Fibroblast-like Synovial Cells Derived From Synovial Fluid

JUDITH A. STEBULIS, RONALD G. ROSSETTI, FRANCISCO J. ATEZ, and ROBERT B. ZURIER

ABSTRACT. ***Objective.*** To obtain fibroblast-like synovial cells (FLS) from synovial fluid (SF).

Methods. SF aspirated from joints of patients with rheumatoid arthritis (RA), other types of inflammatory arthritis, and osteoarthritis (OA) was centrifuged and the resulting cell pellet resuspended in growth medium. After 2 days, nonadherent cells were removed. FLS were also cultured from surgical specimens of synovial tissue (td-FLS). Phenotype characterization of fluid derived FLS (fd-FLS) was accomplished by flow cytometry and immunohistochemistry staining. Tumor necrosis factor-α (TNF-α) induced interleukin 6 (IL-6), IL-8, and cyclooxygenase 2 (COX-2) mRNA levels were assessed.

Results. Second and later passage fd-FLS exhibited uniform fibroblast-like morphology. Fd-FLS and td-FLS expressed a similar profile of cell surface antigens including the fibroblast marker Thy-1. Less than 2% of either cell type expressed surface markers characteristic of dendritic cells, phagocytic cells, T cells, or leukocytes. Immunohistochemistry staining revealed the presence of fibroblast products prolyl-4 hydroxylase, procollagen I, and procollagen III in both culture types. TNF-α induced increases in IL-6, IL-8, and COX-2 mRNA were suppressed by dexamethasone in both fd-FLS and td-FLS.

Conclusion. FLS can be cultured from SF. The fibroblast phenotype was confirmed by analysis of surface antigens and intracellular proteins. Inflammatory mediators produced after stimulation of both fd-FLS and td-FLS were suppressed by dexamethasone. In addition to providing a more accessible source of FLS, fd-FLS may also facilitate study of synovial cells in early RA when tissue specimens are not readily available. (J Rheumatol 2005;32:301–6)

Key Indexing Terms:
SYNOVIAL CELLS SYNOVIAL FLUID FIBROBLASTS RHEUMATOID ARTHRITIS

Pannus formation is the hallmark pathologic change seen in joints of patients with rheumatoid arthritis (RA). The proliferating, highly activated synovial lining develops redundant folds of tissue that invade and destroy cartilage and bone at the joint margins. Synovial proliferation occurs in other forms of inflammatory arthritis, but pannus formation and marginal erosions are unique to RA. Therapies that control inflammation limit pain and swelling but do not necessarily prevent synovial proliferation and progressive joint damage. Agents that block tumor necrosis factor-α (TNF-α) or interleukin 1ß (IL-1ß) have shown promise in terms of joint preservation, but symptoms recur when therapy is withdrawn[1]. Thus, while effective in controlling disease in many patients, these therapies may not address the underlying pathology. Synovial cells from patients with RA exhibit characteristics of transformed cells, including unregulated growth, loss of contact inhibition, and a pattern of oligo- or monoclonal expansion[2-5], but they exhibit little evidence of active cell division[6,7]. The precise origin of proliferating RA synovial cells and mechanisms underlying their transformation to an aggressive, invasive phenotype remain areas of active study. These investigations would be facilitated by better access to synovial cells. The difficult logistics often involved in obtaining tissue are well known, and commercially available cell lines are not well accepted substitutes for fresh tissue. We initiated studies to determine whether fibroblast-like cells (FLS) could be obtained from synovial fluid (SF).

From the Department of Medicine, Rheumatology Division, University of Massachusetts Medical School, Worcester, Massachusetts, USA.

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J.A. Stebulis, MD, Fellow; R.G. Rossetti, MPH, Research Associate; F.J. Atez, BS, Medical Student; R.B. Zurier, MD, Professor of Medicine.

Address reprint requests to Dr. J.A. Stebulis, Department of Medicine, Rheumatology Division, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655.
E-mail: judith.stebulis@umassmed.edu

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MATERIALS AND METHODS

Culture of cells from SF. SF was aspirated from joints of patients with RA, inflammatory polyarthritis, and osteoarthritis (OA). Fluid was collected in heparinized syringes, then centrifuged at 1200 rpm for 15 min. The resulting cell pellet was resuspended in 7 ml of growth medium [minimal essential medium (MEM) with 15% heat inactivated fetal bovine serum (FBS), 1% nonessential amino acids, 1% penicillin/streptomycin solution] and plated in 25 ml tissue culture flasks. Cultures were incubated at 37°C with 5% CO_2 for 24 to 48 h, after which medium was aspirated and cultures

were washed with phosphate buffered saline (PBS) to remove nonadherent cells. Growth medium was replaced every 3 to 4 days. After 10 to 14 days adherent cells were removed from flasks by trypsinization, washed, and transferred to 6 well tissue culture plates in fresh growth medium. Fluid derived FLS (fd-FLS) were passaged (split 1:3) when they reached confluence, generally at 10 to 14 days. Passages 2 through 6 were used for experiments.

Tissue derived FLS (td-FLS). Synovial tissue was obtained from knee joints of patients with RA or OA at surgery for joint replacement. Synovial tissue was minced and placed in tissue culture dishes with growth medium. After 2 to 4 days tissue was removed and adherent cells were washed with PBS. Td-FLS were maintained in growth medium at 37°C with 5% CO_2 and passaged (split 1:3) when they reached confluence. Td-FLS from passages 3 through 8 were used in experiments.

Flow cytometry. Fd-FLS and td-FLS (passages 2 through 4) were released from culture by trypsinization, washed once, and resuspended in MEM with 1% FBS. Cells were then incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated antibodies at 37°C for 1 h [CD33-FITC: SC-19660, Santa Cruz Biotechnology, Santa Cruz, CA, USA,; CD86-FITC: SC-19617, Santa Cruz; CD14-FITC: SC-1182, Santa Cruz; CD90 (Thy-1)-FITC: AHU0058, BioSource International, Camarillo, CA, USA; CD3-FITC: PN IM1281, Immunotech, Wildwood, MO, USA; CD11B-FITC: PN IM0530, Immunotech; CD32-PE: PN IM1935, Immunotech). After 3 washes the cells were fixed in a 1.25% paraformaldehyde solution and analyzed by flow cytometry. Isotype matched IgG1 labeled cells (IgG1-FITC: Iotest 679.1Mc7; IgG1-PE: Iotest 679.1Mc7) and unlabeled cells were used for negative controls.

Immunohistochemistry. Passages 3 through 6 fd-FLS (3 RA and 2 OA cultures) and td-FLS (2 RA and 1 OA culture) in 12 well culture plates were fixed with acetone for 15 min, washed twice with PBS, then incubated for 1 h in a humid chamber with antibodies to prolyl-4-hydroxylase, procollagen I, or procollagen III (prolyl-4-hydroxylase mAb to 5B5 subunit: ab8737, Abcam, Cambridge, UK; procollagen I: MAB1912, Chemicon, Temecula, CA, USA; procollagen III: AB764, Chemicon). Cells were washed 3 times with PBS, then incubated for an additional hour with isotype matched horseradish peroxidase (HRP) conjugated secondary antibodies (anti-rat IgG: A5795, Sigma, St. Louis, MO, USA; anti-rabbit IgG: A0545, Sigma; anti-mouse IgG: ab6728-1, Abcam). After 3 additional washes the HRP reaction was developed with diaminobenzadine per manufacturer's instructions (DAB Enhanced Liquid Substrate System, Sigma D 3939). The primary antibody was omitted in negative controls.

Functional studies. Cultures of fd-FLS and td-FLS from patients with RA were grown to confluence in 6 well culture plates. Cells rested in low serum medium (MEM with 0.5% FBS) for 24 h before the experiment. Cells were treated overnight with 2 µM dexamethasone, then stimulated with 1 ng/ml TNF-α. Whole-cell lysates were collected 4 h after stimulation and analyzed by ELISA for IL-6, IL-8, and COX-2 gene expression (Quantikine Colorimetric mRNA Quantitation kit, R&D Systems, Minneapolis, MN, USA).

RESULTS

SF cultures. After removal of nonadherent cells on Day 2, primary cultures of SF contained stellate, spindle-shaped, and large round cells consistent with a mixed population of fibroblasts and macrophages (Figure 1A). Smaller numbers of macrophage-like cells remained after the first passage, but second and later passage cultures consisted almost entirely of stellate or spindle-shaped cells (Figure 1B). In most cultures, spindle-shaped cells appeared to grow from dense cellular clusters, but isolated cells were also seen (Figure 1C). FLS in primary culture were frequently seen close to cells with macrophage morphology (Figure 1D). FLS in several rapidly proliferating RA cultures formed aggregates reminiscent of tissue, with well defined borders and nonadherent villous projections that floated freely in the culture medium (Figure 2). Non-RA cultures generally did not form tissue aggregates. Cells failed to proliferate in about 33% of cultures. No differences in SF characteristics were identified that distinguished cultures that proliferated from those that did not, including patient diagnosis and treatment, initial fluid volume, or cell counts. Cultures were established from tenosynovial aspirates of less than 1 ml initial volume (Figure 3). Successful cultures proliferated rapidly in passages 1 through 4, generally growing to confluence in 10 to 20 days, but grew more slowly in later passages. Several cultures have been maintained for more than 1 year, reaching passage 9 or 10.

Phenotype characterization by flow cytometry. Fd-FLS and td-FLS expressed a similar profile of cell surface markers: 80% of fd-FLS and 51% of td-FLS expressed the fibroblast marker Thy-1. Fewer than 2% of either cell type expressed surface markers characteristic of dendritic cells, macrophages, monocytes, or leukocytes (CD3, CD11b, CD14, CD33, CD86, Fc RII; Table 1). In a separate analysis of one fd-FLS culture from a patient with RA, CD34 (endothelial cells and fibrocytes), DC-sign (dendritic cells), and CD11a (macrophages, monocytes, lymphocytes) were not detected, and fewer than 5% of cells expressed the MHC class II antigen (data not shown).

Immunohistochemistry. Positive staining for prolyl-4-hydroxylase and procollagens I and III was evident in the majority of cells in all fd-FLS and td-FLS cultures tested, whereas control cultures did not display a positive reaction (Figure 4).

Functional assessment. To determine whether adherent cells derived from SF are functionally similar to traditional td-FLS, cells were stimulated with TNF-α with or without dexamethasone pretreatment, then assayed for expression of

Table 1. FACS analysis for cell surface antigens on fd-FLS and td-FLS. Results represent means of 3 experiments with cells from passages 2 through 4.

Antigen	fd-FLS, % Positive	td-FLS, % Positive	Specificity
Thy-1 (CD90)	80.1	50.7	Fibroblast, neuron
CD86 (B-7)	0.5	0.3	Dendritic cell, monocyte, lymphocyte
CD33	0.3	0.3	Dendritic cell, PB monocyte, BM granulocyte/macrophage precursor
CD14	0.4	0.3	Dendritic cell, monocyte, granulocyte, macrophage
CD32 (FCγRII)	1.8	0.4	Leukocyte
CD3	2.0	1.2	T cell
CD11b	1.7	1.1	Granulocyte, monocyte, lymphocyte

PB: peripheral blood, BM: bone marrow.

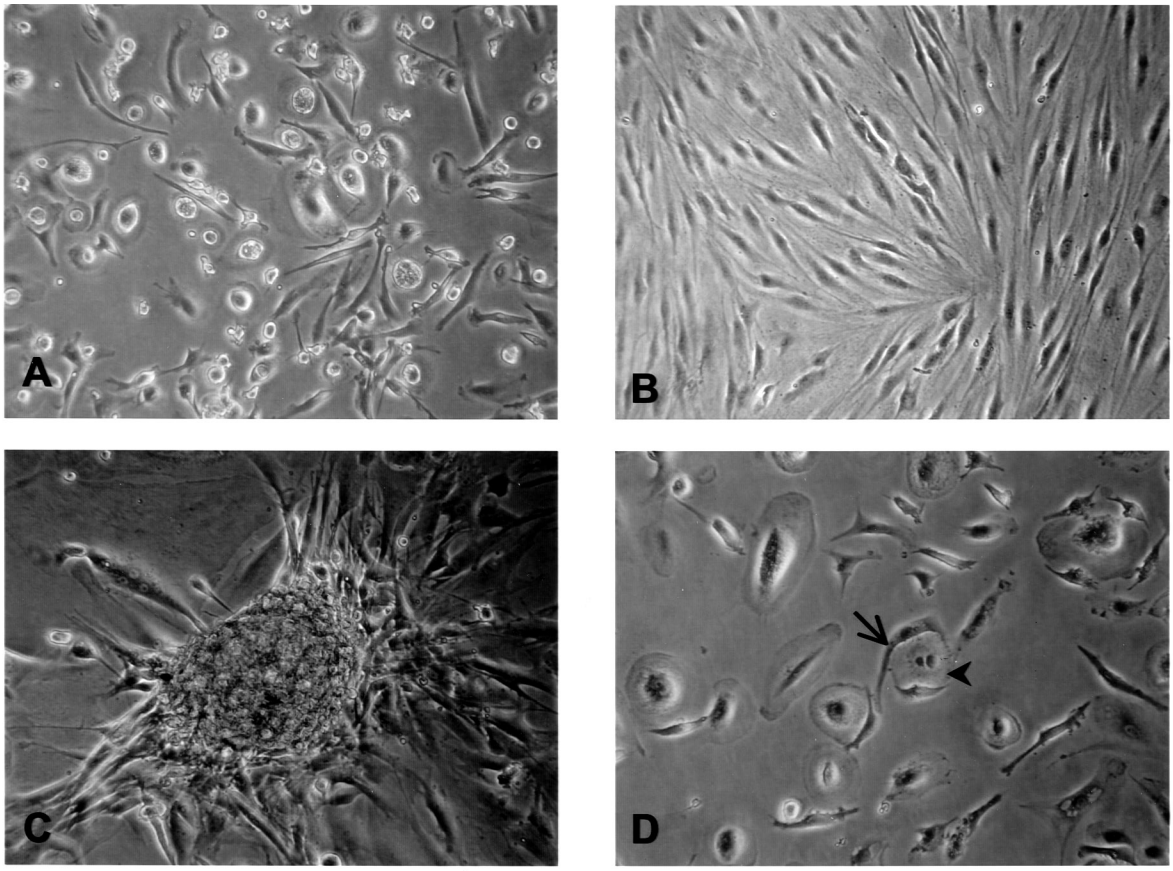

Figure 1. Light microscopic features of SF cell cultures. SF cells isolated and cultured as described in Materials and Methods. A. Primary culture (Day 8) of SF cells from patient with RA. Primary cultures of fd-FLS contain a mixed population of stellate, spindle-shaped, and large round cells. B. Third passage culture of fd-FLS from patient with RA. Second and later passage cultures of fd-FLS consist of a uniform population of spindle-shaped and fibroblast-like cells. Macrophage-like cells were more resistant to trypsinization and were no longer present in cultures after the first passage. C. Spindle-shaped cells emerging from a cellular cluster. D. Contact between fibroblast-like and macrophage-like cells. Spindle-shaped cells (arrow) lie alongside large, round macrophage-like cells (arrowhead) in primary culture of RA SF.

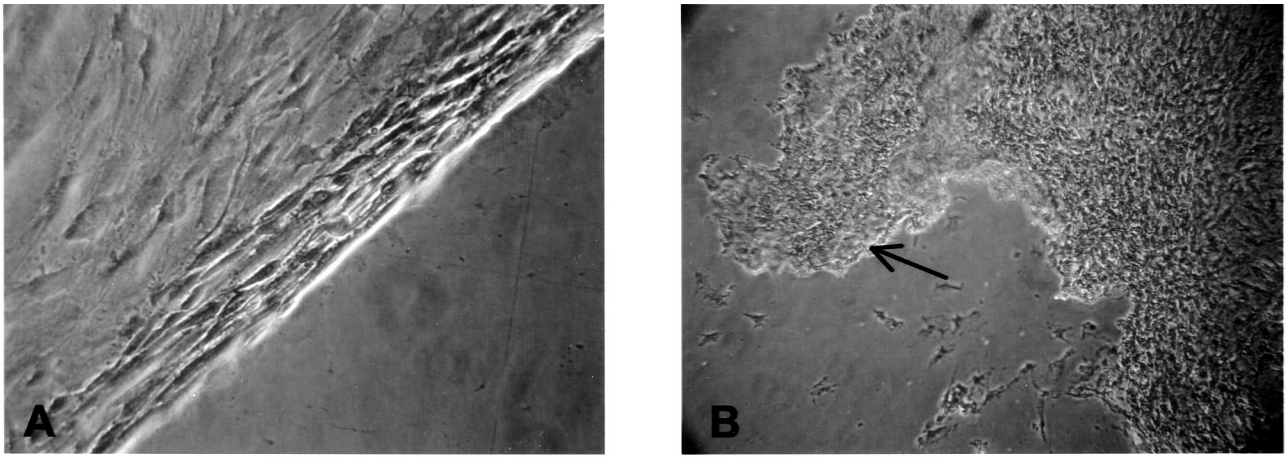

Figure 2. RA fd-FLS culture (2nd passage) forming tissue-like structure. Fd-FLS from patients with RA, but not other forms of arthritis, exhibit the ability to form tissue-like structures. A. The tissue border is composed of 2 to 4 layers of FLS aligned longitudinally. Cells lying behind the border are less dense and less well organized. B. Villous projections of cells float in the culture medium.

inflammatory mediators. Before stimulation, both td-FLS and fd-FLS expressed low levels of IL-6 and COX-2 mRNA (< 40 amol/ml). Baseline expression of IL-8 mRNA was generally higher in both cell types (range 4–478 amol/ml), although there was large variation among individual cultures (data not shown). In every experiment with both cell types expression of IL-6, IL-8, and COX-2 mRNA increased after 4 h exposure to TNF-α. The largest effect was seen in

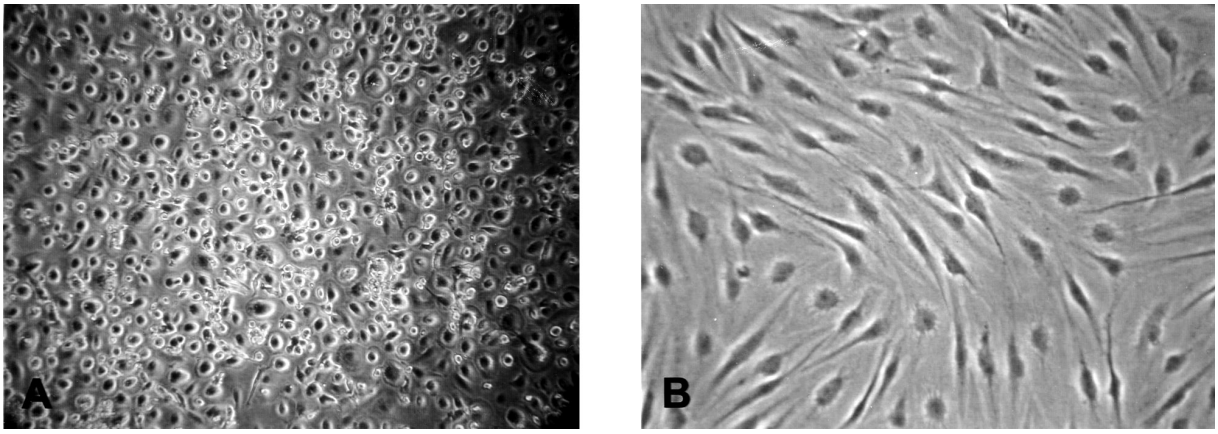

Figure 3. Fd-FLS culture derived from < 1 ml of SF. Large volume synovial aspirates are not required to establish successful fd-FLS cultures. Less than 1 ml of tenosynovial fluid was aspirated from the dorsum of the wrist of a patient with inflammatory polyarthritis. The fluid was plated directly, without centrifugation. A. Primary culture (Day 3). B. Fourth passage.

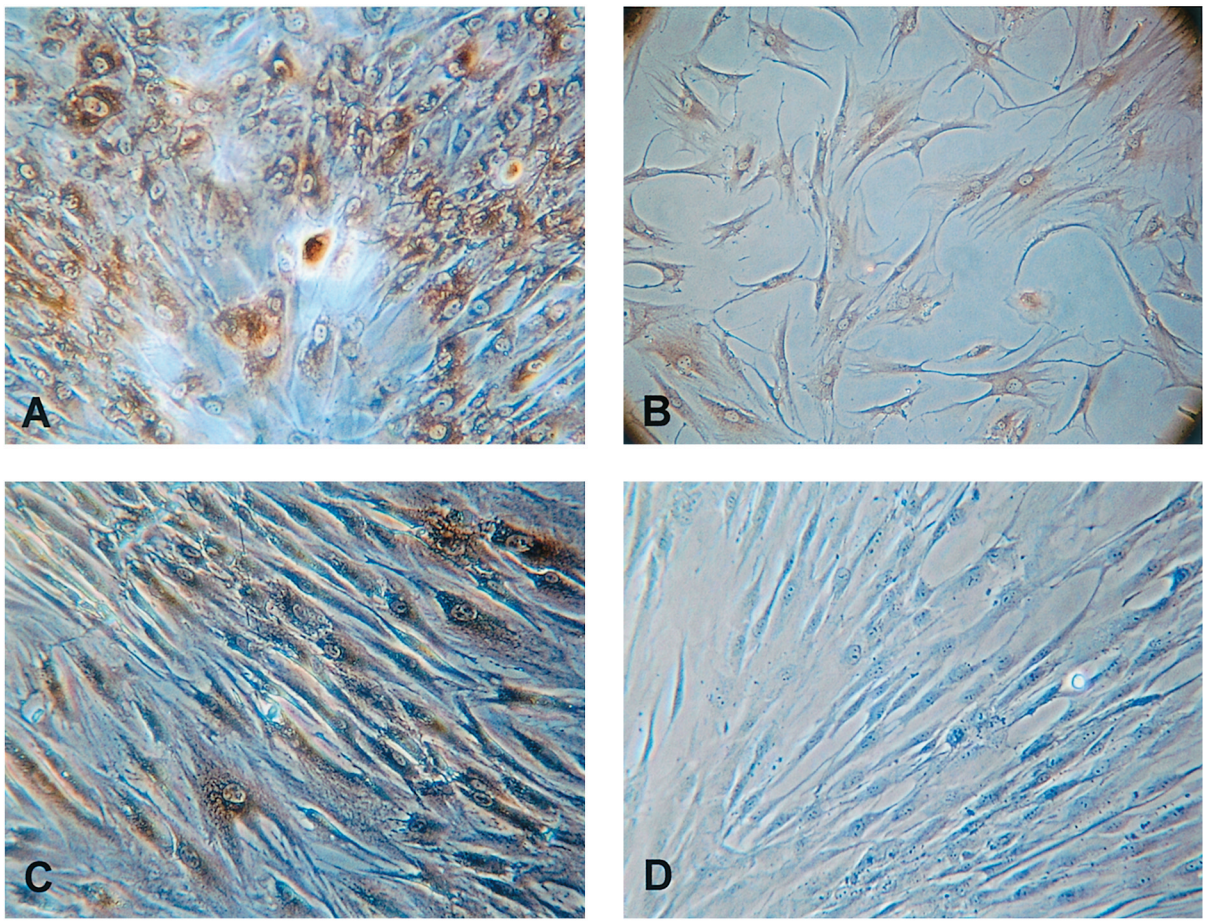

Figure 4. Immunohistochemistry staining of fd-FLS for procollagen I (A), procollagen III (B), prolyl-4-hydroxylase (C), and negative control (D). Positive staining is evident in the majority of cells. Cells were stained as described in Materials and Methods. Primary antibodies were omitted in negative controls. Similar results were obtained with td-FLS cultures (not shown). Results are representative of 3 or more experiments with fd-FLS and td-FLS.

IL-8, with an average 16-fold increase over unstimulated cells in both td-FLS and fd-FLS. More modest increases were observed in IL-6 mRNA (4 to 9-fold) and COX-2 mRNA (2 to 3-fold) after stimulation (Figure 5A). Treatment of cells with dexamethasone reduced TNF-α induced increases in inflammatory mediators (Figure 5B). Suppression by dexamethasone of IL-6 and IL-8 mRNA levels was similar for fd-FLS and td-FLS. Dexamethasone suppression of COX-2 expression was far less in fd-FLS (19%) than in td-FLS (65%).

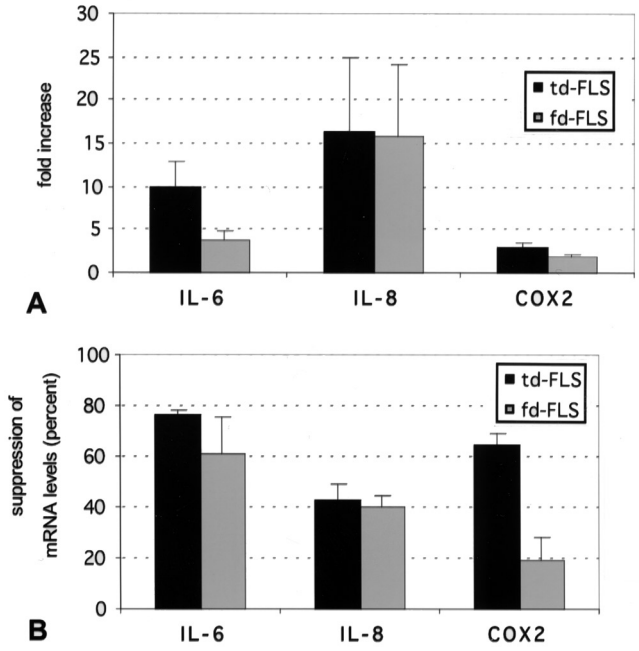

Figure 5. Gene expression of inflammatory mediators in fd-FLS and td-FLS. Fd-FLS and td-FLS cultures from patients with RA were incubated overnight in low serum medium with or without 2 µM dexamethasone, then stimulated with 1 ng/ml TNF-α for 4 h. Results represent mean (± SEM) of 3 experiments with duplicate samples. A. Effect of stimulation on IL-6, IL-8, and COX-2 gene expression in td-FLS and fd-FLS. Results are expressed as fold increase in mRNA levels in stimulated versus unstimulated cells. B. Suppression of IL-6, IL-8, and COX-2 gene expression by dexamethasone. Results are expressed as percentage suppression of mRNA in dexamethasone treated versus untreated cells.

DISCUSSION

Joint tissue injury in patients with RA is likely due to a multicellular assault on articular cartilage and bone. Nonetheless, studies in animals and humans suggest that joint damage can proceed with participation of synovial cells alone[8-14]. Recent advances in management of patients with RA have resulted in reduced access by investigators to synovial tissue. The availability of dendritic cells from SF and peripheral blood has facilitated research on that cell type[15]. Thus, a more proximal source of synovial cells than the replaced joint might be of some use to investigators.

We have shown that adherent cells with fibroblast-like morphology are readily cultured from SF of patients with inflammatory arthritis. Analyses of cell surface antigens by flow cytometry and intracellular products by immunohistochemistry confirm the fibroblast phenotype of these fluid derived cells. Surface antigens characteristic of dendritic cells, monocytes, macrophages, and lymphocytes are expressed at low levels by the third passage, at which time SF cultures consist of a homogeneous fibroblast population. Costly, time-consuming cell separation techniques are not necessary.

FLS cultures from SF from patients with RA, but not from other forms of arthritis, exhibit the ability to form tissue-like structures in culture. Similar to changes observed in cells isolated from RA synovium, fd-FLS from RA patients grow in an anchorage-independent fashion and form villous projections that float freely in the culture medium. Others have reported similar findings in RA fd-FLS, and have shown that these cells are capable of mediating cartilage destruction in an animal model[14].

Gene expression of IL-6, IL-8, and COX-2 is upregulated in activated RA FLS obtained from tissue and cultured *in vitro*[16,17], and treatment of these cells with dexamethasone reduces IL-6, IL-8, and COX-2 mRNA[18-22]. We examined production of inflammatory mediators in fd-FLS and compared the findings to td-FLS. Exposure to TNF-α increased IL-6, IL-8, and COX-2 mRNA levels to a similar extent in td-FLS and fd-FLS cultures. In addition, the pattern of cytokine and COX-2 expression was the same in both cell types both before and after stimulation, with relatively higher levels of IL-8 mRNA compared with IL-6 and COX-2. In both cell types, the response to TNF-α stimulation was most pronounced for IL-8 (16-fold increase), whereas IL-6 was most susceptible to suppression by dexamethasone (40% inhibition). Quantitative differences were observed between td-FLS and fd-FLS in baseline production of IL-6, IL-8, and COX-2 mRNA, as well as the degree of COX-2 inhibition by dexamethasone. This may be due, at least in part, to variation in patient characteristics, including disease activity and treatment, at the time of joint aspiration.

Thus, FLS obtained from SF are phenotypically and functionally the same as FLS derived in the traditional manner from surgical specimens. This will make it possible to study fd-FLS from patients with early RA when tissue specimens usually are not available, and will also allow longitudinal studies to be done in efforts to determine whether FLS function is altered by disease course and/or by therapy.

The question remains: What is the origin of fd-FLS? Whereas pannus formation in RA is thought to result from division of cells within the synovium, the precise origin of the cells is not known[23]. In addition, FLS reappear in joints after synovectomy. Although these cells may emerge from residual synovial tissue, it is possible that cells recruited from the circulation by cytokines produced in the injured joint have the potential to differentiate along a fibroblast pathway. Fibrocytes are circulating progenitor cells of mesenchymal lineage that make up about 0.5% of peripheral blood leukocytes[24]. When cultured *in vitro*, fibrocytes display adherent spindle-shaped morphology, proliferate rapidly, express fibroblast products including collagens I and III, and can be stimulated to produce cytokines, including IL-6 and IL-8[25-27], exactly as we have shown for fd-FLS. Results of wound healing studies in humans and mice indicate that fibrocytes are recruited rapidly to sites of tissue injury, where they develop the ability to synthesize connective tissue matrix, provide antigen-specific T cell stimulation, and promote angiogenesis[28]. Fibrocytes are characterized by a

unique array of cell surface antigens including collagen I, CD34, MHC class II, and costimulatory molecules[24]. The fd-FLS we studied do not express characteristic fibrocyte antigens, perhaps because the cells were analyzed after 6 to 8 weeks in culture at a time when further differentiation into mature synoviocytes had occurred. Alternatively, or in addition, the unique milieu of the rheumatoid joint may promote differentiation along a different pathway. Although the source of fd-FLS may be sloughed synovial lining cells, identification of circulating connective tissue cells capable of fibroblast-like differentiation, T cell stimulation, and promotion of angiogenesis presents an intriguing target for further study of the synovial abnormalities in patients with RA.

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