Cytokine Regulation of Colony-Stimulating Factor (CSF) Production in Cultured Human Synovial Fibroblasts. II. Similarities and Differences in the Control of Interleukin-1 Induction of Granulocyte-Macrophage CSF and Granulocyte-CSF Production


Synovial fibroblasts are likely to be a significant source of granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte-CSF (G-CSF), which could be crucial to the pathogenesis of rheumatoid arthritis. Using specific enzyme-linked immunosorbent assays (ELISAs) and Northern analysis, GM-CSF and G-CSF expression were followed in human synovial fibroblast-like cells in response to a number of agents, either alone or in the presence of an optimal stimulatory concentration of interleukin-1 (IL-1). For both CSFs, interferon-γ (100 U/mL) did not increase their levels but dramatically suppressed the stimulatory action of IL-1, while basic fibroblast growth factor (10^{-4} mol/L), although nonstimulatory by itself, potentiated IL-1 action. The glucocorticoid, dexamethasone (10^{-7} mol/L), inhibited IL-1-stimulated CSF production. However, evidence was obtained for noncoordinated CSF regulation. Cyclooxygenase inhibitors potentiated the action of IL-1 on GM-CSF synthesis but suppressed G-CSF synthesis, suggesting that endogenous cyclooxygenase products can have opposite effects in modulating the levels of each CSF. Also, the lymphokine, IL-4 (250 pmol/L), slightly inhibited GM-CSF formation in the presence of IL-1 but elevated the G-CSF levels in these cultures without having an effect by itself. Transforming growth factor β (≤20 ng/mL) did not modulate levels of either CSF. Mesenchymal cell production of both GM-CSF and G-CSF is generally viewed as being under coordinate control; our findings suggest that their synthesis in IL-1-stimulated human synoviocytes can be modulated by a number of agents, in some cases with divergent actions depending on which CSF is examined.

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This latter observation suggests that the two CSFs might not always be produced coordinately, an observation supported by the finding that cyclooxygenase inhibitors lower GM-CSF production but enhance G-CSF levels in response to IL-1.

MATERIALS AND METHODS

Synovial cell cultures. Human synovial cell explant cultures were established from nonrheumatoid donors, as previously described.49 Passaged cells were functionally and morphologically similar to the synovial fibroblast-like cells.49 In experiments, synoviocytes were plated overnight at 5 x 10^4 cells/0.5 mL/well in 48-well plates (Costar, Cambridge, MA) in a modified minimal essential medium (α-MEM; Commonwealth Serum Laboratories, Parkville, Australia) supplemented with 10% heat-inactivated (at 56°C for 30 minutes) fetal bovine serum (FBS; Flow, Sydney, Australia). After washing with phosphate-buffered saline (PBS), pH 7.4, 50-μL cytotoxicity of actinomycin D-treated L929 murine fibroblast monolayers;19 recombinant human IFN-γ (2.4 x 10^6 antiviral U/mL) (E. Hochuli, Hoffmann-La Roche, Basel, Switzerland) or (2 x 10^7 antiviral U/mL) (G. R. Adolf, Boehringer Ingelheim, Sydney, Australia) (one unit of TNF activity was defined as the amount causing 50% cytotoxicity of actinomycin D-treated L929 murine fibroblast monolayers); recombinant human IFN-γ (2.4 x 10^6 antiviral U/mL) (E. Hochuli, Hoffmann-La Roche, Basel, Switzerland) or (2 x 10^7 antiviral U/mL) (Genentech); recombinant human IL-4 (>400 U/μg) (DNAX, Palo Alto, CA); purified recombinant human TNFβ (Genentech) (ID₅₀ ~ 30 pg/mL for induction of growth of the CCL64 mink epithelial cells) (A. Roberts, National Cancer Institute, Bethesda, MD).29 Dexamethasone and indomethacin were from Sigma (St Louis, MO), while naproxen was from Syntex (Palo Alto, CA); basic FGF was from Promega Corporation (Roselle, NY) and Upstate Biotechnology Incorporated (Lake Placid, NY). Probes. The GM-CSF probes used in this study were either from the Genetics Institute (Cambridge, MA) or Biogen (Geneva, Switzerland) while the G-CSF probes came from Genetics Institute (NY) while the G-CSF probes came from Genetics Institute (NY). The GAPDH and γ-actin probes were from Walter and Eliza Hall Institute and Dr J. Thibodeau (CHUL Research Centre, Québec, Canada), respectively.

Expression of results. Measurements in supernatants are presented as mean values ± SEM from triplicate cultures. The significance of differences was assessed using a two-tailed Student's t-test; results were considered not significantly different if P > 0.05.

RESULTS

IFN-γ lowers synoviocyte GM-CSF and G-CSF levels. We used ELISAs to quantitate CSF levels because, as discussed previously,17 there are limitations to the bioassays used to identify and quantitate them, including interference from other cytokines. As reported previously,17 untreated human synovial fibroblast-like cells produce undetectable or extremely low levels of GM-CSF and G-CSF. When IFN-γ (100 U/mL) was added to these cultures, there was no increase in the amounts of either CSF (see Table 1 for the data from a representative experiment). This same observation was made with different cell lines under the same culture conditions in 27 and 20 experiments for GM-CSF and G-CSF, respectively.

Ten to 20 μg of RNA was electrophoresed on a 1% agarose gel containing 2.2 mol/L formaldehyde and transferred onto Hybond-N+ nylon membranes. The filter was hybridized overnight at 42°C in a standard hybridization buffer containing 2 x 10^6 cpm/mL heat-denatured [32P]-labeled fragments of either GM-CSF or G-CSF cDNA that were labeled by the random primer method.23 After hybridization the membranes were washed under stringent conditions, dried at room temperature, and exposed for autoradiography at ~70°C. Equal loading and integrity of the cytoplasmic RNA in each lane was confirmed by staining the agarose gels with ethidium bromide. Equal loading was also verified by the housekeeping genes, γ-actin, or glyceraldehyde 3-phosphate dehydrogenase.

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Table 1. IFNγ Suppresses Synoviocyte GM-CSF and G-CSF Formation in Response to Both IL-1 and TNFα

<table>
<thead>
<tr>
<th>Agent Added</th>
<th>GM-CSF (ng/mL)</th>
<th>G-CSF (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.007</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.012 ± 0.006</td>
<td>0.41 ± 0.17</td>
</tr>
<tr>
<td>IL-1</td>
<td>0.50 ± 0.01</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td>IL-1 + IFNγ</td>
<td>0.14 ± 0.01*</td>
<td>5.7 ± 0.2*</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.078 ± 0.004</td>
<td>0.91 ± 0.08</td>
</tr>
<tr>
<td>TNFα + IFNγ</td>
<td>0.024*</td>
<td>0.65 ± 0.04*</td>
</tr>
</tbody>
</table>

Synovial cells (from donor KA, passage 5), cultured (5 × 10⁶ cells/well) in triplicate as described in Materials and Methods, were incubated for 24 hours at 37°C with IFNγ (100 U/mL), IL-1α (100 U/mL), or TNFα (10⁻⁸ mol/L), or with a combination of IFNγ with the other cytokines. Supernatants were collected and assayed for GM-CSF and G-CSF by immunosassay (see Materials and Methods). Values are means ± SEM.

*P < .05, when compared with IL-1- or TNFα-treated cultures.

GM-CSF and G-CSF expression in cytokine-stimulated synoviocytes. As found previously and also presented in Table 1, IL-1α and TNFα increased both GM-CSF and G-CSF levels, with more of the latter being produced and with IL-1 yielding a stronger response. IFNγ (100 U/mL) suppressed these increases in response to optimal concentrations of the stimulating cytokines. The dose-response curves for the effect of IFNγ on the stimulatory action of optimal concentrations of IL-1 are presented in Fig 1A and B. The inhibitory action of IFNγ (100 to 1,000 U/mL) on IL-1 action was noted in 18 and 16 other experiments for GM-CSF and G-CSF, respectively, and also found in four other experiments when the effect on the weaker response to TNFα was analyzed. The degree of inhibition of GM-CSF formation in response to IL-1 was often greater than that found for the inhibition of G-CSF formation. The results were the same if the stimulation was prolonged for 48 hours or if the experiments were conducted in 10% FBS. No CSFs were detected in the cell lysates and the modulation by IFNγ could be observed within 6 hours. Our results for GM-CSF differ from those of Alvaro-Gracia et al., who recently reported that IFNγ (100 U/mL) could inhibit GM-CSF formation by human synoviocytes in response to TNFα but not to IL-1; G-CSF regulation was not examined in that study.

The inhibitory action of IFNγ on CSF formation was also mirrored at the mRNA level (Fig 2). IL-1 increased the levels of both steady-state GM-CSF and G-CSF mRNA transcripts, an observation consistent with the inhibitory action of actinomycin D on CSF formation in these cultures, as reported before. IFNγ prevented these increases in steady-state mRNA expression; IFNγ by itself did not raise the CSF mRNAs. The data are from a typical experiment, but were confirmed in six others.

Endogenous cyclooxygenase products can regulate GM-CSF and G-CSF levels. Both IL-1 and TNFα can elevate the synthesis of human synoviocyte cyclooxygenase products, such as PGE₂, which in turn can modulate the levels in the cultures of certain other products resulting from the action of IL-1 and TNFα. In Table 2 we show that the cyclooxygenase inhibitor, indomethacin, potentiates GM-CSF formation in response to IL-1 but inhibits G-CSF formation under the same conditions. These observations, in the presence of IL-1, were made in 24 other experiments for GM-CSF and in 12 other experiments when G-CSF levels were monitored. Reversal of these effects by PGE₂ was consistent with the action of indomethacin being via inhibition of the formation of a cyclooxygenase product. Indomethacin was also active at 10⁻⁶ mol/L. Responses to TNFα were similarly modulated by indomethacin. Similar results were observed using naproxen (10⁻⁵ to 10⁻³ mol/L) and ketoprofen (10⁻⁶ to 10⁻⁸ mol/L) in place of indomethacin. The inhibitory action of IFNγ described above was also apparent in the presence of indomethacin indicating that a cyclooxygenase product was not involved in the action of the IFNγ (data not shown). In fact, IFNγ did not regulate PGE₂ in the synoviocytes either in the presence (data not shown) or absence of IL-1; the lack of effect on IL-1-stimulated PGE₂ formation suggests that IFNγ was not
inhibiting CSF formation (Table 1) by interfering with the action of IL-1 with its receptor.

Effects of IL-4 on synoviocyte CSF levels. The lymphokine, IL-4, which often has actions opposite to those of IFNγ, inhibited IL-1–mediated induction of GM-CSF but potentiated its action on G-CSF formation (Table 3). These observations were repeated with IL-4 (250 pmol/L) in 25 other experiments for GM-CSF and in 16 others for G-CSF. The degree of inhibition of GM-CSF formation by IL-4 was not as dramatic as that by IFNγ (see above). IL-4 did not stimulate GM-CSF or G-CSF formation by itself.

Inhibition of CSF formation by glucocorticoid. The anti-inflammatory glucocorticoid, dexamethasone (10^{-7} mol/L), reversed the stimulatory action of IL-1 on synoviocyte GM-CSF and G-CSF formation (Table 4). This observation was made in a total of seven experiments for GM-CSF induction and 11 for the case of G-CSF. Thus, the action of the steroid is not the same as the nonsteroidal anti-inflammatory drugs (Table 2) because the steroid suppressed the production of both CSFs.

**Table 2. Endogenous Cyclooxygenase Products and Synoviocyte CSF Levels**

<table>
<thead>
<tr>
<th>Agent Added</th>
<th>GM-CSF (ng/mL)</th>
<th>G-CSF (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Indo</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-1α</td>
<td>0.11 ± 0.01</td>
<td>1.95 ± 0.02</td>
</tr>
<tr>
<td>IL-1α + Indo</td>
<td>0.34 ± 0.02*</td>
<td>1.23 ± 0.01*</td>
</tr>
<tr>
<td>IL-1α + Indo + PGE2</td>
<td>0.06 ± 0.01</td>
<td>1.45 ± 0.02</td>
</tr>
</tbody>
</table>

| Synovial cells (from donor GK, passage 3), cultured as in Table 1, were incubated for 24 hours with indomethacin (Indo) (10^{-7} mol/L), IL-1α (100 U/mL), or a combination of these in the absence or presence of PGE2 (10^{-7} mol/L), as indicated, and supernatant GM-CSF and G-CSF measured by immunoassay. Values are means ± SEM. |

*P < .05, when compared with IL-1–treated cultures.

**Table 3. Effect of IL-4 on Synoviocyte GM-CSF and G-CSF Levels**

<table>
<thead>
<tr>
<th>Agent Added</th>
<th>GM-CSF (ng/mL)</th>
<th>G-CSF (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-1α</td>
<td>1.7 ± 0.1</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>IL-1α + IL-4</td>
<td>1.1 ± 0.1*</td>
<td>6.4 ± 0.2*</td>
</tr>
</tbody>
</table>

Synovial cells (from donor VM, passage 1), cultured as in Table 1, were incubated for 24 hours with IL-4 (250 pmol/L), IL-1α (100 U/mL), or their combination, and supernatant GM-CSF and G-CSF measured by immunoassay. Values are means ± SEM.

*P < .05, when compared with IL-1–treated cultures.

**Table 4. Inhibition of GM-CSF and G-CSF Formation by Glucocorticoid**

<table>
<thead>
<tr>
<th>Agent Added</th>
<th>GM-CSF (ng/mL)</th>
<th>G-CSF (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dex</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-1α</td>
<td>0.57 ± 0.04</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>IL-1α + Dex</td>
<td>0.09 ± 0.01*</td>
<td>1.2 ± 0.1*</td>
</tr>
</tbody>
</table>

Synovial cells (from donor JB, passage 5), cultured as in Table 1, were incubated for 24 hours with dexamethasone (Dex) (10^{-7} mol/L), IL-1α (100 U/mL), or their combination, and supernatant GM-CSF and G-CSF measured by immunoassay. Values are means ± SEM.

*P < .05, when compared with IL-1–treated cultures.

**Effects of FGF and TGFβ on CSF formation.** We have reported previously that a number of cytokines, namely M-CSF (CSF-1), IL-3, IL-2, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor α (TGFα), did not stimulate GM-CSF or G-CSF formation either alone or in the presence of an optimal concentration of IL-1 or TNFα. We now demonstrate that basic FGF (10^{-8} mol/L), although inactive by itself, could potentiate to some extent the stimulatory effect of optimal IL-1 (Table 5). Similar findings were also made in 21 other experiments for GM-CSF and 18 others for G-CSF. The enhancement due to FGF could be measured at 6 hours for both CSFs (data not shown). Northern analysis (Fig 3) illustrates that the changes in antigen levels due to FGF were paralleled at the mRNA level.

TGFβ (0.02 to 20 ng/mL), which we have reported to stimulate urokinase-type PA activity in the synovial fibroblasts, failed in several experiments to regulate CSF formation in the absence or presence of an optimal IL-1 concentration (data not shown).

**DISCUSSION**

We have previously shown that both IL-1 and TNFα stimulate GM-CSF and G-CSF formation in human synoviocytes with the former cytokine being more potent in terms of the degree of enhancement of CSF formation and also of the concentration at which it was active. It was concluded that synovial fibroblast-like cells may be a source of GM-CSF and other as yet uncharacterized CSFs present in rheumatoid synovial fluid. The GM-CSF present in these fluids is considered to be important in the maintenance of such lesions because, for example, it can induce class II
were incubated for 24 hours with FGF (10^-15 mol/L) and IL-1α (100 U/mL), either alone or in combination, and supernatant GM-CSF and G-CSF measured by immunoassay. Values are means ± SEM. *P < .05, when compared with IL-1–treated cultures.

MHC expression on monocytes and the expression of the serine proteinase, PA. In vitro, GM-CSF enhances monocyte adherence to the endothelium, which could account for the very rapid and transient monocytopenia in evidence after intravenous injection into patients. This enhanced monocyte-endothelial adhesion by locally produced GM-CSF may also be involved in the close apposition of monocytes to the blood vessel wall and in the altered transendothelial migration and intrasynovial accumulation of monocytes into rheumatoid joints. Furthermore, it has been reported that GM-CSF, when administered to a patient with Felty’s syndrome, caused a flare-up of the rheumatoid arthritis in the affected joints. Synoviocyte G-CSF may also contribute by activating the neutrophils that are present in the synovial fluids of such patients. CSF production by cells, such as fibroblasts, endothelial cells, and monocytes, must be tightly regulated in vivo to induce rapid changes in leukocyte numbers but also possibly to limit the magnitude of the inflammatory response and prevent tissue damage.

Most studies involving the actions of cytokines on synoviocytes have analyzed those of individual cytokines. However, given that a number of cytokines have been detected in the rheumatoid synovium, it is likely that they will be acting in concert on joint cells, such as synoviocytes. For example, we have reported before that IL-1 and TNFα can synergize in stimulating in vitro GM-CSF and G-CSF formation by human synoviocytes and chondrocytes. In the present report we have shown that IFNγ suppresses IL-1-induced (and TNFα-induced) GM-CSF and G-CSF production, and concomitantly alters steady-state mRNA levels. Others have recently reported that IFNγ inhibited GM-CSF production induced by TNFα in human synovial fibroblast-like cells, but not that induced by IL-1. The reasons for this discrepancy with regard to the modulation of IL-1 action are unclear as is the mechanism of the inhibitory action of IFNγ on CSF gene regulation. It would not appear to involve prostaglandin production nor interference with the interaction of IL-1 with its receptor because IFNγ does not modulate PGE2 levels of IL-1–treated synoviocytes. It is possible that the findings in the present study have clinical significance. Only low levels of IFNγ are measurable in synovial effusions. It could be, therefore, that the reported efficacy of IFNγ administration in rheumatoid arthritis may be due in part to its antagonistic actions on IL-1– and TNFα-mediated CSF production. It has been proposed recently that the combined presence and opposing actions of IFNγ and TNFα might be one method by which articular inflammation is normally attenuated. From our prior results and from results of the present study referred to above, where we found that IL-1 was a more potent stimulus of CSF production than TNFα and that the two cytokines interact synergistically to stimulate CSF production, it could be that the suppression by IFNγ of the IL-1 action may be more significant than its previously reported inhibition of TNFα action.

We and others have found that, as for GM-CSF and G-CSF formation, IL-1 was more potent than TNFα in stimulating the PGE2 and collagenase levels of human synovial fibroblasts and that both cytokines acted synergistically when their combined effects were judged by the same criteria. However, when the effects of IFNγ have been examined on the cytokine-treated synoviocytes, different results have emerged. IFNγ inhibited both IL-1– and TNFα-stimulated collagenase formation in one report, but only the effect of TNFα in another. In addition, we have found no effect of IFNγ on IL-1– and TNFα-stimulated PGE2 formation, in contrast to the literature. There have also been discrepancies in the literature when IL-1 and TNFα have been tested for their actions on IFNγ-induced class II MHC antigen expression on synovial fibroblasts; in human synovial cells, TNFα, but not IL-1, reversed the induction, while in rat synovial cells only IL-1 was also effective.

We also showed that IL-4 could inhibit the GM-CSF production by IL-1–stimulated synoviocytes but stimulate the corresponding G-CSF production. The inhibitory action on GM-CSF formation was generally not as dramatic as that resulting from the presence of IFNγ. The differing result with IL-4, depending on which CSF was being examined, illustrates that the two CSFs are not always...
coordinately regulated (see below). The actions of IFNγ and IL-4 on cellular function are sometimes opposite\textsuperscript{14,27}; this would seem to apply only for G-CSF formation but not for the case of GM-CSF formation in the stimulated synoviocytes.

The suppressive effect of the cyclooxygenase inhibitors on IL-1–stimulated GM-CSF formation but their potentiation of the corresponding G-CSF levels shows (1) that endogenous cyclooxygenase products can modulate GM-CSF and G-CSF levels, and (2) that once again that the production of the two CSFs is not always coordinately regulated. In contrast to the nonsteroidal anti-inflammatory drugs, the glucocorticoid, dexamethasone, inhibited the production by IL-1 of both CSFs. This means that the CSFs join the list of products from cytokine-stimulated synoviocytes for which steroids inhibit the action of IL-1,\textsuperscript{19} including PA,\textsuperscript{40} collagenase,\textsuperscript{41} hyaluronic and PGE\textsubscript{2}.\textsuperscript{43}

Such control of CSF formation by this class of steroid may represent a part of their action during the treatment of inflammatory joint disease.

There is evidence for both transcriptional and posttranscriptional control of GM-CSF and G-CSF formation in IL-1– and TNFα-treated connective tissue cells, including human synovial fibroblast-like cells.\textsuperscript{17,22,24,25,44} Also, the data presented so far in the literature suggest that their expression is coordinated in such cytokine-treated cells.\textsuperscript{22,24} There appears to be some common aspects involved in their regulation in the synoviocytes because (1) IL-1 and TNFα, either alone or in combination with each other,\textsuperscript{17} and IL-1, in combination with FGF (Table 5 and Fig 3), raise their levels, and (2) IFNγ (Table 1, Figs 1 and 2) and dexamethasone (Table 4) downregulate expression of both CSFs. However, as shown above, there are differences highlighted by the differing responses to cyclooxygenase inhibitors (Table 2) and IL-4 (Table 3). The reasons for these similarities and differences in the control of CSF formation will require an analysis of the relevant signaling pathways, including those controlling transcriptional and posttranscriptional events.\textsuperscript{35,44}

We showed above that FGF, while being inactive by itself, was able to potentiate the stimulatory action of IL-1 (and also TNFα) on GM-CSF and G-CSF production. It should be mentioned, however, that the potentiating effects of FGF on the actions of IL-1 and TNFα were generally not as dramatic as the interaction between the latter two cytokines;\textsuperscript{17} these observations also having been made in the same experiment (data not shown). Because FGF, IL-1, and TNFα have been identified in rheumatoid fluids,\textsuperscript{34,44} these cytokines may be contributing the joint CSF levels via a concerted action on the synoviocytes. TGFβ\textsubscript{3}, which elevates urokinase-type PA activity and mRNA transcripts in the synoviocytes, and potentiates the action of IL-1 in this regard,\textsuperscript{26} did not modulate either CSF expression alone or in the presence of IL-1; therefore, the regulation of urokinase-type PA activity can be dissociated from that of the CSFs. A number of other cytokines, namely M-CSF (CSF-1), IL-3, IL-2, PDGF, EGF, and TGFα, did not stimulate GM-CSF or G-CSF production either alone or in the presence of IL-1,\textsuperscript{17} nor did they inhibit the enhancing action of IL-1 (data not shown).

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Cytokine regulation of colony-stimulating factor (CSF) production in cultured human synovial fibroblasts. II. Similarities and differences in the control of interleukin-1 induction of granulocyte-macrophage CSF and granulocyte-CSF production

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