Granulocyte-Macrophage Colony-Stimulating Factor Expression by Human Fibroblasts Is Both Upregulated and Subsequently Downregulated by Interleukin-1

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Interleukin-1 (IL-1) treatment of human WI-38 lung fibroblasts results in granulocyte-macrophage colony-stimulating factor (GM-CSF) expression as well as a delayed increase in prostaglandin E\(_2\) (PGE\(_2\)) production that closely correlates with the decline of GM-CSF mRNA levels. Pretreatment with PGE\(_2\) reduces the IL-1 induced GM-CSF mRNA and protein expression to 10% to 15% of control values at concentrations of PGE\(_2\) that are endogenously produced after IL-1 stimulation. Inhibition of PGE\(_2\) synthesis by indomethacin prolongs the IL-1 induced GM-CSF mRNA expression and increases the cumulative GM-CSF protein secretion. Exposure of WI-38 fibroblasts to PGE\(_2\) results in an increase in intracellular cyclic adenosine monophosphate (cAMP) levels. The inhibition of GM-CSF expression by PGE\(_2\) can be mimicked by stable cAMP analogs as well as cAMP elevating agents such as cholera toxin, forskolin, and isobutylmethylxanthine. Thus the inhibition exerted by PGE\(_2\) is mediated via cAMP. Taken together, these results suggest that IL-1 stimulation of human fibroblasts provides not only the upregulatory signal for GM-CSF expression but also a delayed and indirect downregulatory signal that serves to limit GM-CSF expression in the continued presence of IL-1.

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MATERIALS AND METHODS

Cell culture. Normal human WI-38 lung fibroblasts (American Type Culture Collection, Rockville, MD) were obtained at passage 13 and were used between passages 25-35. Human bone marrow cells were aspirated from posterior iliac crests of normal healthy male volunteers and transferred to heparinized blood collection tubes. Bone marrow–derived fibroblasts were obtained by several passages of bone marrow–adherent cells in McCoy’s medium (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS; HyClone, Logan, UT) until markers for macrophages (CD14, CD68) and for endothelial cells (von Willebrand factor) were no longer detectable. These studies were approved by the Research Subjects Review Board of the University of Rochester. Both cell types were routinely cultured in McCoy’s medium with 10% FCS at 37°C in a humidified atmosphere containing 5% CO\(_2\). For drug treatment, 5 × 10\(^5\) cells were plated onto 100-mm plates (Corning, Corning, NY) and grown to confluence over the next 3 days. Medium was then aspirated and replaced with fresh medium for 24 hours. All drug treatments were performed in fresh complete medium containing the appropriate agent for the indicated time.

Reagents. Recombinant human IL-1α (100 U/\(\mu\)g), IL-1β (5 × 10^3 U/\(\mu\)g), and TNF-α (2 × 10^5 U/\(\mu\)g) (Genzyme, Cambridge, MA), cholera toxin and dibutyryl cyclic adenosine monophosphate (cAMP) (Calbiochem, San Diego, CA) were dissolved in McCoy’s.
medium. Prostaglandins E\(_1\), E\(_2\), and F\(_{2\alpha}\), TPA, Actinomycin D and cycloheximide (Sigma, St Louis, MO), forskolin (Calbiochem, San Diego, CA) and isobutylmethylxanthine (IBMX; Calbiochem) were dissolved in dimethylsulfoxide (DMSO) as vehicle. The DMSO concentration never exceeded 0.2% in the culture medium. At these concentrations DMSO alone had no discernible effect. \(^{32}\)P-dATP was obtained from Dupont NEN, Boston, MA.

**Analysis of cytokine levels in cell culture supernatants.** GM-CSF, G-CSF, and IL-6 measurements were made directly on cell culture supernatants by enzyme linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN).

**PGE\(_2\) measurements.** PGE\(_2\) levels in the supernatants were measured by a radioimmunoassay kit (Amersham, Arlington Heights, IL). Brieﬂy, confluent ﬁbroblasts were treated with or without IL-1β in McCoy’s medium with 10% dialyzed FCS. For these experiments serum was dialyzed extensively against phosphate-buffered saline containing activated charcoal to remove low molecular weight lipids that might otherwise interfere with the PGE\(_2\) measurements. Aliquots of supernatants were removed at hourly intervals and methyl oximated before use in a standard radioimmunoassay.

**cAMP measurements.** Measurement of intracellular cAMP was performed by a radioimmunoassay kit (Amersham). Brieﬂy, confluent WI-38 and bone marrow-derived ﬁbroblasts were treated with 1 μmol/L PGE\(_2\) in serum-free McCoy’s medium. At various time points, two volumes of absolute ethanol were added. The cells were scraped, spun to remove debris, evaporated to dryness, and processed for radioimmunoassay.

**Northern-blot analyses.** Total cellular RNA was isolated by guanidine isothiocyanate denaturation followed by phenol-chloroform extraction. Equal amounts (25 μg/lane) were fractionated on formaldehyde-agarose gels and transferred to Nytran membranes (Schleicher and Shuell, Keene, NH). These were sequentially probed with \(^{32}\)P-labeled probe obtained by random priming of full-length GM-CSF cDNA (provided by Dr Frank Lee, DNAX Institute) and a 0.9-kb HindIII-EcoRI fragment of glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA (provided by Dr Ray Wu, Cornell University). For detection of G-CSF and IL-6 transcripts, 5' kinased \(^{32}\)P-labeled oligonucleotide probes (R&D Systems) were used. The Nytran ﬁlters were exposed to X-AR ﬁlm (Eastman Kodak, Rochester, NY) at -70°C. Quantitation and normalization was performed by a scanning densitometer (GS300; Hoefer Scientiﬁc, San Francisco, CA).

**Data analysis.** Values are reported as mean ± S.E.M. The signiﬁcance of difference between groups was calculated using Student’s t-test, P < .05.

**RESULTS**

**Effect of IL-1 on GM-CSF and PGE\(_2\) production.** Before proceeding to study the negative regulatory aspect of GM-CSF expression, the stimulatory effect of IL-1 in this system was characterized. WI-38 cells and normal human bone marrow-derived ﬁbroblasts grown to conﬂuence were cultured in the presence of IL-1α and IL-1β at concentrations of 1 to 100 U/mL. Supernatants were harvested at 6, 12, 18, 24, and 48 hours and the release of GM-CSF was measured. In the absence of IL-1 no GM-CSF was produced by either cell type. In the presence of IL-1α or IL-1β maximal stimulation was observed at concentrations >25 U/mL. GM-CSF was detectable in the supernatants at six hours; cumulative GM-CSF release in the culture medium was maximal at 24 hours and remained constant over the 24 to 48 hours of culture. WI-38 cells produced much larger quantities of GM-CSF (1.830 ± 130 pg/mL) than bone marrow-derived ﬁbroblasts (170 ± 40 pg/mL). A supramaximal stimulatory concentration of 100 U/mL of IL-1 and a 24-hour treatment period were used for subsequent experiments.

For the study of GM-CSF mRNA levels, WI-38 cells were cultured in the presence of 100 U/mL of IL-1β and cells were harvested hourly for RNA isolation. GM-CSF mRNA was detectable within 1 to 2 hours. The message steadily increased and reached a peak at 4 to 6 hours and then declined to undetectable levels within 12 to 18 hours (Fig 1A).

IL-1 has been shown to increase prostaglandin production in many cell types including ﬁbroblasts. WI-38 cells grown to conﬂuence were cultured with 100 U/mL IL-1α and aliquots of culture supernatants were harvested every hour for the next 24 hours. IL-1α-treated cells produced much larger quantities of PGE\(_2\) than the untreated cells (Fig 1B). The
increase in PGE$_2$ production became apparent after 5 to 6 hours of treatment and subsequently increased over the next 10 to 12 hours before stabilizing at a level 8- to 10-fold higher than control cells.

**Effect of PGE$_2$ modulation on IL-1-inducible GM-CSF production in fibroblasts.** The delayed release of PGE$_2$ in response to IL-1 concomitant with the downregulation of GM-CSF suggested that production of PGE$_2$ might play a role in the deinduction of GM-CSF. To test this hypothesis, the effect of PGE$_2$ on the initial rapid upregulation of GM-CSF was studied. Co-incubation with 10 µmol/L PGE$_2$, completely abolished GM-CSF production by bone marrow-derived fibroblasts and reduced GM-CSF production by WI-38 cells to 10% to 15% of control values (Fig 2). The effect was seen both with IL-1α and IL-1β. Dose response experiments showed that the PGE$_2$-mediated suppression of IL-1β-induced GM-CSF production was maximal at 10 µmol/L, with an IC$_{50}$ of 0.2 to 0.3 µmol/L. To determine whether prostaglandins other than PGE$_2$ had an effect on GM-CSF expression, PGE$_1$ and PGE$_3$, were tested. PGE$_3$, (0.1 to 10 µmol/L) had no significant effect on IL-1-induced GM-CSF expression, whereas the dose response to PGE$_1$ was essentially identical to that of PGE$_2$ (data not shown).

The diminution of secreted GM-CSF protein reflected the expression of intracellular GM-CSF mRNA. Cells exposed for 6 hours to IL-1β (100 U/mL) and PGE$_2$ (10 µmol/L) expressed <20% as much GM-CSF mRNA as compared with cells exposed to IL-1 alone (Fig 3A). A dose response of PGE$_2$ on GM-CSF mRNA surprisingly showed significant suppression at levels as low as 30 nmol/L; in bone marrow–derived fibroblasts a similar dose response was observed (data not shown). Addition of PGE$_2$ at 6 hours produced a significant decline in the mRNA levels at 8-, 9-, and 10-hour time points; the levels at 7 hours were not affected. Similarly, if cells were initially exposed to IL-1 and PGE$_2$
To determine if PGE₂ is able to inhibit specifically GM-CSF expression, WI-38 fibroblasts were cultured with or without IL-1β (10 ng/mL) for 24 hours. From 3 hours to 6 hours the cells were exposed to either PGE₂ (10 μmol/L) or cycloheximide (10 μg/mL), or both in the continued presence of IL-1β. Cells were harvested at 6 hours and analyzed for GM-CSF mRNA.

was then added at 1 to 4 hours, the mRNA levels at 6 hours were significantly lower than the controls. However, the addition of PGE₂ at 5 hours was not effective in reducing the 6-hour mRNA level (data not shown). There seems to be a lag period of 1 to 2 hours before the PGE₂-induced suppression comes into effect.

To determine if a protein synthesis step is partly responsible for the lag period, the effect of inhibition of nascent protein synthesis was studied. Addition of cycloheximide (10 μg/mL) inhibited >95% of new protein synthesis as measured by 3H-leucine incorporation (data not shown). At this concentration cycloheximide alone caused the appearance of detectable mRNA levels in the unstimulated WI-38 cells. Cells treated with IL-1 in the presence of cycloheximide expressed much higher levels of GM-CSF mRNA than cells treated with cycloheximide alone. Cells treated with IL-1 and PGE₂ in the presence of cycloheximide showed no suppression of mRNA levels (Fig 4). Thus, protein synthesis is essential for PGE₂-mediated inhibition of GM-CSF.

To determine whether inhibition of GM-CSF expression is specific in the context of induction of multiple cytokines by IL-1, we tested the effect of PGE₂ on G-CSF and IL-6. IL-1 induction of G-CSF and IL-6 was not affected by pretreatment with PGE₂. In the absence of IL-1 stimulation, WI-38 cells did not release detectable quantities of G-CSF and IL-6 in the supernatants. After 24-hour culture with 100 U/mL of IL-1, 19 ± 4 ng/mL of G-CSF, and 36 ± 3 ng/mL of IL-6 were released in the supernatant. On co-culture with IL-1 and 0.1 to 10 μmol/L of PGE₂ no significant change from these levels was observed. The G-CSF and IL-6 mRNA levels induced by IL-1 at 6 hours were not affected by PGE₂.

To determine if PGE₂ is able to inhibit specifically GM-CSF induced by stimuli other than IL-1, the effect of PGE₂ on TNF-α and TPA-induced GM-CSF expression was tested. WI-38 cells stimulated with 1,000 U/mL of TNF-α produced 950 ± 120 pg/mL of GM-CSF over 24 hours; however, coincubation with 10 μmol/L PGE₂ reduced GM-CSF levels below 100 pg/mL. Similarly, treatment with 30 nmol/L TPA resulted in production of 1,440 ± 320 pg/mL of GM-CSF in 24 hours and co-incubation with 10 μmol/L PGE₂ and 30 nmol/L TPA reduced the GM-CSF production to 230 ± 115 pg/mL. In contrast, measurement of G-CSF and IL-6 in the supernatants showed that the production of these cytokines in response to TPA and TNF-α was not affected by PGE₂ (data not shown). When TNF-α (1,000 U/mL) and TPA (30 n) were co-incubated with 10 μmol/L PGE₂, the GM-CSF mRNA levels at 6 hours were several fold less than with TNF-α and TPA alone (Fig 5). Thus, GM-CSF induction by TNF-α and TPA could be effectively suppressed by PGE₂.

The inhibitory effect of exogenously added PGE₂ on GM-CSF expression suggested that intrinsically generated PGE₂ might play a role in negatively regulating GM-CSF expression. To test this hypothesis, WI-38 cells were cultured with IL-1β in the presence of 1 nmol/L to 1 μmol/L indomethacin. The presence of 300 nmol/L indomethacin caused near complete inhibition of PGE₂ production with an IC₅₀ of 3 nmol/L (Fig 6A). For subsequent experiments indomethacin was used at a concentration of 1 μmol/L. The cumulative GM-CSF release was twofold higher in the presence of indomethacin (4,130 ± 275 pg/mL) than when it was absent (1,945 ± 330 pg/mL).

To investigate the inhibitory effect of intrinsic PGE₂s on GM-CSF mRNA expression, WI-38 fibroblasts were cultured with IL-1β (100 U/mL) and indomethacin (1 μmol/L) for 3, 6, 9, 12, and 24 hours, at which time cells were harvested for RNA isolation. Northern analyses showed that mRNA levels at 3 and 6 hours were not significantly different when treated with IL-1β in the presence or absence of indomethacin. However, the rate of decline in mRNA levels was
To determine whether cAMP was able to mimic the effects of PGEs, the co-incubation of IL-1 and dibutyryl cAMP was studied. Dibutyryl cAMP is a weak suppressor of GM-CSF production in WI-38 cells, requiring 1 mmol/L concentration to achieve significant suppression. In contrast, dibutyryl cAMP (100 μmol/L) achieved significant reduction of GM-CSF in bone marrow fibroblasts that was comparable to the effect of PGEs (Fig 2). Agents that elevate cAMP (cholera toxin (0.1 μg/mL), forskolin (10 μmol/L) and IBMX (100 μmol/L) effectively blocked GM-CSF release in the culture supernatants by both cell types (Fig 2). Furthermore, these agents were able to suppress significantly the IL-1-induced increase in GM-CSF mRNA levels and mimic the downregulatory effect of PGEs (Fig 3B). Thus, the inhibition of GM-CSF by PGEs is at least partially mediated by an increase in intracellular cAMP.

**DISCUSSION**

The results reported here show that IL-1 stimulation causes a time-dependent release of PGEs by WI-38 cells. While these studies were in progress, Lin et al reported that IL-1α stimulates PGE2 synthesis in WI-38 fibroblasts. They did not detect significant PGE2 levels at time points up to 5 hours after stimulation, but observed significantly higher levels in the IL-1–treated group at later time points. Our results are in accord with this report. IL-1 is known to induce cyclooxygenase as well as phospholipase A2, and the time course of the reported increase in phospholipase A2 correlates well with PGE2 production. Thus, there is a strong probability that IL-1 induces increased synthesis/activity of cyclooxygenase and/or phospholipase A2 that indirectly elevates levels of PGE2 by increasing arachidonic acid release and/or metabolism. These intermediate steps may be responsible for the delayed increase of PGE2. IL-1–induced PGE2 formation is 10-fold higher in the presence of calcium ionophore A23187 than it has been suggested that IL-1 induces PGDF, which in turn increases intracellular calcium in an autocrine manner. Thus, it is possible that the need for calcium ions is responsible for a delayed activation of PGE2 production.

GM-CSF mRNA becomes detectable within an hour of IL-1 stimulation, accumulates steadily over 4 to 6 hours, and then slowly declines over 24 hours. The decline in GM-CSF mRNA levels is concomitant with the increase in PGE2.

**Table 1. Effect of PGE2 on Intracellular cAMP Levels in WI-38 and Normal Bone Marrow–Derived Fibroblasts**

<table>
<thead>
<tr>
<th></th>
<th>cAMP (mmol/protein)</th>
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<tbody>
<tr>
<td><strong>WI-38 cells</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>130 ± 70</td>
</tr>
<tr>
<td>PGE2 (1 μmol/L)</td>
<td>12,210 ± 2,060</td>
</tr>
<tr>
<td><strong>Bone marrow–derived fibroblasts</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;25</td>
</tr>
<tr>
<td>PGE2 (1 μmol/L)</td>
<td>1,020 ± 450</td>
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</tbody>
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Cells grown in 24-well plates were washed with serum-free medium before the addition of 0.1 mL of serum-free medium with or without 1 μmol/L PGE2. Quenching was performed 5 minutes later by addition of absolute ethanol. The supernatants were spun, evaporated to dryness, resuspended in assay buffer, and assayed by radioimmunoassay. The results are expressed as mean ± SEM (n = 5).
GM-CSF REGULATION BY IL-1

production. This time course is seen despite the continued presence of IL-1 in the medium. IL-1 receptor is not downregulated by continued IL-1 stimulation over a period of 6 hours. In fact, previous reports suggest that prostaglandins of the E series increase IL-1 receptor expression by fibroblasts. However, we cannot rule out the possibility that downregulation, possibly of receptor function, is at least partly responsible for the termination of IL-1 induction.

GM-CSF induction by TNF-α and TPA was suppressed by PGE2. TNF-α, although binding to a different receptor from IL-1, evokes a similar phosphorylation pattern to that of IL-1 in fibroblasts and is known to stabilize GM-CSF mRNA. Moreover, it is also known to induce PGE2 production. TPA, on the other hand, evokes a very dissimilar pattern of phosphorylation and in addition to stabilizing GM-CSF mRNA, increases transcriptional activity of the GM-CSF gene as well. These data are consistent with either a direct inhibitory effect of PGE2 on GM-CSF expression or an indirect effect involving inhibition downstream to a point where IL-1, TNF, and TPA signaling pathways converge.

GM-CSF mRNA levels are reinduced in a concentration-dependent manner by PGE2. The inhibition is achieved at concentrations that are well within the range evoked by IL-1 stimulation, especially when it is considered that PGE2 is an autocrine agent, and effective intracellular and pericellular concentrations are likely to be much higher than those detected in the supernatants. Furthermore PGE2 is as potent as PGE2 for inhibition of GM-CSF expression, and a concordant increase in the levels of PGE2 would accentuate the GM-CSF inhibition.

In these experiments indomethacin was used to inhibit IL-1–induced endogenous PGE2 production. In the presence of indomethacin a significant increase in GM-CSF protein secretion as well as a marked prolongation of GM-CSF mRNA expression was observed. As discussed earlier, IL-1–induced endogenous PGE2 release closely correlates with the decrease of GM-CSF mRNA levels. Taken together, these observations support the hypothesis that IL-1 stimulation results in a positive as well as a delayed negative regulatory signal for GM-CSF expression, and the negative regulation is partially achieved via PGE2. Downregulation of GM-CSF mRNA, although delayed, occurs even in the absence of PGE2, indicating that additional negative regulatory mechanisms exist. Such negative regulatory signals may be safeguards for preventing uncontrolled expression of GM-CSF.

PGE2 can elicit large elevations in intracellular cAMP, and cAMP analogs as well as cAMP elevating agents can mimic the effects of PGE2 on GM-CSF mRNA and protein expression. Evidence is conclusive that the inhibition of GM-CSF expression caused by PGE2 is mediated through cAMP. Because cycloheximide can block the PGE2 and cAMP-mediated suppression, it is probable that PGE2–induced elevation of cAMP results in synthesis of one or more proteins that in turn affect GM-CSF gene expression. The delay observed in the onset of PGE2–induced decline of GM-CSF mRNA levels is also consistent with such a hypothesis. Cycloheximide has also been postulated to prevent synthesis of one or more proteins that degrade GM-CSF mRNA. It cannot be ascertained at this point whether the protein synthesis step involved in the PGE2–cAMP inhibition is the same one that is involved in the superinduction phenomenon evoked by cycloheximide. If so, endogenous PGE2 does not seem to play a role at least in the basal synthesis of these proteins, because the presence of cycloheximide leads to an increased level of basal- and IL-1–induced GM-CSF mRNA, whereas indomethacin does not significantly affect the mRNA levels over the same period.

It has been reported that PGE2/cAMP induce IL-6 expression. In these experiments PGE2 failed to elicit IL-6 or G-CSF expression by itself. A significant inhibitory effect on IL-1-induced IL-6 or G-CSF expression was not observed. Regulation of IL-6 expression seems to depend on a complex interaction of a number of different cellular processes. It has been shown that dexamethasone downregulates GM-CSF mRNA levels in an almost identical manner as PGE2. However, dexamethasone suppresses IL-6, whereas PGE2 does not. It is interesting that glucocorticoids have been shown to inhibit IL-1–induced phospholipase A2 induction and PGE2 production in WI-38 cells. Thus, one negative regulatory mechanism itself seems to suppress another negative regulatory mechanism. On the other hand, elevation of intracellular calcium ions leads to upregulation of GM-CSF expression as well as potentiation of PGE2 production, which is a downregulatory mechanism. Because these positive and negative stimuli are likely to come from different sources during the physiologic processes of inflammation and hematopoiesis, GM-CSF expression could be governed by multiple interacting pathways that tightly regulate it over a temporal and possibly a spatial frame.

Summarizing, it seems that PGE2–mediated downregulation of GM-CSF is part of an autoregulatory process, is dependent on protein synthesis, and is exerted via an elevation in intracellular cAMP level. It is reasonable to speculate that PGE2 exposure results in synthesis of one or more proteins that are responsible for downregulation of GM-CSF mRNA.

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