10**, *line 2A (companion RA-FLS to PC line 2C) (passage 7);* **lane 14**, *PC line 2C (passage 5). PCs were stimulated with either IL-1 (5 ng/ml), shown in* **lanes 2, 5, 8, 11, and 14** *), or with IL-1 (5 ng/ml) and cycloheximide (50 μg/ml), shown in* **lanes 3, 6, 9, 12, and 15**. **Lane 16** *is a positive control.*

and primary chondrocytes. The morphology of adherent cells that were removed from lesions in RA cartilage was intermediate between the thin, biopolar shape of long-term cultured FLSs and the initially spheroid and then flat, polygonal appearance of primary chondrocytes in culture. Much like chondrocytes, they have a discrete, small nucleus with one or two dark-staining nucleoli. The nucleoli of FLSs on the other hand, were less discrete and more plentiful.

Six PC lines were initially established. One (PC-1) was maintained through 25 passages without much change in morphology, doubling time, or VCAM-1 staining. The remaining lines were stored in liquid nitrogen at various passages and used in the studies reported herein. Such prolonged stable growth is not characteristic for chondrocytes, which become fibroblastic and then senescent after the 7th or 8th passage,[23,24] whereas FLSs seldom survive beyond 12 to 15 passages. It should be stressed, however, that as yet there has been no attempt to demonstrate clonality in the PC lines.

The growth factor responsiveness of primary cultured human articular chondrocytes and FLSs are well defined and can be summarized as follows: in the former, TGF-β is the most potent mitogen, whereas PDGF, bFGF, and insulin-like growth factor lag behind.[26] DNA synthesis by FLSs, in contrast, is usually greatest with PDGF and somewhat less with the other growth factors. As chondrocytes dedifferentiate (less than four passages), they develop a profile of proliferative responses that is more like FLSs. Guerne et al[27] showed that chondrocytes, dedifferentiated chondrocytes, and FLSs exposed to IL-1β proliferate in a predictable manner. With FLSs, for instance, IL-1β is often mitogenic by itself and acts in an additive or synergistic fashion with PDGF or TGF-β to enhance DNA synthesis. Dedifferentiated chondrocytes act the same, but the response of early-passage chondrocytes is just the opposite; namely, IL-1β significantly down-regulates proliferation in the presence of the primary mitogen TGF-β. When PCs are cultured with comparable amounts of PDGF or TGF-β, their proliferation is approximately the same, but the addition of IL-1β reduces proliferation by 50% or more. In this regard, therefore, PCs are not like either FLSs or dedifferentiated chondrocytes; rather, they behave like primary chondrocytes.

Another distinguishing feature that was used to discriminate PCs from FLSs was their constitutive expression of large amounts of surface VCAM-1. Most of the PC lines showed >75% of cells staining brightly for VCAM-1. VCAM-1 is a 90 to 110-kd glycoprotein, first identified and characterized on cytokine or LPS-treated cultured human umbilical vein endothelial cells.[28,29] It mediates the adhesion of inflammatory cells to activated vascular endothelium through an interaction with its counter-receptor $\alpha 4\beta 1$.[22,30] VCAM-1 is present on vascular endothelial cells in the inflamed synovium, but the immunohistological staining of synovial lining cells is even more intense.[21] FACS analysis of cultured FLSs show only 15 to 25% of cells consitutively expressing small amounts of VCAM-1. This contrasts with dermal fibroblasts, which express little or no VCAM-1.[21] Primary chondrocytes show a biphasic staining pattern with anti-VCAM-1; the majority (more than two-thirds) are low (VCAM-1 dull) in FACS analysis, whereas a small percentage are bright staining (unpublished observation). In previous studies using FLSs, antibodies to $\alpha 4\beta 1$ and VCAM-1 inhibit the binding of Jurkat cells (which express $\alpha 4\beta 1$) to cytokine-stimulated but not to resting FLSs.[21] In identical experiments with PCs (see Figure 5), VCAM-1-mediated Jurkat cell binding occurs in the absence of cytokine treatment, presumably because of the significantly greater amount of consitutively expressed VCAM-1.

The major fibrillar collagens are types I and II, which are made both *in vivo* and in culture by mesenchymal cells.[31] Early in ontogeny, type I is the predominant collagen produced by chondrocytes; mature chondrocytes make type II collagen. With dedifferentiation, chondrocytes assume a fibroblast-like morphology and a type I collagen phenotype.[32] The demonstration that PCs express only the mRNA for type I collagen could be interpreted as 1) they are most like FLSs, 2) they are chondrocytes that have dedifferentiated, or 3) they represent chondrocytes at a very early stage of development.

An argument for assigning PCs to a chondrocyte lineage comes from the genotyping with ILA and iNOS. The former is a recently identified member of the human nerve growth factor/TNF receptor family. ILA was originally identified as a mRNA present in activated but not resting T lymphocytes.[15] It was later found that among mesenchymal cells only primary cultured chondrocytes expressed the gene for ILA, not skin or synovial fibroblasts.[25] The most potent inducers of ILA in chondrocytes are IL-1β, TNF-α, leukemia inhibitory factor, and LPS, but after four to six passages, these cytokines can no longer induce the expression of ILA mRNA, except in the presence of a protein synthesis inhibitor, suggesting that mRNA destabilization is responsible for the lack of ILA in late-passage cells. A similar pattern of ILA mRNA expression is observed in early- and late-passage PCs.

Table 3. *Comparison of* in vitro *Characteristics of Cells Likely Involved in RA Cartilage Destruction*

			Function				Genotype			
	Morphology	Phenotype, VCAM-1	Primary growth factor	Growth factor plus IL-1	NO production	Collagenase	ILA	iNOS	Collagen type I	Collagen type II
Synovial fibroblasts	Spindle	Dull (25%)	PDGF	↑	0	+	0	0	+	0
Primary chondrocytes	Spheroid, polygonal	Dull (60%)	TGF-β	↓	+	+	+	+	+	+
PCs	Rhomboid	Bright (>70%)	PDGF/TGFβ	↓	0	+	+	+	+	0

FLSs do not synthesize or secrete NO constitutively or when stimulated with IL-1β, TNF-α, leukemia inhibitory factor, or LPS, whereas these cytokines induce NO synthesis in primary chondrocytes.[26] The mRNA for the iNOS gene is present in early-passage chondrocytes but not FLSs. Both iNOS gene expression and NO production are lost in late-passage chondrocytes.[33] PCs are distinctive because they do not produce NO, despite expressing iNOS mRNA.

In considering the lineage of PCs, a number of factors need to be weighed (see Table 3). First, PCs are remarkably stable and have a longer life span than either FLSs or chondrocytes. Second, PCs express type I collagen genes and fail to make NO, which could be cited as evidence of a link with FLSs. However, the negative influence of IL-1β on PC growth and the inducible expression of mRNA for ILA and iNOS are not consistent with a FLS lineage and favor the idea that PCs are more like chondrocytes, but not typical primary chondrocytes. PCs might be dedifferentiated chondrocytes, but this is not likely because they retain susceptibility to down-regulation of proliferation by IL-1β and maintain a distinctive appearance in culture. A third alternative is that PCs are chondrocyte precursors.

Two recognized repositories for mesenchymal stem cells are the bone marrow and the periosteum.[34] This could be relevant to cartilage destruction in RA because, although detailed morphological information about the cellular events at the cartilage-pannus junction is abundant, unfortunately, these observations come almost exclusively from well established or advanced disease. The initial events in RA, however, are obscure. In the knee, and perhaps other joints, an avascular tongue of fibroblasts normally extends from the synovium onto the cartilage surface. With the onset of disease, cells of unknown origin are thought to arise from the perichondral (or periosteal) synovial membrane at the joint margin and dissect beneath the layer of FLSs. The cartilage immediately below them appears digested and depleted of proteoglycans, and there is a paucity of chondrocytes.[35] These earliest events are believed to prepare the cartilage for a subsequent invasion by blood vessels, FLSs, macrophages, and occasional neutrophils and lymphocytes, ie, the granulation tissue usually referred to as pannus.

Some investigators have attributed the initial invasion of cartilage to primitive mesenchymal cells, distinct from conventional synoviocytes, with morphological features reminiscent of the invading cells in the spontaneous arthritis of MRL/lpr mice[36,37] and the PCs described in this report. This process, called mesenchymoid transformation, is invasive, destructive, and claimed to be unique to RA. The responsible elements arise from the synovial stroma and appear as a homogeneous population of cytoplasm-rich cells with conspicuous vesicular nuclei containing one to two prominent nucleoli and a prominent rough endoplasmic reticulum.[38] Several additional properties of PCs make them attractive candidates for RA tissue injury. These include the ability to produce collagenase and, as compared with FLSs, they bind to cartilage matrix proteins more avidly and migrate faster in an *in vitro* experimental wound assay (manuscript in preparation). Alternatively, they could represent cells arising from the adjacent bone marrow, trying unsuccessfully to reconstitute cartilage. However, our failure to demonstrate PCs in atrophic and destructive lesions of cartilage in osteoarthritis does not support this idea.

PCs have some features in common with FLSs and chondrocytes but can clearly be distinguished from these cell types. The more rapid growth and prolonged life span *in vitro* suggest that PCs may represent an earlier stage in mesenchymal cell differentiation.

Acknowledgments

Aung Lwin, David Boyle, Michelle Yeo, and Jacqueline Quach provided excellent technical support.

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