Constitutive Production of Inflammatory and Mitogenic Cytokines by Rheumatoid Synovial Fibroblasts

By Richard Bucala, Christopher Ritchlin,* Robert Winchester,* and Anthony Cerami

*From the Rockefeller University; and *The Hospital for Joint Diseases, New York University Medical Center, New York, New York 10021*

Summary

Conditioned media obtained from fibroblasts cultured from rheumatoid and certain other inflammatory synovia were observed to stimulate [^{3}H]thymidine incorporation in an indicator murine fibroblast line. Synovial fibroblasts derived from the joints of patients with osteoarthritis did not display this property. This effect persisted in culture for many weeks and occurred in the absence of co-stimulatory immune cells. Antibody neutralization studies implicated a role for basic fibroblast growth factor (bFGF), transforming growth factor β (TGF-β), granulocyte/macrophage colony-stimulating factor (GM-CSF), and interleukin 1β (IL-1β) in the increased proliferative activity of synovial fibroblast–conditioned media. Synovial cell synthesis of bFGF, TGFβ1, GM-CSF, IL-1β, and IL-6 was confirmed by ^{35}S-methionine labeling and immunoprecipitation. The constitutive production of inflammatory and mitogenic cytokines by synovial fibroblasts may represent the result of long-term, phenotypic changes that occurred in vivo. Persistent cytokine production by synovial fibroblasts may play an important role in the continued recruitment and activation of inflammatory cells in chronic arthritis and in the formation of rheumatoid pannus.

Chronic inflammatory arthritis is characterized by a relapsing and remitting course of joint inflammation that leads ultimately to joint destruction and crippling (1). The overlying joint capsule or synovium may undergo profound hypertrophic and proliferative changes during this process. In rheumatoid arthritis, for example, these changes result in the formation of synovial pannus, which invades periarticular tissue and causes severe bony and ligamentous destruction (1).

The distinctive molecular features that characterize the synovial response to inflammation are poorly understood. Monocyte-derived products such as IL-1 and TNF/cachectin stimulate synovial fibroblasts to secrete collagenases and prostaglandin E_2 (PGE_2)[1] (2, 3). Additional inflammatory mediators that have been detected either in situ or in synovial fluid include IL-6 (4, 5), IFN-γ (6), transforming growth factor β (TGF-β) (7, 8), granulocyte/macrophage colony-stimulating factor (GM-CSF) (9), platelet-derived growth factor (PDGF) (10), and fibroblast growth factors (FGF) (10). The precise interplay of the diverse mediators in the pathophysiology of chronic synovitis has yet to be elucidated.

[1] *Abbreviations used in this paper:* EGF, epidermal growth factor; FGF, fibroblast growth factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; PDGF, platelet-derived growth factor; PGE_2, prostaglandin E_2; TGF-β, transforming growth factor β.

The infiltration and activation of immune cells within the joint space occurs in many forms of arthritis. In certain inflammatory arthritides such as rheumatoid arthritis, however, the invasive and proliferative changes of synovial pannus become a dominant feature. The degree to which the synovial fibroblast contributes directly to these changes in unknown. Observations that may be relevant to this question include reports that in vitro, rheumatoid synovial fibroblasts grow at a faster rate and achieve a higher saturation density (11, 12).

In the present study, we demonstrate that fibroblasts derived from human rheumatoid and chronic inflammatory synovia continually produce inflammatory cytokines and growth factors during long-term culture.

Materials and Methods

Isolation and Propagation of Synovial Fibroblasts. Synovial fibroblasts were isolated from tissue obtained during reconstructive surgery using established methods (13). Cells were cultured in medium consisting of 80% DMEM, 10% NCTC-109 (Gibco Laboratories, Grand Island, NY), 10% heat-inactivated FCS, ITS supplement (Collaborative Research, Bedford, MA), and gentamicin (50 μg/ml). Cell cultures were trypsinized at ~80% confluence and passaged by a threefold division of cultures. At passage three (~2 wk for rheumatoid cultures, and 2–3 wk for osteoarthritis cultures), synovial fibroblast cultures were found to be completely free of lym-

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phoid and monocytic cells as assessed by morphology (Wright-Giemsa staining) and specific immunostaining with anti-CD2, CD3, CD11b, and CD14 mAbs (14, 15). Contamination by mycoplasma species was excluded by testing antibiotic-free culture supernatant for adenosine phosphorylase activity (Mycotest; Gibco Laboratories).

Synovial fibroblasts were derived from patients with the diagnosis of osteoarthritis (n = 7) and rheumatoid arthritis (n = 6) (American Rheumatism Association Criteria [16]). Pathological examination revealed histopathological changes consistent with these diagnoses. Inflammatory synovial fibroblasts were obtained from patients (n = 4) with arthritic conditions other than rheumatoid arthritis. These synovia showed marked inflammatory cell infiltration on pathological examination.

Mitogenesis Assay. Synovial fibroblast–conditioned media was generated by growing cells at 60% confluence in 2 ml of medium containing 89% DME, 10% NCTC-109, and 1% FCS for 48 h. For the assay of mitogenic factors, 96-well flat-bottomed microtiter plates were seeded with 0.1 ml of NIH-3T6 cells (4×10^3/ml) in DMEM and 10% FCS. After 48 h, the cells were growth arrested by replacement of culture medium with DMEM containing 1% FCS. 48 h later, this medium was aspirated and 0.1 ml of synovial cell conditioned media added. 1 μCi of [^{3}H]methyl-thymidine (2 Ci/mmol; New England Nuclear, Boston, MA) was added after 24 h and incubation continued for an additional 30 h. The amount of [^{3}H]thymidine incorporated into DNA was measured by collecting cell lysates onto glass fiber filters (PhD cell harvester; Cambridge Technology, Watertown, MA) and analyzing radioactivity by liquid scintillation spectrometry. All assays were performed in triplicate. Results are expressed as percent [^{3}H]thymidine incorporation relative to a 10% serum supplemented control, in which total incorporated radioactivity typically ranged from 3,500 to 12,000 cpm.

Antibody Neutralization. The immunoglobulin fractions of specific polyclonal antisera to the following human proteins were used: anti-IL-1β (Genzyme, Boston, MA), anti-IL-6 (R & D Systems, Minneapolis, MN), anti-GM-CSF (R & D Systems), anti-epidermal growth factor (EGF) (Collaborative Research), anti-basic FGF (bFGF) (R & D Systems), anti-TGF-β1 (the generous gift of Dr. Martin Turner, The Charing Cross Sunley Research Center, London, UK) (17), and anti-PDGF (Genzyme). For neutralization studies, 0.3 ml of conditioned media was incubated with specific antibodies for 90 min at 37°C before the addition, in triplicate, of 0.1-ml samples to the mitogenesis assay. Antibodies were titrated against an "index"-conditioned media that displayed the highest level of mitogenic activity and were subsequently used in excess of an amount that maximally inhibited mitogenesis.

TNF/Cachectin Assay. TNF/Cachectin in synovial fibroblast–conditioned media was measured by specific ELISA of concentrated conditioned media (10×) and by determining cytotoxicity for actinomycin D–treated L929 cells as described previously (18).

Metabolic Labeling and Immunoprecipitation. Synovial fibroblasts were grown to 50% confluence in 6-well plates and labeled with 0.5 mCi of ^{35}S-methionine (1,026 Ci/mmol; Trans^{35}S-Label; ICN, Irvine, CA) following a standard protocol (19). Equivalent amounts of radioactive protein (determined by TCA precipitation) were then incubated with specific antibody overnight at 4°C, followed by incubation with protein-A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). Protein-A Sepharose–bound material was washed sequentially in buffer containing 150 mM NaCl, 20 mM Tris, pH 7.4, 2 mM EDTA, 1% deoxycholate, 1% NP-40, 0.1% SDS, 0.2 mM PMSF (2×), buffer containing 10 mM Tris, pH 7.5, 1% Triton X-100 (2×), buffer containing 10 mM Tris, pH 7.5, 1 M NaCl (1×), and buffer containing 10 mM Tris, pH 7.5, 0.1% SDS (1×). Washed resin was resuspended in 75 μl of Laemmli polyacrylamide gel electrophoresis sample buffer containing dithiothreitol and heated to 90°C for 2 min. 4-μl aliquots were analyzed for ^{35}S incorporation by liquid scintillation spectrometry. 25-μl aliquots were subjected to electrophoresis in 15% polyacrylamide mini-gels (19), and radioactive proteins were analyzed by fluorography.

Results

Recent studies have reported an increased growth rate in vitro of synovial fibroblasts derived from rheumatoid and inflammatory arthropathies when compared with fibroblasts derived from noninflammatory arthropathies (11, 12). Initial studies of cell growth kinetics in our laboratory supported these findings. To address the possibility that the increased proliferative capacity of rheumatoid synovial fibroblasts might be the result of an enhanced production of autocrine growth factors, a sensitive growth factor assay was used that tested the ability of conditioned media to stimulate [^{3}H]thymidine incorporation in an "indicator" murine fibroblast cell line (NIH-3T6). Fig. 1 illustrates the result of this analysis for 17 synovial fibroblast lines derived from patients with osteoarthritis (n = 7), rheumatoid arthritis (n = 6), and inflammatory (nonrheumatoid) arthritis (n = 4). It is apparent that in contrast to the osteoarthritis synovial fibroblasts, fibroblasts derived from either inflammatory or rheumatoid synovium constitutively produce soluble factors that stimulate [^{3}H]thymidine incorporation in murine fibroblasts.

Fig. 2 demonstrates that the growth stimulatory activity of synovial fibroblast–conditioned media slowly diminishes with passage number, however, significant activity persists at passages 6–8 (corresponding to 8–12 wk in culture). This result complements our observations that synovial fibroblast

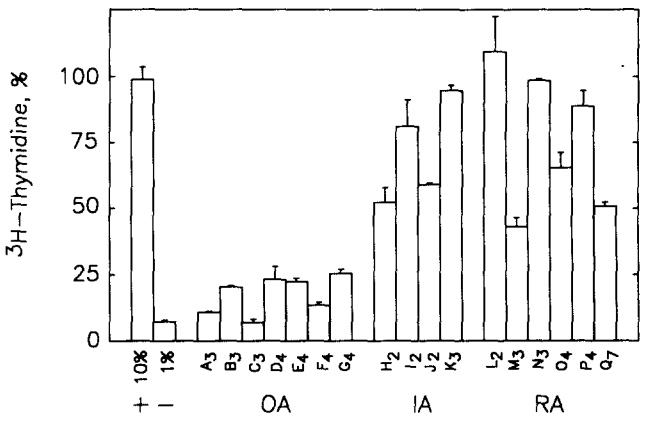

Figure 1. Induction of [^{3}H]thymidine incorporation in quiescent NIH-3T6 cells by conditioned media obtained from seven osteoarthritis (OA), four inflammatory arthritis (IA), and six rheumatoid arthritis (RA) synovial fibroblast lines. Letters designate individual patient-derived cell lines, and subscripts designate the cell passage number at the time of assay. 10% and 1% refer to media containing either 10% or 1% FCS, which served as positive and negative controls, respectively. Percent [^{3}H]thymidine refers to incorporation induced by synovial fibroblast–conditioned media relative to the 10% serum supplement positive control. Error bars represent the SEM of triplicate assays.

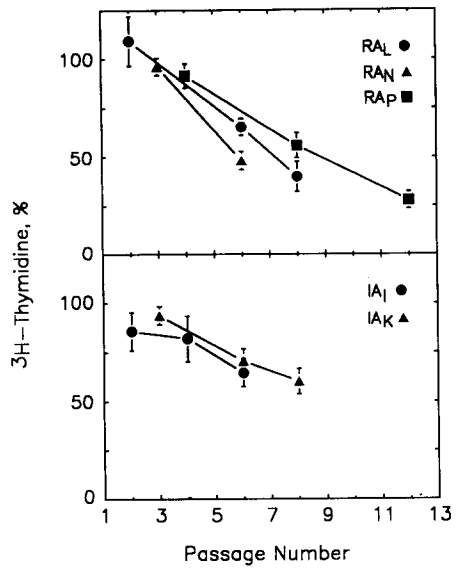

Figure 2. Diminution of the proliferative capacity of conditioned media from three rheumatoid synovial fibroblast lines (RA) and two inflammatory arthritis synovial fibroblast lines (IA) as a function of increasing cell passage number. Error bars represent the SEM of triplicate assay determinations.

proliferation progressively decreases as a function of time in culture (data not shown). The growth-promoting effect of synovial fibroblast–conditioned media was then studied in a series of antibody neutralization experiments. Fig. 3 (*left*) shows the effect of anticytokine antibody incubation on [^{3}H]thymidine incorporation for five of the rheumatoid synovial fibroblast lines (rheumatoid arthritis) that were studied. The proliferative capacity of this conditioned media was found to be dependent on the presence of EGF (4/5), bFGF (4/5), TGF-β1 (3/5), GM-CSF (3/5), and IL-1β (5/5). Evidence against the possibility that these cytokines were unique to rheumatoid arthritis was obtained by assaying media conditioned by synovial fibroblasts obtained from three patients with chronic, inflammatory arthritis that was clinically distinct from rheumatoid arthritis. In these cases, a similar pattern of constitutive cytokine production was observed when compared with the rheumatoid synovial fibroblast cells (EGF, 2/3; bFGF, 3/3; TGF-β1, 3/3; GM-CSF, 1/3; and IL-1β, 3/3). In separate experiments, the production of cachectin/TNF also was examined. This cytokine could not be detected in any of the conditioned media tested by either a specific ELISA or by the L929 cell cytotoxicity assay (18).

The synthesis of specific cytokines by synovial fibroblasts was confirmed in vitro by metabolic labeling with ^{35}S-methionine. Quantitative immunoprecipitation with anti-bFGF, anti-TGF-β1, anti-GM-CSF, and anti-IL-1β antibodies demonstrated increased synthesis of cytokines in inflammatory and rheumatoid synovial fibroblast lines when compared with two osteoarthritis fibroblast cell lines (Fig. 4). Gel electrophoresis and fluorography showed protein bands that correspond to the molecular masses of bFGF (~18 kD) (20), TGF-β (12.5 kD, monomer) (21), and IL-1β (17.5 kD) (22). Immunopre-

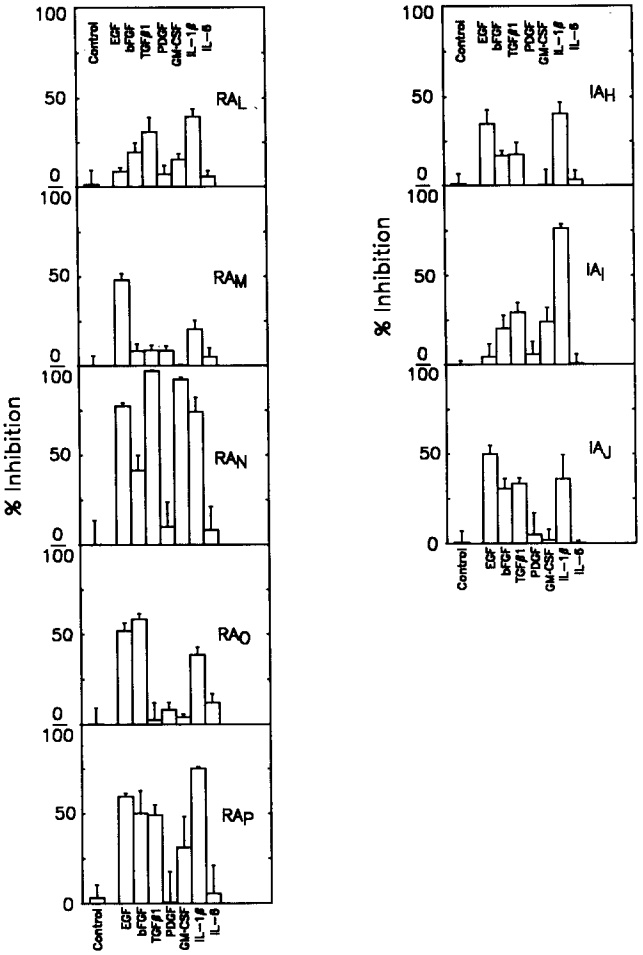

Figure 3. Effect of specific antibody neutralization on the proliferative capacity of conditioned media from five rheumatoid (RA) and three inflammatory (IA) arthritis synovial fibroblast lines. Conditioned media was used from the same passage number as shown in Fig. 1. Error bars represent the SEM of three to six assay determinations.

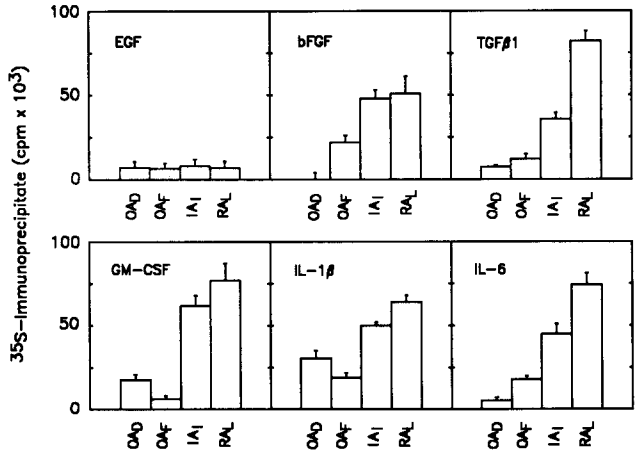

Figure 4. Quantitative immunoprecipitation by specific antibody of ^{35}S-labeled protein from two osteoarthritis (OA), one inflammatory (IA), and one rheumatoid (RA) synovial fibroblast cell lines.

cipitation studies with anti-EGF failed to demonstrate any bands in the 7–8 kD (23) region, despite labeling to high specific activity and prolonged exposure by fluorography (20 d). We suggest that the inhibitory effect of anti-EGF antiserum on conditioned media in the 3T6 cell proliferation assay is most likely due to the inactivation of EGF present in the 1% serum supplement in the assay (24). IL-6, although not implicated as a growth factor in the 3T6 fibroblast assay (Fig. 3), also was found to be synthesized in increased amounts by inflammatory and rheumatoid synovial fibroblasts (Fig. 4). This finding supports previous studies of enhanced IL-6 production by rheumatoid synoviocytes (25).

Discussion

Media conditioned by fibroblasts cultured from rheumatoid and inflammatory synovia were found to stimulate [^{3}H]thymidine incorporation in an indicator fibroblast cell line. Synovial fibroblasts derived from noninflammatory, osteoarthritis synovia did not display this property. This effect appeared to represent a long-term, constitutive property of synovial fibroblasts derived from rheumatoid and inflammatory synovia because it occurred in the absence of immune cells and persisted for >12 wk in culture. Antibody neutralization and immunoprecipitation studies implicated a role for bFGF, TGF-β1, GM-CSF, and Il-1β in the increased proliferative activity of synovial fibroblast–conditioned media.

IL-1β production was observed in all the synovial fibroblast lines that were studied. This finding complements a report of the spontaneous production of an IL-1-like factor by cloned synovial cells (26). The long-term, constitutive production of Il-1β by synovial fibroblasts may perpetuate an autocrine loop that continuously stimulates fibroblast mitogenesis (27). Even after the resolution of acute inflammatory changes, fibroblast-derived Il-1β would promote synovial hyperplasia as well as the continued recruitment and activation of additional infiltrating immune cells.

The other growth factors that were detected in synovial fibroblast–conditioned media included bFGF, TGF-β1, and GM-CSF. The induction of bFGF production by synovial fibroblasts (28, 29) may promote neovascularization and contribute further to the proliferative and invasive properties of rheumatoid synovium. TGF-β1 mediates a diverse set of biological functions (30) and has been detected within synovial tissue in situ, particularly in areas of active fibrosis (8). GM-CSF activity was detected in two of the cell lines, and has been implicated in the pathogenesis of chronic inflammatory synovitis in a previous study (9). Of significance, GM-CSF augments monocyte IL-1 production, thus creating a positive feedback loop (31). The observation that GM-CSF can be produced autonomously in long-term synovial fibroblast cultures further implicates synovial fibroblasts in a central role in synovial inflammation, even after the egress of inflammatory cells (32).

The antibody neutralization studies defined specific "cytokine" profiles for eight synovial fibroblast lines. Cytokine production by the inflammatory (nonrheumatoid) group did not appear to differ significantly from the rheumatoid group. The differences in the identity and the level of individual cytokines that were detected in the synovial fibroblast lines may reflect differential cytokine expression due to variations in disease activity, concurrent antiinflammatory therapy, or genetic background. These possibilities are under investigation; however, variability in the level of specific cytokine production points to the difficulties in attributing complex inflammatory processes to single, dominantly acting inflammatory mediators. Cytokines interact and synergize in their growth-promoting effects (27, 30, 31). TGF-β for example, may exert either a proliferative or an antiproliferative effect, depending on the interplay of additional growth factors (30). Synergy most likely accounts for the observation that in some cases, the addition of more than one individual anticytokine antibody inhibited proliferation by >50%.

A central question posed by our findings regards the mechanism by which synovial fibroblasts can be induced to undergo such prolonged phenotypic changes that result in constitutive cytokine production. One possibility is that the infiltration of activated inflammatory cells and local production of monocytic and lymphocytic inflammatory products creates an appropriate milieu that drives synovial fibroblasts into a long-term, activated state. The identity of such inciting products as well as the precise sequence of activation is unknown at the present time. It is likely that monocytic products such as TNF/cachectin, IFN-γ, and macrophage-inflammatory proteins (33), are involved in effecting fibroblast activation. The fibroblast, in turn, participates in the inflammatory response by secreting the cytokines we have described, as well as proteases, matrix proteins, and prostaglandins (1–3). This scheme does not exclude the possibility that constitutive differences in the responsiveness of synovial fibroblasts to inflammatory mediators may exist, thus rendering some individuals more susceptible to the sequelae of chronic arthritis. The present study emphasizes the role of the synovial fibroblast in modulating joint inflammation, and suggests novel strategies for approaching the etiopathogenesis and therapy of some forms of chronic arthritis.

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R. Bucala is a Brookdale National Fellow.

Address correspondence to Richard Bucala, Laboratory of Medical Biochemistry, Box 277, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

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