Rapid Priming of Human Monocytes by Human Hematopoietic Growth Factors: Granulocyte-Macrophage Colony-Stimulating Factor (CSF), Macrophage-CSF, and Interleukin-3 Selectively Enhance Superoxide Release Triggered by Receptor-Mediated Agonists

By Akira Yuo, Seiichi Kitagawa, Kazuo Motoyoshi, Eriko Azuma, Masaki Saito, and Fumimaro Takaku

The effects of hematopoietic growth factors on human monocyte superoxide (O$_2^-$) release were investigated by using purified human monocytes in suspension. Among growth factors studied, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage-CSF (M-CSF), and interleukin-3 (IL-3) primed human monocytes and enhanced O$_2^-$ release stimulated by the receptor-mediated agonists, N-formyl-methionyl-leucyl-phenylalanine (FMLP) and concanavalin A (Con A), but not by phorbol myristate acetate, which bypasses the receptors to stimulate the cells. The optimal priming was obtained by pretreatment of cells with 1 to 5 ng/mL (0.07 to 0.34 nmol/L) GM-CSF, 50 to 100 ng/mL (0.5 to 1.1 nmol/L) M-CSF, or 10 to 20 ng/mL (0.6 to 1.3 nmol/L) IL-3 for 10 minutes at 37°C. Potency of the maximal priming effects on FMLP- or Con A-induced O$_2^-$ release was GM-CSF > M-CSF = IL-3. The combination of the optimal concentrations of any two CSFs resulted in the effect of more potent priming agent alone. Enhancement of O$_2^-$ release by GM-CSF was observed over the complete range of effective concentrations of FMLP (10$^{-10}$ to 10$^{-4}$ mol/L). The pretreatment of monocytes with granulocyte-CSF (50 ng/mL), interferon-γ (1,000 U/mL), or IL-4 (20 ng/mL) for 10 minutes at 37°C had no effect on O$_2^-$ release stimulated by FMLP or Con A. These findings show that GM-CSF, M-CSF, and IL-3 selectively enhance O$_2^-$ release in human monocytes triggered by receptor-mediated agonists after short-term preincubation.

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**MATERIALS AND METHODS**

**Reagents.** Highly purified (> 98%) recombinant human hematopoietic growth factors and cytokines were used in the present experiments. Endotoxin contamination of each preparation was less than 100 pg/mg protein. GM-CSF produced by Escherichia coli (molecular weight [MW] 14.7 Kd) was provided by Schering-Plough Co, Ltd (Osaka, Japan). M-CSF produced by Chinese hamster ovary (CHO) cells (MW 85 to 90 Kd) was provided by Morinaga Milk Industry Co (Tokyo, Japan). IL-3 (MW 15 Kd) and GM-CSF (MW 18.8 Kd) produced by E coli were provided by Kirin Brewery Co, Ltd (Tokyo, Japan). Interferon-γ (IFN-γ) and IL-4 produced by E coli were provided by Shionogi Pharmaceutical Co (Osaka, Japan) and DNAX (Palo Alto, CA), respectively. Cytochrome C type III, FMLP, Con A, and PMA were purchased from

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Sigma Chemical Co (St Louis, MO); Conray from Mallinckrodt Inc (St Louis, MO); and Ficoll from Pharmacia Fine Chemicals, Inc (Piscataway, NJ).

Preparation of cells. Neutrophils and mononuclear cells were prepared from heparinized venous blood of healthy adult donors as described, using dextran sedimentation, centrifugation with Conray-Ficol, and hypotonic lysis of contaminated erythrocytes. Monocytes were further purified from mononuclear cells by centrifugal elutriation in a Hitachi SRE6 elutriation rotor (Hitachi Ltd, Tokyo, Japan). Neutrophil fractions contained greater than 95% neutrophils. Mononuclear cell fractions contained 15% to 25% monocytes, 75% to 85% lymphocytes, and less than 1% neutrophils. Monocyte fractions contained 90% to 95% monocytes and 5% to 10% lymphocytes. All cell fractions were suspended in Hanks' Balanced Salt Solution (HBSS).

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Determination of \( \text{O}_2^- \) release. \( \text{O}_2^- \) was assayed by superoxide dismutase-inhibitable reduction of ferricytochrome C, and the continuous assay was performed in a Hitachi 557 spectrophotometer (a double wavelength spectrophotometer; Hitachi Ltd, Tokyo, Japan), equipped with thermostatted cuvette holder (37°C), as described. The final concentration of cytochrome C was 110 \( \mu \)mol/L and the final cell concentrations of neutrophils, mononuclear cells, and monocytes were 2 \( \times 10^6 \), 1 \( \times 10^6 \), and 2 \( \times 10^6 \) cells/mL, respectively. The reduction of cytochrome C was measured at 550 nm with a reference wave length at 540 nm, and the time-course of cytochrome C reduction was followed on the recorder. The amount of \( \text{O}_2^- \) release was calculated from cytochrome C reduced for 5 minutes after the addition of FMLP or Con A. When mononuclear cells were used, the amount of \( \text{O}_2^- \) release was adjusted to the value per 2 \( \times 10^5 \) monocytes according to the percentage of monocytes in the mononuclear cell fraction.

Statistical analysis. The Student's t-test was used to determine statistical significance.

RESULTS

Effects of GM-CSF, M-CSF, and IL-3 on \( \text{O}_2^- \) release in human monocytes stimulated by FMLP or Con A. Human monocytes, like human neutrophils, release \( \text{O}_2^- \) in response to FMLP, Con A, or PMA. The representative time-courses of \( \text{O}_2^- \) release in human monocytes stimulated by these agonists are shown in Fig 1. For a comparison, the time-courses of \( \text{O}_2^- \) release in human neutrophils stimulated by FMLP or Con A are also shown in Fig 1. When human monocytes were preincubated with GM-CSF (5 ng/mL), M-CSF (100 ng/mL), or IL-3 (20 ng/mL) for 10 minutes at 37°C, FMLP- or Con A-induced \( \text{O}_2^- \) release was significantly potentiated (Fig 1). The initial rate as well as the total amount of \( \text{O}_2^- \) release were enhanced by GM-CSF, M-CSF, or IL-3. As shown in Fig 2, the priming effects of GM-CSF, M-CSF, and IL-3 were dependent on the concentrations of factor used and the preincubation time. The maximal effects were obtained at 1 to 5 ng/mL (0.07 to 0.34 nmol/L) GM-CSF, 50 to 100 ng/mL (0.5 to 1.1 nmol/L) M-CSF, and 10 to 20 ng/mL (0.6 to 1.3 nmol/L) IL-3, respectively. The potency of the priming effects was GM-CSF > M-CSF = IL-3, when assessed on the basis of the maximal effects and the optimal molar concentrations. The priming effect of GM-CSF, M-CSF, and IL-3 was very rapid and the optimal effect was obtained by pretreatment of cells with each factor for 10 minutes at 37°C. Preincubation of human monocytes with each CSF for up to 2 hours did not show further enhancement of FMLP-induced \( \text{O}_2^- \) release (data not shown). Enhancement of \( \text{O}_2^- \) release by GM-CSF was observed over the complete range of effective concentrations of FMLP (10^-4 to 10^-6 mol/L) (Fig 3).

In contrast to the results obtained with FMLP and Con A, pretreatment of monocytes with GM-CSF (5 ng/mL), M-CSF (100 ng/mL), or IL-3 (20 ng/mL) for 10 minutes at 37°C failed to enhance \( \text{O}_2^- \) release stimulated by PMA (100 ng/mL), a direct activator of protein kinase C (Fig 1). Prolonged incubation of cells with each CSF for up to 2 hours gave the same results (data not shown). GM-CSF enhanced FMLP-induced \( \text{O}_2^- \) release in human monocytes by approximately twofold. These findings contrast well with our previous studies with human neutrophils, in which

![Fig 1. Time-courses of \( \text{O}_2^- \) release in human neutrophils and monocytes stimulated by FMLP, Con A, or PMA. Cells (2 \( \times 10^7 \) cells/mL) were preincubated with GM-CSF (5 ng/mL), M-CSF (100 ng/mL), or IL-3 (20 ng/mL) for 10 minutes at 37°C before FMLP (10^-7 mol/L for neutrophils and 10^-8 mol/L for monocytes), Con A (100 \( \mu \)g/mL), or PMA (100 ng/mL) was added.]
GM-CSF enhanced FMLP-induced \( O_2^- \) release by fourfold to sevenfold (Fig 1). These differences may reflect the difference of cell types or the difference of cell preparation procedures. In the present experiments, we used monocytes isolated by the centrifugal elutriation system. The cell preparation procedures might reduce the responsiveness of cells to GM-CSF. To clarify this problem, the studies were performed by using mononuclear cells, containing 15% to 25% monocytes and 75% to 85% lymphocytes, isolated by Ficoll-Conray method, and gave the same results (data not shown), indicating that the difference of cell types is responsible for the difference of enhancing effect by GM-CSF.

In contrast to human monocytes, FMLP-induced \( O_2^- \) release in human neutrophils was not affected by pretreatment of cells with M-CSF (100 ng/mL) or IL-3 (20 ng/mL) for 10 minutes at 37°C (data not shown).

Combined effects of GM-CSF, M-CSF, and IL-3 on \( O_2^- \) release in human monocytes. To find out whether GM-CSF, M-CSF, and IL-3 act synergistically on human mono-

cytcs, the cells were pretreated with the optimal concentrations of any two CSFs in combination for 10 minutes at 37°C, and were then stimulated with FMLP or Con A to release \( O_2^- \). As summarized in Table 1, neither synergistic nor additive effects were obtained in any combinations and the effect of more potent CSF alone was observed.

Effects of G-CSF, IFN-\( \gamma \), and IL-4 on \( O_2^- \) release in human monocytes. The priming effects of G-CSF, IFN-\( \gamma \), and IL-4 on human monocytes were tested under the optimal conditions for GM-CSF-induced priming. As shown in Table 1, pretreatment of human monocytes with G-CSF (50

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>FMLP</th>
<th>Con A</th>
</tr>
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<tbody>
<tr>
<td>IL-3</td>
<td>0.90 ± 0.08*</td>
<td>1.26 ± 0.14*</td>
</tr>
<tr>
<td>M-CSF</td>
<td>0.78 ± 0.05*</td>
<td>1.23 ± 0.13*</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>1.24 ± 0.10*</td>
<td>1.80 ± 0.31*</td>
</tr>
<tr>
<td>IL-3 + M-CSF</td>
<td>1.01 ± 0.03*</td>
<td>1.24 ± 0.16*</td>
</tr>
<tr>
<td>GM-CSF + GM-CSF</td>
<td>1.24 ± 0.08*</td>
<td>1.53 ± 0.07*</td>
</tr>
<tr>
<td>M-CSF + GM-CSF</td>
<td>1.22 ± 0.07*</td>
<td>1.83 ± 0.05*</td>
</tr>
<tr>
<td>G-CSF</td>
<td>0.49 ± 0.02</td>
<td>0.67 ± 0.04</td>
</tr>
<tr>
<td>IFN-( \gamma )</td>
<td>0.46 ± 0.04</td>
<td>0.65 ± 0.03</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.50 ± 0.02</td>
<td>0.67 ± 0.03</td>
</tr>
</tbody>
</table>

Monocytes (2 \( \times 10^6 \) cells/mL) were preincubated with IL-3 (20 ng/mL), M-CSF (100 ng/mL), GM-CSF (5 ng/mL), any two of these CSFs, G-CSF (50 ng/mL), IFN-\( \gamma \) (1,000 U/mL), or IL-4 (20 ng/mL) for 10 minutes at 37°C, and were then stimulated with FMLP (10^{-6} \text{ mol/L}) or Con A (100 \( \mu \)g/mL) to release \( O_2^- \). The data are expressed as mean ± SD of three to six experiments.

*Significantly increased as compared with control cells (\( P < .01 \)).

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Fig 2. Effects of GM-CSF, M-CSF, and IL-3 on \( O_2^- \) release in human monocytes stimulated by FMLP. The data are expressed as mean ± SD of three to five experiments. (Top) Human monocytes (2 \( \times 10^6 \) cells/mL) were preincubated with indicated concentrations of GM-CSF, M-CSF, or IL-3 for 10 minutes at 37°C and were then stimulated with FMLP (10^{-6} \text{ mol/L}). (Bottom) Human monocytes were preincubated with GM-CSF (5 ng/mL), M-CSF (100 ng/mL), or IL-3 (20 ng/mL) for indicated periods at 37°C and were then stimulated with FMLP (10^{-6} \text{ mol/L}).

Fig 3. \( O_2^- \) release as a function of the concentration of FMLP used as stimulus. Human monocytes (2 \( \times 10^6 \) cells/mL) were preincubated with or without GM-CSF (5 ng/mL) for 10 minutes at 37°C and were then stimulated with indicated concentrations of FMLP. The data are expressed as mean ± SD of three experiments.
ng/mL), IFN-γ (1,000 U/mL), or IL-4 (20 ng/mL) for 10 minutes at 37°C had no effect on O₂⁻ release stimulated by FMLP or Con A.

DISCUSSION

In the present experiments, we studied the priming effects of hematopoietic growth factors on O₂⁻ release in human monocytes by using purified monocyte fractions. To exclude the possible activation or priming of monocytes by adherence to plastic surface, we isolated human monocytes by the centrifugal elutriation system and assessed the priming effects after short-term preincubation of suspended monocytes with appropriate factors. The results show that, among the hematopoietic growth factors tested, GM-CSF, M-CSF, and IL-3 enhance O₂⁻ release in human monocytes stimulated by the receptor-mediated agonists (FMLP and Con A), but not by PMA, which bypasses the receptors to stimulate the cells. The priming effects were dependent on the concentrations of CSFs used and the preincubation time. The optimal priming was obtained by pretreatment of cells with each CSF for 10 minutes at 37°C. The dose-response curves for priming of monocytes by GM-CSF, M-CSF, and IL-3 were almost identical to those for enhancement of adherence by GM-CSF, enhancement of tumor cell killing by M-CSF, and enhancement of adherence by IL-3, respectively, and were compatible with the affinity of receptors for each CSF. In addition, the potency of maximal priming effect by these CSFs (GM-CSF > M-CSF = IL-3) was consistent with the potency of stimulatory effect of these CSFs on the release of virus protein from human monocytes infected with human immunodeficiency virus. The pretreatment of monocytes with G-CSF, IFN-γ, or IL-4 for 10 minutes had no effect on O₂⁻ release stimulated by FMLP or Con A. These findings taken together suggest that GM-CSF, M-CSF, and IL-3 prime human monocytes via its specific receptors and enhance selectively O₂⁻ release triggered by receptor-mediated agonists after short-term preincubation.

Although it is likely that GM-CSF, M-CSF, and IL-3 prime human monocytes via its own receptors, neither additive nor synergistic effects were observed when the optimal concentrations of any two CSFs were used in combination, ie, the combination of the optimal concentrations of any two CSFs resulted in the effect of more potent priming agent alone. Similar findings were observed in the priming of human neutrophils by G-CSF and GM-CSF, ie, combined stimulation of cells with the optimal concentrations of G-CSF and GM-CSF always resulted in the effects of GM-CSF alone. These findings suggest that the common mechanisms for priming are shared by GM-CSF, M-CSF, and IL-3. In addition, these findings indicate that the presence of multiple CSFs at the inflammatory sites does not necessarily cause excessive priming of monocytes. Our previous and present experiments show that in priming obtained after short-term (10 minutes) preincubation of neutrophils and monocytes with appropriate CSFs, namely short-term priming, PMA-induced O₂⁻ release is not affected. On the contrary, in priming obtained after long-term (2 to 3 days) preincubation of macrophages with GM-CSF or M-CSF, namely long-term priming, PMA-induced O₂⁻ release is markedly enhanced. Furthermore, pretreatment of human suspended monocytes with IFN-γ for 3 days is reported to potentiate O₂⁻ release stimulated by PMA, but not by FMLP. The present experiments show that IFN-γ had no short-term effect on human suspended monocytes. These findings taken together suggest that the different mechanisms are involved in the short-term and the long-term priming of respiratory burst in monocytes or macrophages and that the common mechanisms are involved in the short-term priming of neutrophils and monocytes. CSFs appear to activate the pathways for both short-term and long-term priming, whereas IFN-γ appears to activate only the pathway for long-term priming. The similar phenomenon has been recently reported in tumor cell killing by human eosinophils; GM-CSF and IL-3 induce both short-term and long-term priming and IFN-γ induces only long-term priming.

The present experiments show rapid priming of the respiratory burst in human monocytes by GM-CSF, M-CSF, and IL-3. The rapid stimulatory effects of these CSFs on monocyte or macrophage functions are also reported. These effects include stimulation of pinocytosis by M-CSF (20 to 30 minutes), upregulation or downregulation of cell-surface molecules by GM-CSF (10 to 30 minutes) and enhancement of adherence by GM-CSF and IL-3 (10 to 45 minutes). In addition, it has been reported that in vivo administration of GM-CSF induces transient monocyteopenia (evident within 10 to 20 minutes), which may reflect increased adherence of circulating monocytes by GM-CSF. Signaling pathways used in these rapid effects of CSFs on monocytes remain to be determined. Imamura et al reported that human M-CSF translocates and activates protein kinase C in human monocytes within 5 minutes. It is well known that the receptor for M-CSF is by itself tyrosine kinase. Thus, it is likely that phosphorylation of some intracellular proteins induced by protein kinase C and/or tyrosine kinase plays an important role in stimulation of monocytes by M-CSF, although the causal relationship between phosphorylation and functional activation remains to be clarified. Signaling pathways used in rapid activation of monocytes by GM-CSF and IL-3 await further investigation.

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Rapid priming of human monocytes by human hematopoietic growth factors: granulocyte-macrophage colony-stimulating factor (CSF), macrophage-CSF, and interleukin-3 selectively enhance superoxide release triggered by receptor-mediated agonists

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