Expression of the Cytokine RANTES in Human Rheumatoid Synovial Fibroblasts

DIFFERENTIAL REGULATION OF RANTES AND INTERLEUKIN-8 GENES BY INFLAMMATORY CYTOKINES*

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A chronic inflammatory disease may be characterized by an accumulation of activated leukocytes at the site of inflammation. Since the chemokine RANTES may play an active role in recruiting leukocytes into inflammatory sites, we investigated the ability of cultured human synovial fibroblasts isolated from patients suffering from rheumatoid arthritis to produce this chemokine and compared its regulation to that of the closely related chemokine gene, interleukin-8 (IL-8). In unstimulated synovial fibroblasts, the expression of mRNA for both chemokines was undetectable, but was increased in both a time- and dose-dependent manner upon stimulation with the monokines tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β). Preincubation of the cells with cycloheximide "superinduced" the level of IL-8 mRNA stimulated by TNFα and IL-1β, but decreased the expression of RANTES mRNA in response to TNFα. In addition, differential regulation of these genes was noted when synovial fibroblasts were stimulated with a combination of cytokines. IL-4 down-regulated and IFNγ enhanced the TNFα- and IL-1β-induced increase in RANTES mRNA, whereas the induction of IL-8 mRNA by TNFα or IL-1β was inhibited by IFNγ and augmented by IL-4. Moreover, a combination of TNFα and IL-1β synergistically induced IL-8 mRNA expression, whereas under the same conditions, the level of expression of RANTES mRNA was less than that induced by TNFα alone. These observations were also reflected at the level of chemokine secretion. These studies demonstrate that by expressing the chemokines RANTES and IL-8, synovial fibroblasts may participate in the ongoing inflammatory process in rheumatoid arthritis. In addition, the observation that these chemokine genes are differentially regulated, depending upon the presence of different cytokines, indicates that the type of cellular infiltrate and the progress of the inflammatory disease is likely to depend on the relative levels of stimulatory and inhibitory cytokines.

Various cytokines have been implicated as important mediators of inflammation and joint destruction in rheumatoid arthritis (RA) and other inflammatory processes. Recently, a group of small molecular weight proinflammatory cytokines, known variously as the platelet factor 4 (PF4), the interocrine, or most recently the "chemokine" superfamily, has been described (2, 3). They are related by having primary structural similarities and a conserved 4-cysteine motif and are subdivided into two groups, namely C-X-C and C-C, depending on whether or not there is an intervening amino acid between the first two cysteines (2, 3). In general, the chemokines of the C-X-C class, of which IL-8 is a member, are reported to have proinflammatory functions through their actions mainly on neutrophils, whereas the chemokines in the C-C class of the superfamily appear to act on mononuclear cells with various degrees of specificity. For example, RANTES has been shown to be a chemotactic factor for monocytes and T lymphocytes of the memory phenotype in vitro (4). To date, RANTES expression has been primarily observed in cultured T cell lines that are antigen-specific and growth factor-dependent (5).

Culturing human cells in vitro has shown that IL-8 expression is up-regulated in a variety of cells such as monocytes, tissue macrophages, endothelial cells, epithelial cells, and dermal fibroblasts in response to stimuli including lipopolysaccharide, TNFα, and IL-1β (3). However, the lymphocyte-derived cytokines IL-4 and IFNγ are reported to down-regulate the lipopolysaccharide, TNFα-, or IL-1-stimulated IL-8 expression in monocytes (3, 6) and in human thymic epithelial cells (7), respectively. In contrast to the known distribution and regulation of IL-8 in many cell types, the cellular distribution or factors controlling the regulation of the RANTES gene have not yet been characterized with the exception that, in situ hybridization, RANTES mRNA has been shown to be present in synovial lining cells of patients with RA (8).

Synovial fibroblasts are believed to play an important role in the pathogenesis of RA (1). These cells are known to produce a number of putative mediators of inflammation such as plasminogen activator, prostaglandin E2, collagenase,stromelysin (9–11), and several leukocyte-activating cytokines including granulocyte/macrophage- and granulocyte-colony.

1 The abbreviations used are: RA, rheumatoid arthritis; IFNγ, interferon-γ; IL-1β, interleukin-1β; IL-4, interleukin-4; IL-8, interleukin-8; RANTES, Regulated upon Activation, Normal T Expressed, and presumably Secreted; TNFα, tumor necrosis factor α; ELISA, enzyme-linked immunosorbent assay.

2 The term "chemokines" was decided upon at the Third International Symposium on Chemotactic Cytokines, September 1992, Paden-by-Vienne, Austria.
stimulating factors and IL-6 (12, 13) in response to the macrophage-derived cytokines TNFα and IL-1. In addition, recent studies in our laboratory and others (14) have also demonstrated that synovial fibroblasts produce the chemokines IL-8 and MCP-1 (15) in response to stimulation by either TNFα or IL-1β, or upon engagement of major histocompatibility complex class II molecules with superantigen (16). It is therefore possible that these fibroblasts may play a major role in leukocyte recruitment to, and activation in, the rheumatoid synovium by producing chemokines. Moreover, the type of leukocyte infiltrate present in the RA synovium could depend on the relative levels of chemokines such as RANTES and IL-8, which in turn may depend on various stimuli present in the synovial environment. Hence, we have also studied potential similarities and differences in the regulation of these two genes by a variety of cytokines that are reported to be present in the rheumatoid synovium.

**MATERIALS AND METHODS**

**Reagents—**TNFα (recombinant human TNFα, specific activity 10^7 units/mg, in the actinomycin D-free I-929 cytotoxicity assay), was a generous gift from Knoll Pharmaceuticals (Whippany, NJ). TNFα stock was stored at -80°C in phosphate-buffered saline containing 0.1% sodium azide. Recombinant human IL-1β and IPN-γ were a generous gift from Genentech Inc. (South San Francisco, CA). Recombinant human IL-4 was a generous gift from the Genetics Institute (Cambridge, MA). Tests on these solutions using the Limulus amoebocyte assay for the presence of endotoxin were negative. Cycloheximide and actinomycin D were purchased from Sigma. Collagenase, trypsin-EDTA, RPMI 1640, and Dulbecco’s modified Eagle’s medium were purchased from GIBCO (Burlington, Ontario, Canada). Hyclone fetal calf serum was purchased from Professional Diagnostics (Edmonton, Alberta, Canada). Hybond N membranes, [γ-32P]dCTP, [α-32P]dCTP, and RNAguard (RNase inhibitor) and ribosomal RNA standards (28 and 18 S) were purchased from Pharmacia (Montréal, Québec, Canada). All other reagents used in this study were of molecular biological grade.

**Cell Culture—**The synovial fibroblast cultures used in this study were derived from patients who were diagnosed as suffering from either definite or classical RA according to the American Rheumatism Association 1987 criteria (17) and who were undergoing total knee replacement due to joint deterioration. The cultures were prepared as described previously (18, 19). Primary cultures were released by treatment with 0.2% trypsin, 0.05% EDTA at 37°C for 4 min and subcultured into 75-cm² flasks. Morphologically, the passaged cells resembled the previously reported “fibroblast-like” synovial cells (18, 19). The cells were used between passages 3 and 14 (20). Routine FACS analysis of cell surface markers revealed that after the third passage, the cultures were consistently major histocompatibility complex class II-negative, Mo-1-negative (the latter indicating absence of myeloid cells and the type A “macrophage-like” synoviocytes) and factor VIII-negative (endothelial cell marker).

**Isolation of Cytoplasmic RNA and Northern Blot Analysis—**Cultured synovial fibroblasts were grown to confluence in 75-cm² culture flasks and treated as described in the figure legends. After the appropriate stimulation time with the cytokines, the cells were harvested using trypsin-EDTA, the cytoplasmic RNA was prepared, and Northern blots were performed as described in the figure legends. The supernatants were collected and analyzed for chemokine content. Levels of IL-8 were measured using an IL-8 monoclonal antibody sandwich ELISA employing two anti-IL-8 monoclonal antibodies recognizing different, non-competing determinants. Briefly, microtiter plates were coated with the first anti-IL-8 antibody, and then the samples and the second anti-IL-8 antibody conjugated to horseradish peroxidase were added. After washing, substrate was added and the absorbance was measured at 490 nm in a Vmax plate reader (Molecular Devices Corporation, Menlo Park, CA). A standard curve was generated by plotting A_{490} cm versus log rhIL-8 concentration, using a four-parametric logit curve fitting program (developed at Genentech Inc.). The specificity of the assay for IL-8 was verified using 13 other soluble immune proteins, including interleukins 1-6, and was found to have no cross-reactivity with any of these proteins tested. RANTES levels were assessed by a similar assay, the details of which are to be published elsewhere.4

**RESULTS**

The Effect of TNFα and IL-1β on Steady State Levels of RANTES and IL-8 mRNA in Synovial Fibroblasts—Rheumatoid synovial fibroblasts were grown to confluence in 75-cm² culture flasks and stimulated either for increasing periods of time with fixed concentrations of TNFα or IL-1β or with increasing concentrations of TNFα or IL-1β for 24 h, and Northern blots were performed. No RANTES mRNA was detected, regardless of the time point (data not shown), unless the cells were stimulated with cytokines (Figs. 1 and 2). However, RANTES mRNA was first detected after a stimulation of 6 h with TNFα or 2 h with IL-1β and the maximum levels were reached at 24 h and sustained with up to 48 h (Figs. 1A and 2A, respectively). At 72 h, the level was decreased to one-half of the maximum level (results not shown). The filters were then stripped and reprobed with an IL-8 cDNA fragment. A similar time course of induction of IL-8 mRNA in response to the monokines was observed. We then examined the effect of concentration of TNFα and IL-1β on RANTES gene expression following a 24-h incubation. The steady state levels of RANTES mRNA were observed to be concentration-dependent upon incubation with either TNFα or IL-1β (Figs. 1B and 2B, respectively). A similar dose response to the monokines was observed for IL-8 mRNA expression (Figs. 1B and 2B).

**The Effect of Cycloheximide and Actinomycin D on the Monokine-induced Up-regulation of RANTES and IL-8 Gene Expression—**To determine whether the TNFα- or IL-1β-induced up-regulation of RANTES and IL-8 genes was protein synthesis-dependent, we conducted experiments in presence of the protein synthesis inhibitor cycloheximide. Pretreatment with cycloheximide had no effect on the levels of RANTES or IL-8 mRNA in unstimulated synovial fibroblasts (Figs. 3, A and B). In contrast, pretreatment with cycloheximide led to a “superinduction” of IL-1β-induced RANTES and IL-8 gene expression. In addition, superinduction of TNFα-stimulated IL-8 gene expression was also observed in presence of cycloheximide (Fig. 3B). In contrast, cycloheximide pretreatment caused varying degrees of inhibition of the TNFα-induced RANTES mRNA expression (Fig. 3B). In this experiment, cycloheximide exerted only a small inhibitory effect. We also conducted experiments by preincubating the

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4 M. Sadick, manuscript in preparation.
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A

RANTES

IL-8

28S

0 2 6 12 24 48

TIME (hrs)

B

RANTES

IL-8

28S

0 0.1 1.0 10 100

TNFα [ng/ml]

FIG. 1. Effect of TNFα on steady state level of RANTES and IL-8 mRNA in human rheumatoid synovial fibroblasts. Cells were grown to confluence in 75-cm² culture flasks and stimulated with 100 ng/ml of TNFα for increasing periods of time (A) or with increasing concentrations of TNFα for 24 h (B). The cells were harvested, cytoplasmic RNA was prepared, and RANTES mRNA was then detected as described under “Materials and Methods.” The filters were then stripped and reprobed successively with an IL-8 cDNA fragment and an oligonucleotide probe for 28 S ribosomal RNA. The data presented in these figures are representative of at least seven other experiments performed with similar results.

B

RANTES

IL-8

28S

0 0.1 1.0 10 100

TNFα [ng/ml]

FIG. 2. Induction of steady state level of RANTES and IL-8 mRNA by IL-1β in human rheumatoid synovial fibroblasts. Cells were grown to confluence in 75-cm² culture flasks and stimulated with 3 ng/ml of IL-1β for increasing periods of time (A) or increasing concentrations of IL-1β for 24 h (B). RANTES and IL-8 mRNA and 28 S ribosomal RNA were then detected as described in Fig. 1. The data presented in these figures are representative of at least seven other experiments performed with similar results.

Differential Regulation of RANTES and IL-8 Gene Expression in Synovial Fibroblasts—Since the synovial environment is likely to contain a mixture of several cytokines, we investigated the effect of combinations of various cytokines on the expression of RANTES and IL-8 genes. Cultured synovial fibroblasts were therefore stimulated either with TNFα or IL-1β in combination with IL-4 or IFNγ or with TNFα in combination with IL-1β. As shown in Figs. 4 and 5, the expression of RANTES and IL-8 mRNA was differentially regulated by different combinations of these cytokines. IFNγ had no direct effect on the level of either RANTES or IL-8 mRNA (Fig. 4, lanes 2–4). However, IFNγ synergistically enhanced the stimulatory effect of TNFα and IL-1β on the level of RANTES mRNA but inhibited that on IL-8 mRNA in a dose-dependent manner (Fig. 4, lanes 6–8 and 10–12). Similarly to IFNγ, IL-4 exerted no direct effect on the level of either RANTES or IL-8 mRNA (Fig. 5A, lanes 2–4). However, in contrast to IFNγ, IL-4 decreased the stimulating effect of TNFα on the RANTES mRNA level but synergistically increased the effect of TNFα on the level of IL-8 mRNA in a dose-dependent manner (Fig. 5A, lanes 6–8). An identical result was obtained for RANTES and IL-8 gene regulation when the cells were stimulated with IL-1β in combination with IL-4 (data not shown). The effect of combined presence of TNFα and IL-1β on synovial fibroblasts was also examined
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**FIG. 3. The effect of cycloheximide and actinomycin D on IL-1β- or TNFα-induced steady state level of RANTES and IL-8 mRNA in human synovial fibroblasts.** Confluent cells were prerinoculated with 10 μg/ml cycloheximide or with 5 μg/ml actinomycin D for 1 h as indicated. The cells were then incubated with either diluent (lanes 1–3 in A and lanes 1 and 2 in B) or 3 ng/ml IL-1β (lanes 4–6 in A) or 100 ng/ml TNFα (lanes 3 and 4 in B) for 24 h, harvested, and analyzed for RANTES and IL-8 mRNA and 28 S ribosomal RNA as described under "Materials and Methods." This figure is from one experiment, which is representative of five others performed with similar results.

Incubation of cells with IL-1β alone (Fig. 5B, lanes 2–4) or TNFα alone (Fig. 5B, lane 5) stimulated the expression of both RANTES and IL-8 mRNA as described above. However, IL-1β in combination with TNFα dose-dependently decreased the TNFα-induced RANTES mRNA level but synergistically enhanced the TNFα-induced IL-8 mRNA level (Fig. 5B, lanes 6–8).

**Inflammatory Cytokines Differentially Regulate the Secretion of RANTES and IL-8 in Synovial Fibroblasts**—The observations reported above were also verified at the level of protein secretion. Cultured human synovial fibroblasts were incubated for 24 h with either diluent, TNFα, IL-1β, or TNFα and IL-1β. The cells were also incubated with IFNγ or IL-4 in the presence or absence of TNFα or IL-1β (Fig. 6). At the end of incubation, the supernatants were collected and analyzed for the presence of RANTES and IL-8 by ELISA. It was observed that the effect of the cytokines on RANTES and IL-8 secretion, either alone or in combination, followed an identical pattern to their effect on the expression of RANTES and IL-8 mRNA.

**DISCUSSION**

RANTES and IL-8 are members of a recently discovered superfamily of low molecular weight immunoregulatory mediators, known as "chemokines." This superfamily includes platelet factor 4 (PF4), monocyte chemotactic protein-1 (MCP-1), and the macrophage inflammatory proteins (MIP) 1α and 1β (2, 3, 23–25). Studies on the chemotactic activity of several of these proteins in vitro have indicated relatively rigid patterns of target cell selectivity. For example, IL-8 is chemotactic for neutrophils, as well as some T cell subsets (26, 27), but not for monocytes. In contrast, MCP-1 is highly specific for monocytes (28, 29), whereas RANTES is a chemotactic factor for monocytes and T lymphocytes of the memory phenotype (4). Since the accumulation of activated T cells and macrophages at an inflamed site such as the synovial tissue of patients with RA may lead to significant structural damage to the joints, we examined the regulation of RANTES gene expression and its production in synovial fibroblasts, in response to stimulation by cytokines that are likely to be present in the synovial environment.

RANTES was originally characterized as a T cell-specific gene, which was expressed in cultured CD8+ CTL and also in Ag− or mitogen-activated T cells (5). Recent in situ hybridization studies have shown that RANTES mRNA is present in synovial lining cells (8). In the present study, we show that cultured human synovial fibroblasts express and secrete RANTES in response to activation by the monokines TNFα and IL-1β. This observation, combined with the fact that these cells are also capable of producing other chemotactic cytokines such as IL-8 (Refs. 3, 14, and 76, footnote 3, and present study) and MCP-1 (15, 30) in response to various stimuli including TNFα or IL-1β, may be of major relevance.
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FIG. 5. Effect of IL-4 and IL-1β on TNFα-induced steady state level of RANTES and IL-8 mRNA in human synovial fibroblasts. The cells were grown to confluence and incubated with increasing concentrations of IL-4 (A) or increasing concentrations of IL-1β (B) either in combination with or without 100 ng/ml TNFα for 24 h as shown. The cells were then harvested and analyzed for RANTES and IL-8 mRNA and 28S ribosomal RNA as described under "Materials and Methods." This figure is from one experiment, which is representative of four others performed with similar results. Different times of exposure of the autoradiographic images for each mRNA species in B were chosen to clearly demonstrate the effect of the combined treatment of monokines.

to the pathogenesis of RA, particularly in the early stages of the disease, where such chemokines could play a major role in recruiting and activating peripheral blood leukocytes.

Studies to date indicate that chemokine gene expression is controlled at several levels, including the transcriptional and post-transcriptional levels. The results of our studies in synovial fibroblasts using actinomycin D and cycloheximide support previous observations made in various other cell types concerning the regulation of the IL-8 gene (31-33). It has been shown that IL-8 promoter activity is positively driven by nuclear factor κB and CREB-like trans-acting factors (33) and possibly in a negative manner by (an) as yet unidentified repressor(s) (33). Moreover, IL-8 mRNA contains several AUUUA sequences (34) and may therefore be controlled at
the level of mRNA stabilization (35). Although the mechanism of IL-1β-induced RANTES gene expression appears to closely resemble that of the IL-8 gene, the RANTES gene may be regulated by mechanisms other than (de)stabilization, since unlike IL-8, the 3'-untranslated region of RANTES mRNA does not possess AUUUA sequences (5). Our results also suggest that TNFα- but not IL-1β-induced RANTES mRNA expression involves the synthesis of one or more proteins. We are presently conducting experiments to determine which of these mechanisms (transcriptional or post-transcriptional) are predominantly involved in regulating the expression of these two chemokine genes.

The differential regulation of the two chemokine genes in synovial fibroblasts by inflammatory cytokines may determine the type of cellular infiltrate present in the rheumatoid synovium. As we have shown in the present report for IL-8 production, TNFα and IL-1β synergistically up-regulate the production of colony stimulating factors (granulocyte and granulocyte/macrophage) (12) and prostaglandins (36) in synovial fibroblasts and in other human cellular systems (37,38). However, a relative inhibitory effect of this monokine combination on the expression of other cytokine genes, as observed for RANTES gene expression, has not yet been reported. This may be an important observation with respect to the progress of RA. When a critical number of monocytes have accumulated and been activated in the synovium, a certain level of TNFα and IL-1β may accumulate in the synovial tissue. The combined level of these two monokines may alter the balance of RANTES to IL-8 production, resulting in a relative enhancement of neutrophil recruitment and a relative decrease in mononuclear cell accumulation. This observation may partly explain the transient "acute flares" involving a massive neutrophil infiltration into the rheumatoid joint often observed during the disease.

The monokine-stimulated expression of the RANTES and IL-8 genes and the secretion of two chemokines were differentially regulated by IFNγ and IL-4. TNFα- and IL-1β-
induced secretion and mRNA expression of IL-8 were inhibited and those of RANTES were synergistically enhanced by IFNγ, whereas IL-4 exerted the opposite effect. These combined observations are in keeping with previous results showing that the T cell products, IL-4 and IFNγ, have opposing effects on TNF-α- or IL-1β-induced cellular functions (39, 40) including the production of cytokines such as IL-1β, IL-6, IL-8, and granulocyte-colony stimulating factor (43). Thus, the present study extends the observation of opposing effects of IFNγ and IL-4 on monokine-induced cytokine expression in human synovial fibroblasts to include the regulation of IL-8 and RANTES. Our results also suggest that considerable caution should be taken when arguing a general anti-inflammatory role for IFNγ and IL-4 in chronic inflammation, as they differentially regulate chemokine gene expression.

In view of the different spectra of activity of the various chemokines, the observations in the present study showing differential regulation of the genes coding for RANTES and IL-8 may have important ramifications with respect to the control of the type of leukocyte infiltrate observed in the rheumatoid joint or other inflammatory sites. Overall, we can hypothesize that the combined presence and opposing effects of these monokines and lymphokines on chemokine gene expression might be a method by which leukocyte trafficking to inflammatory sites is normally regulated.

REFERENCES
