

Brief report

Autocrine production of epithelial cell–derived neutrophil attractant-78 induced by granulocyte colony-stimulating factor in neutrophils

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Whereas mobilization to inflammatory sites is an important function of neutrophils, it remains to be determined whether granulocyte colony-stimulating factor (G-CSF) stimulates the mobilization of neutrophils to the inflammatory sites. This study compared the expression of more than 9000 genes in neutrophils treated with and without G-CSF with the use of a DNA microarray system to determine the

effects of G-CSF on the function of neutrophils. It was found that messenger RNA expression of epithelial cell–derived neutrophil attractant-78 (ENA-78), which has been reported to be a chemotactic factor for neutrophils, was induced by G-CSF in neutrophils. The study demonstrated that the supernatant of G-CSF–treated neutrophils induced the chemotaxis of neutrophils and that anti–ENA-78 antibody and

anti–CXCR-2 antibody inhibited the chemotaxis. These data suggest that G-CSF may enhance the mobilization of neutrophils and consequently augment the accumulation of neutrophils in the inflammatory sites through the secretion of ENA-78. (Blood. 2002;99:1863-1865)

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Introduction

Granulocyte colony-stimulating factor (G-CSF) is now widely used for the treatment of neutropenic patients with severe infection.¹⁻³ It is not only a specific growth factor for the granulocyte colony-forming unit (CFU-G) but also an activator for mature neutrophils.⁴⁻⁶ G-CSF enhances the survival of neutrophils and stimulates superoxide production and phagocytosis.^{2,7} These findings indicate that G-CSF exerts its effects in the treatment of severe infection through the increase of neutrophils and activation of effector functions. Whereas mobilization to inflammatory sites is known to be an important function of neutrophils, the effect of G-CSF on the mobilization of neutrophils is controversial.⁸⁻¹⁰

In this study, we sought to demonstrate the effects of G-CSF on the function of neutrophils with the use of a DNA microarray system. By comparing the expression of more than 9000 genes by screening messenger RNAs (mRNAs) extracted from untreated neutrophils and those treated with G-CSF *in vitro*, we found that epithelial cell–derived neutrophil attractant-78 (ENA-78) was expressed 17-fold higher in the neutrophils treated with G-CSF than in those untreated. As ENA-78 is an attractant for neutrophils,^{11,12} we examined the expression of ENA-78 in the neutrophils treated with or without G-CSF by a Northern blot analysis and further examined the motility-stimulating activities for neutrophils in the supernatants of the neutrophils treated with or without G-CSF.

human G-CSF,¹³ the plasma level of this G-CSF reaches 20 ng/mL after a drip infusion at 2 µg/kg. Anti–ENA-78 antibody, anti–CXCR-2 antibody, and recombinant ENA-78 were purchased from R&D Systems (Minneapolis, MN).

Cells

Neutrophils were separated from peripheral blood obtained from healthy volunteers. Neutrophils were separated from mononuclear cells by centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). After removal of mononuclear cells, precipitated cells were separated into a red blood cell fraction and a neutrophil fraction by mixing with 2% methylcellulose. After contaminated red blood cells were removed by hypotonic shock, neutrophils were washed with phosphate-buffered saline twice. Purity of the neutrophils recovered by this method was higher than 99%.

DNA microarray system

Total RNA was extracted from the neutrophils incubated for 24 hours with or without G-CSF at 10 ng/mL with the use of Trizol Reagent (Life Technologies, Tokyo, Japan). mRNA was purified from the total RNA with the use of a Quickprep mRNA purification kit (Amersham Pharmacia Biotech). Differentially expressed genes were screened with the use of a DNA microarray system, UniGEM human (Kurabo Industries, Osaka, Japan)

Northern blot analysis

Northern blot analysis was performed according to the method described previously.¹⁴ Briefly, total RNA (20 µg) was separated by electrophoresis in 1.2% denaturing formaldehyde-agarose gels and transferred to nylon membrane (Hybond N⁺, Amersham). After prehybridization, the membrane was hybridized with the complementary DNA (cDNA) probes for ENA-78 labeled with ³²P by using a random primer DNA labeling kit (Takara Biomedicals,

Study design

Reagents

Modified G-CSF was kindly supplied from Kyowa Hakko (Tokyo, Japan). Because this G-CSF is stabler in human plasma than intact

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Table 1. Genes induced by granulocyte colony-stimulating factor in neutrophils

Genes	Fold induction	Accession no.
Epithelial cell–derived neutrophil attractant-78 (ENA-78)	17.0	NM_002994
Plasminogen activator, urokinase	16.5	D11143
Plasminogen activator inhibitor, type II	15.1	J02685
LIM and senescent cell antigenlike domains 1	13.5	NM_004987
Solute carrier family 21 member 3	12.6	U21943
Interleukin 1, alpha	12.3	M28983
EGF-like module containing, hormone receptor-like sequence	9.9	X81479
Tumor necrosis factor, alpha-induced protein 6	9.5	M31165
CD44 antigen	8.7	X66733
Protein geranylgeranyltransferase type I	8.1	AA481712
Leukocyte-associated immunoglobulinlike receptor 1	7.0	AF013249
Chemokine (C-C motif) receptor-like 2	6.7	AF015524
Complement component 3	6.6	K02765
Complement component 3a receptor 1	6.3	Z73157
Activating transcription factor B	5.8	AF016898
Insulin-induced gene 1	5.5	AW663903
Thyroid receptor interacting protein 10	4.2	D63485
Granulocyte chemotactic protein-2 (GCP-2)	4.0	NM_002993
Kynureninase (L-kynurenine hydrolase)	3.8	U57721
Orosomucoid 1	3.7	X02544
Basic helix-loop-helix domain containing, class B	3.7	NM_003670
Glycerol kinase	3.7	AJ252550
Interleukin 6 signal transducer (gp130)	3.6	NM_002184
cAMP responsive element-binding protein-like 2	3.6	AF039081
CD48 antigen (B-cell membrane protein)	3.5	NM_001778
Ubiquitin-activating enzyme E1-like protein	3.5	NM_006395
RAN-binding protein 2	3.4	D42063
Toll-like receptor 2	3.4	U88878
Fc fragment of IgE, high affinity I, receptor	3.3	NM_004106
Adenosine A2a receptor	3.3	S46950
Peptidylprolyl isomerase F	3.2	M80254
Flotillin 1	3.2	NM_005803
TNF receptor–associated factor 3	3.2	U19260
Annexin A7	3.2	J04543
Sialyltransferase 4B	3.1	U63090
Hepatoma-derived growth factor	3.1	A1720570
Tissue inhibitor of metalloproteinase 1	3.0	AW261830

Tokyo, Japan). The probed membrane was then washed and exposed to radiographic film. cDNA fragments for ENA-78 were amplified by reverse transcriptase–polymerase chain reaction, cloned into a TA cloning vector, and then used as probes for Northern blot assay. Polymerase chain reaction primers were as follows: ENA-78 forward, GGTTGGATGCTCTGTCCAA; reverse, CCTTCCAGAAAGTCTTCTAT.

Flow-activated cell sorter analysis

After staining with anti-CXCR-2 antibody and fluorescence-conjugated second antibody, the cells were analyzed with a FACScaliber (Becton Dickinson, Mountain View, CA).

Transwell chamber assay

For examination of the chemotactic activities in the supernatants of neutrophils, the Transwell chamber (Coster, Cambridge, MA) assay was performed according to the previously described method.¹⁵ Supernatants were prepared by incubating the neutrophils for 24 hours after incubation with G-CSF for 24 hours and washing them twice with RPMI-1640 medium. Supernatants (600 μ L) diluted to 20% and neutrophils suspended in 100 μ L RPMI-1640 medium were placed in the lower and upper chambers, respectively. After 4 to 8 hours of incubation, transmigrated cells in the lower chambers were counted under an inverted microscope at least

in 20 areas. As a positive control, recombinant ENA-78 was added in the lower chambers at 10 ng/mL. In some experiments, anti-ENA-78 antibody or anti-CXCR-2 antibody was added in the lower chambers at up to 2 μ g/mL.

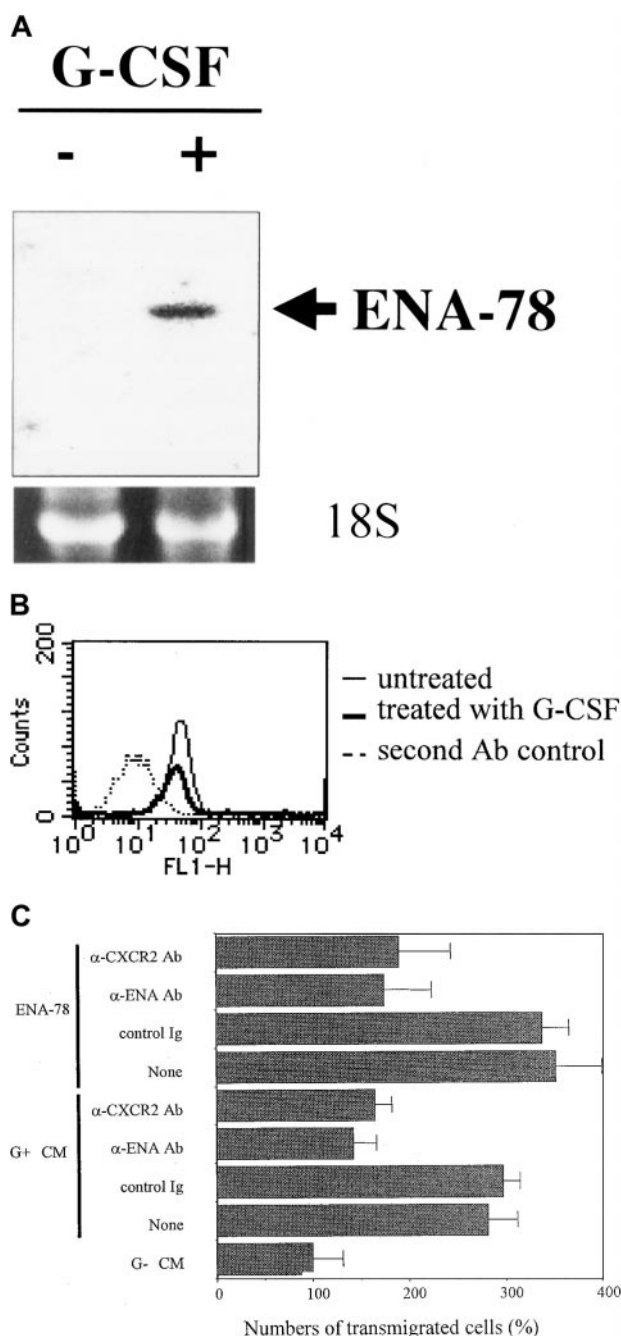


Figure 1. Expression of ENA-78 and CXCR-2 and chemotaxis of neutrophils. (A) mRNA expression of ENA-78 in neutrophils treated with or without G-CSF. Total RNAs were obtained from the untreated neutrophils and those treated with 10 ng/mL G-CSF for 24 hours and then used for Northern blot analysis. Representative result of 3 different experiments is shown. (B) Expression of CXCR-2 on neutrophils. The untreated neutrophils and those treated with 10 ng/mL G-CSF for 24 hours were stained with anti-CXCR-2 antibody and fluorescein isothiocyanate–conjugated second antibody and then analyzed for expression of CXCR-2 with a FACScaliber. Representative result of 2 different experiments is shown. (C) Chemotaxis of neutrophils stimulated by conditioned medium of neutrophils. Supernatants of neutrophils treated with or without G-CSF (G⁺ or G⁻, respectively) at 10 ng/mL were placed at 20% in the lower chambers. Anti-ENA-78 or anti-CXCR-2 antibody was added in the lower chambers at 2 μ g/mL. Recombinant ENA-78 (10 ng/mL) was used as a positive control. Results are presented as percentage of numbers of transmigrated neutrophils treated with G CM. Data present the mean \pm SD of 3 different experiments.

Results and discussion

Differentially expressed genes

One hundred twenty-two and 50 genes, including EST clones, were expressed 2-fold and 3-fold higher, respectively, in the neutrophils treated with G-CSF than in those untreated. Table 1 shows 37 known genes among those expressed 3-fold higher in the neutrophils treated with G-CSF than in those untreated. In particular, ENA-78 and granulocyte chemotactic protein-2 (GCP-2), which were reported to be chemotactic factors for neutrophils,^{11,12,16,17} were expressed 17-fold and 4-fold higher, respectively, in the neutrophils treated with G-CSF than in those untreated. Previous reports demonstrated that mast cells, nonsmall cell lung cancer cells, monocytes, and osteosarcoma cells produced ENA-78 and GCP-2, respectively, whereas there has been no report demonstrating the production of ENA-78 and GCP-2 by neutrophils.^{12,16,18,19} Therefore, we focused on these molecules in this study. Urokinase-type plasminogen activator, which has been reported to play an important role in the recruitment of neutrophils to the inflammatory sites,²⁰ was also expressed 16.5-fold higher in the neutrophils treated with G-CSF than in those untreated. The role of plasminogen activator is now under examination.

ENA-78 mRNA expression and chemotaxis of neutrophils

As shown in Figure 1A, the untreated neutrophils did not express ENA-78 mRNA, but the neutrophils treated with G-CSF expressed ENA-78 mRNA. ENA-78 mRNA expression was induced by G-CSF in a dose-dependent manner (data not shown). mRNA expression of GCP-2 was not observed in the treated or untreated neutrophils (data not shown). Figure 1B shows that CXCR2, a

receptor for ENA-78, was expressed at the same level both on the neutrophils treated with and without G-CSF. As shown in Figure 1C, chemotaxis of neutrophils enhanced by recombinant ENA-78 was abrogated by anti-ENA-78 and anti-CXCR-2 antibodies. Chemotaxis was also enhanced by the supernatants of the G-CSF-treated neutrophils. Reversely, anti-ENA-78 and anti-CXCR-2 antibodies abrogated the enhanced chemotaxis. These results indicate that the neutrophils stimulated by G-CSF secrete ENA-78 and consequently stimulate the chemotaxis of neutrophils, suggesting that neutrophils accumulated in the inflammatory sites secrete ENA-78 and then augment the accumulation of neutrophils when treated with G-CSF.

Several previous reports demonstrating that the chemotaxis of neutrophils was decreased by the treatment with G-CSF seem to be inconsistent with our present result.^{8,9} However, in those previous studies they examined the chemotaxis of newly developed neutrophils after the *in vivo* treatment with G-CSF; it is not surprising that the chemotaxis of newly developed immature neutrophils was low. In contrast, we demonstrated that the treatment with G-CSF induced the production of a chemotactic factor, ENA-78, in the neutrophils. Our present results, in combination with the findings of survival-inducing activity of G-CSF, suggest that G-CSF is effective for the treatment of infection through the augmentation of the neutrophil accumulation in the inflammatory sites in addition to the increase of neutrophils and the activation of effector functions.

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