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STIMULATION AND PRIMING OF HUMAN NEUTROPHILS BY GRANULOCYTE COLONY-STIMULATING FACTOR AND GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR: QUALITATIVE AND QUANTITATIVE DIFFERENCES

Akira Yuo*+, Seiichi Kitagawa#, Akimichi Ohsaka#, Masaki Saito#, and Fumimaro Takaku*+#

*Clinical Research Institute and Department of Medicine, National Medical Center, Tokyo 162, Japan

+Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan

#Division of Hemopoiesis, Institute of Hematology and Department of Medicine, Jichi Medical School, Tochigi 329-04, Japan

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SUMMARY: Granulocyte colony-stimulating factor(G-CSF) and granulocyte-macrophage colony-stimulating factor(GM-CSF) increased neutrophil C3bi-receptor expression and adherence and rapidly(<10 min) primed neutrophils to enhance O_2^- release and membrane depolarization stimulated by chemotactic peptide. Direct triggering of O_2^- release in suspended neutrophils was also provoked by GM-CSF but not by G-CSF. GM-CSF-induced O_2^- release was inhibited by cyclic AMP agonists and cytochalasin B. The biological activity was greater in non-glycosylated GM-CSF than in glycosylated GM-CSF, whereas it was identical in glycosylated and non-glycosylated G-CSFs. Direct stimulation and priming by GM-CSF were consistently greater than those by G-CSF and the combined addition of the optimal concentrations of G-CSF and GM-CSF resulted in the effects of GM-CSF alone. These findings indicate that the effects of G-CSF and GM-CSF on neutrophil functions are qualitatively and quantitatively different from each other.

Granulocyte colony-stimulating factor(G-CSF) and granulocyte-macrophage colony-stimulating factor(GM-CSF) are members of a family of hematopoietic growth factors required for proliferation and differentiation of hematopoietic progenitor cells. In addition to their effects on hematopoiesis, G-CSF and GM-CSF have been demonstrated to stimulate or enhance *in vitro* human neutrophil functions including C3bi-receptor expression (1,2), adherence(1,2), superoxide(O_2^-) release(1,3,4) and

Abbreviations: CHO, Chinese hamster ovary; cAMP, cyclic AMP; di-O-C_5(3), 3,3'-dipentyloxacarbocyanine; *E. coli*, *Escherichia coli*; FMLP, N-formyl-methionyl-leucyl-phenylalanine; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HBSS, Hanks' balanced salt solution; O_2^-, superoxide; PGE_1, prostaglandin E_1.

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membrane potential changes(1,5). The clinical trials of G-CSF and GM-CSF have revealed that these CSFs are also active in vivo in terms of activation of neutrophil functions(6,7). However, the differences in their effects on neutrophils are poorly understood and the interaction between these factors remains to be determined. In the present study, we compared the effects of G-CSF and GM-CSF on human neutrophil functions and investigated the interaction between G-CSF and GM-CSF.

MATERIALS AND METHODS

Reagents. Highly purified(>98%) recombinant human CSFs were used in the present experiments. G-CSFs produced by Escherichia coli(E. coli)(mol wt 18,800) and Chinese hamster ovary(CHO) cells(mol wt 19,000) were provided by Kirin Brewery Co. Ltd., Tokyo, Japan and Chugai Pharmaceutical Co., Tokyo, Japan, respectively. GM-CSFs produced by E. coli(mol wt 14,700) and CHO cells(mol wt 20,500-33,400) were provided by Schering-plough Co. Ltd., Osaka, Japan and Genetics Institute, Cambridge, MA, respectively. G-CSF and GM-CSF produced by E. coli were used in the present experiments unless otherwise indicated. Cytochrome C type III, N-formyl-methionyl-leucyl-phenylalanine(FMLP), dibutyryl cyclic AMP(cAMP), prostaglandin E_1(PGE_1) and cytochalasin B were purchased from Sigma Chemical Co., St. Louis, MO; di-O-C_5(3)(3,3'-dipentyloxacarbocyanine) from Japanese Research Institute for Photosensitizing Dyes, Okayama, Japan; anti-Mol monoclonal antibody from Coulter Electronics, Hialeah, FL; and nylon fiber from Wako Pure Chemical Industries Ltd., Tokyo, Japan.

Preparation of cells. Human neutrophils were prepared from healthy adult donors as described(1), and neutrophil fractions(>95% neutrophils) were suspended in Hanks' balanced salt solution(HBSS).

Determination of O_2^- release. O_2^- was assayed by superoxide dismutase-inhibitable reduction of ferricytochrome C and a continuous assay was performed in a Hitachi 557 spectrophotometer(a double-wavelength spectrophotometer; Hitachi Ltd., Tokyo, Japan) equipped with a thermostatted cuvette holder as described(1).

Determination of membrane potential changes. Changes in the transmembrane potential were measured by using di-O-C_5(3) as described(1). The fluorescence was measured with a Hitachi MPF-4 fluorescence spectrophotometer(Hitachi Ltd.) equipped with a thermostatted cuvette holder(37°C).

Determination of C3bi-receptor expression. C3bi-receptor expression was assessed by cytofluorometry in an Ortho Spectrum III(Ortho Diagnostic System, Westwood, MA) using anti-Mol monoclonal antibody as described(1).

Determination of adherence. Neutrophil adherence to nylon fiber was tested by using a Pasteur pipette into which 40 mg nylon wool was packed as described(1).

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

RESULTS

Effects of G-CSF and GM-CSF on O_2^- release. One of the remarkable reported differences between G-CSF and GM-CSF is the difference in the preincubation time required for maximal enhancement of O_2^- release stimulated by FMLP, a chemotactic peptide, i.e. 10 min for G-CSF(3) and 2 hr for GM-CSF(4). This difference might reflect the difference in priming mechanisms, or might result from the difference in the preparation of CSFs used. Therefore, we re-evaluated the preincubation time required for maximal

enhancement of O_2^- release by using G-CSFs and GM-CSFs produced by E. coli and CHO cells, and the data are summarized in Fig. 1. We confirmed our previous observations that (a)pretreatment of cells with G-CSF for 10 min at 37°C was sufficient for maximal priming, (b)the biological activity of G-CSFs produced by E. coli and CHO cells was identical, and (c)the optimal effect was obtained at 25-50 ng/ml(1.3-2.6 nM) G-CSF from E. coli or CHO cells(Fig. 1)(1,3). We also found, in contrast to previous observations(4), that (a)pretreatment of cells for 10 min at 37°C with GM-CSF produced by E. coli or CHO cells was sufficient for maximal priming, (b)the biological activity of GM-CSF from E. coli was much greater than that of GM-CSF from CHO cells on a molar basis, whereas the magnitude of maximal enhancement was identical in both preparations of GM-CSFs, and (c)the optimal effect was obtained at 1-2 ng/ml(0.07-0.14 nM) GM-CSF from E. coli and 10-20 ng/ml(0.45-0.91 nM) GM-CSF from CHO cells(Fig. 1). The enhancing effect of GM-CSF on FMLP-induced O_2^- release was greater than that of G-CSF when assessed on the basis of the optimal concentrations and maximal effects(Fig. 1).

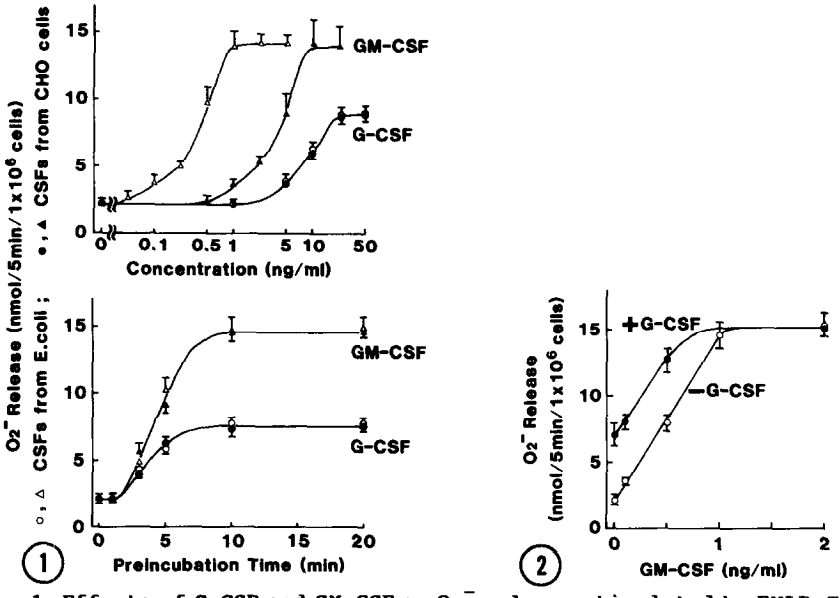

Fig. 1. Effects of G-CSF and GM-CSF on O_2^- release stimulated by FMLP. The data are expressed as mean±SD of three to five experiments. (Upper) Cells($1x10^6$/ml) were preincubated with indicated concentrations of G-CSF (○, ●) or GM-CSF(△, ▲) produced by E. coli(○, △) or CHO cells(●, ▲) for 10 min at 37°C before FMLP(10^{-7} M) was added. (Lower) Cells($1x10^6$/ml) were preincubated with G-CSF(25 ng/ml) from E. coli(○) or CHO cells(●), GM-CSF(2 ng/ml) from E. coli(△), or GM-CSF(20 ng/ml) from CHO cells(▲) for indicated periods at 37°C before FMLP(10^{-7} M) was added.

Fig. 2. Combined effects of G-CSF and GM-CSF on O_2^- release stimulated by FMLP. Cells($1x10^6$/ml) were preincubated with indicated concentrations of GM-CSF with(●) or without(○) G-CSF(25 ng/ml) for 10 min at 37°C before FMLP(10^{-7} M) was added. The data are expressed as mean±SD of three to five experiments.

To find out whether G-CSF stimulates neutrophils in concert with GM-CSF, the cells were pretreated with G-CSF and GM-CSF in combination for 10 min and were then stimulated with FMLP to release O_2^-. As shown in Fig. 2, when lower concentrations of GM-CSF(< 1 ng/ml) were combined with the optimal concentration of G-CSF(25 ng/ml) for priming cells, an almost additive effect was obtained. However, as the concentration of GM-CSF increased, the combined effect of G-CSF and GM-CSF gradually decreased and approached the effect of GM-CSF alone(Fig. 2). To further evaluate the combined effect of G-CSF and GM-CSF on O_2^- release stimulated by FMLP, the optimal concentrations of G-CSF(25 ng/ml) and GM-CSF(2 ng/ml) were added sequentially at an interval of 10 min. Neutrophils pretreated with G-CSF for 10 min at 37°C were further primed by the subsequent addition of GM-CSF, whereas neutrophils pretreated with GM-CSF for 10 min at 37°C did not respond to further addition of G-CSF. The amount of O_2^- release from neutrophils primed with the sequential addition of G-CSF(25 ng/ml) and GM-CSF(2 ng/ml) was identical to that from cells primed with GM-CSF(2 ng/ml) alone(data not shown).

Effects of G-CSF and GM-CSF on membrane potential changes, C3bi-receptor expression and adherence. Membrane depolarization in neutrophils stimulated by FMLP was enhanced by pretreatment of cells with G-CSF or GM-CSF for 10 min at 37°C. The enhancing effect of GM-CSF was greater than that of G-CSF when the optimal concentration of each CSF was used. The magnitude of membrane depolarization in G-CSF- and GM-CSF-primed cells were 130±3% and 155±7% of control, respectively. The magnitude of membrane depolarization in neutrophils primed with G-CSF(25 ng/ml) plus GM-CSF(2 ng/ml) was identical to that in cells primed with GM-CSF(2 ng/ml) alone(data not shown).

The pretreatment of neutrophils with G-CSF or GM-CSF for 30 min at 37°C increased surface expression of C3bi-receptors and enhanced cell adherence to nylon fiber(Table I). The stimulatory effect of GM-CSF on both functions was greater than that of G-CSF when the optimal concentration of each CSF was used. The increment of C3bi-receptor expression and adherence by G-CSF(50 ng/ml) plus GM-CSF(5 ng/ml) was identical to that by GM-CSF(5 ng/ml) alone(Table I).

Effects of cAMP agonists and cytochalasin B on O_2^- release triggered by GM-CSF. In contrast to previous observations(4,5,8), we found that GM-CSF provoked O_2^- release directly in suspended neutrophils in a dose-dependent manner(Fig. 3). GM-CSF-induced O_2^- release required a lag time of 1-2 min and continued for 120 min. The stimulatory effect of GM-CSF from *E. coli* was greater than that of GM-CSF from CHO cells on a molar basis, whereas the magnitude of maximal effect was identical in both preparations of GM-CSF(Fig. 3). On the other hand, G-CSF neither induced any detectable release of O_2^- in suspended neutrophils(Fig. 3)(1), nor had any effect on O_2^-

Table I

Effects of G-CSF and GM-CSF on C3bi-receptor expression and adherence

Treated with	C3bi-Receptor Expression# (Mean Fluorescence Channel)	Adherence* (%)
Control	15	9
G-CSF	57	32
GM-CSF	73	56
G-CSF+GM-CSF	69	51

#Neutrophils($2x10^6$ cells/ml) in HBSS were incubated with G-CSF(50 ng/ml), GM-CSF(5 ng/ml) or G-CSF(50 ng/ml) plus GM-CSF(5 ng/ml) for 30 min at 37°C, and then the binding of anti-Mol monoclonal antibody was analyzed by cytofluorometry. The data are expressed as means of three experiments.

*Neutrophils($1x10^6$ cells/ml) in RPMI 1640 medium containing 0.1% human serum albumin were incubated with G-CSF(50 ng/ml), GM-CSF(5 ng/ml) or G-CSF(50 ng/ml) plus GM-CSF(5 ng/ml) for 30 min at 37°C, and then neutrophil adherence to nylon fiber was tested. The data are expressed as means of three experiments.

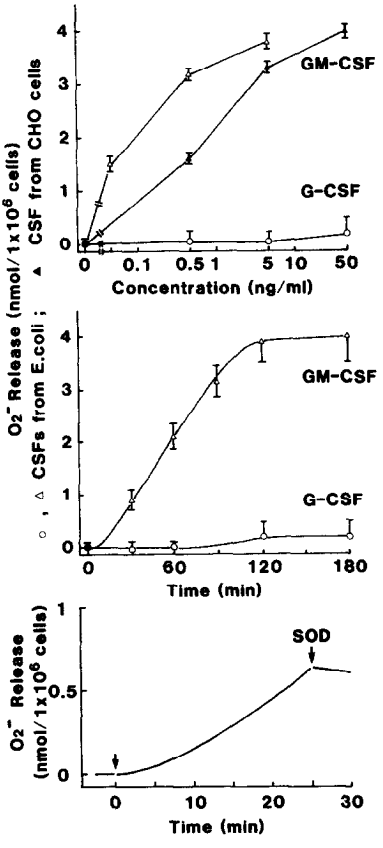

Fig. 3. O_2^- release triggered by G-CSF or GM-CSF. The data are expressed as mean±SD of three experiments. (Upper) Cells($1x10^6$/ml) were stimulated to release O_2^- with indicated concentrations of G-CSF(○), or GM-CSF produced by *E. coli*(△) or CHO cells(▲) for 120 min at 37°C. (Middle) Cells($1x10^6$/ml) were stimulated for release of O_2^- with G-CSF(50 ng/ml) or GM-CSF(5 ng/ml) for indicated periods at 37°C. (Lower) Continuous assay of O_2^- release triggered by GM-CSF. GM-CSF(5 ng/ml) was added at time 0, and reduction of ferricytochrome C was followed on the recorder. The tracing is drawn from a representative experiment of three experiments. SOD; superoxide dismutase, 40 μg/ml.

Table II

Effect of cAMP agonists and cytochalasin B on O_2^- release triggered by GM-CSF#

Pretreatment		O_2^- Release(% Control)
Prostaglandin E_1	10 nM	50 ± 9
	100 nM	30 ± 15
Dibutyryl cAMP	0.5 mM	33 ± 10
	1.5 mM	15 ± 12
Cytochalasin B	5.0 µg/ml	5 ± 2

#Neutrophils($1x10^6$ cells/ml) were preincubated with indicated concentrations of prostaglandin E_1 or dibutyryl cAMP in the presence of theophylline(0.2 mM), or cytochalasin B for 10 min at 37°C, and then stimulated with GM-CSF(5 ng/ml) for 120 min at 37°C. The data are expressed as means±SD of three experiments.

release triggered by GM-CSF(5 ng/ml)(data not shown). As shown in Table II, GM-CSF-induced O_2^- release was inhibited by PGE_1 and dibutyryl cAMP in a dose-dependent manner. In addition, GM-CSF-induced O_2^- release was almost completely abolished by cytochalasin B.

DISCUSSION

The results presented here show that one major difference between the actions of G-CSF and GM-CSF on human neutrophils is the ability to directly trigger O_2^- release from suspended neutrophils. In this regard, it is of interest that various side effects have been reported in clinical trials of GM-CSF(7), whereas no side effects have been noted in clinical trials of G-CSF(6). The direct triggering of O_2^- release from suspended neutrophils by GM-CSF might be, at least in part, responsible for the side effects observed in patients receiving GM-CSF. The qualitative difference in G-CSF and GM-CSF action was also noted in the effect on FMLP-receptors, i.e. the number and affinity of FMLP-receptors were affected by GM-CSF(9), but not by G-CSF(1).

Both G-CSF and GM-CSF increased C3bi-receptor expression and adherence, and enhanced O_2^- release and membrane depolarization stimulated by FMLP. The preincubation time required for maximal enhancement of O_2^- release was 10 min for both CSFs. Both CSFs were also shown not to induce any changes in cytoplasmic free Ca^{2+} and transmembrane potential(1,5). On the other hand, the effects of GM-CSF were consistently greater than those of G-CSF in all parameters tested. In addition, the combination of suboptimal concentrations of G-CSF and GM-CSF resulted in additive effects, whereas the combination of the optimal concentrations of G-CSF and GM-CSF resulted in the effects of GM-CSF alone. These findings taken together suggest that the intracellular signals produced by G-CSF and GM-CSF are partly similar, and that the signals produced by maximal stimulation of GM-CSF-receptors encompass the signals produced by G-CSF-receptors. Additional signals produced by GM-CSF

may be responsible for the qualitative and quantitative differences observed in G-CSF and GM-CSF actions. The present experiments also show that the steps sensitive to cAMP and cytochalasin B are involved in the direct triggering of O_2^- release from suspended neutrophils by GM-CSF.

The biological activity of G-CSF was identical in glycosylated and non-glycosylated preparations. On the other hand, the biological activity of GM-CSF was greater in non-glycosylated than in glycosylated preparations on a molar basis. These findings are consistent with the observations that the proliferative activity of GM-CSF on progenitor cells and the binding affinity of GM-CSF to its receptors increase with decreasing glycosylation(10,11).

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