Effect of recombinant granulocyte colony-stimulating factor on the chemiluminescence response of human polymorphonuclear leukocytes

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The effect of recombinant human granulocyte colony-stimulating factor (rhG-CSF) on the function of human polymorphonuclear leukocytes (PMNs), in terms of chemiluminescence (CL) response, were investigated *in vitro*. Incubation of rhG-CSF in concentrations of more than 4 ng/ml with PMNs obtained from healthy adults, at 37°C for 10 minutes, followed by stimulation with non-opsonized zymosan, significantly enhanced the CL response of the PMNs, compared with the response in untreated PMNs. rhG-CSF exhibited a less powerful priming effect on the CL response of PMNs than recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF). The priming effect of rhG-CSF on PMNs obtained from patients with lung cancer and elderly persons was almost the same as in PMNs obtained from healthy adults. On the other hand, the priming effect of rhG-CSF on activated PMNs from patients with bacterial infections tended to be strong at lower levels and weak at higher levels, in contrast to the effect on PMNs from healthy adults.

Key words: rhG-CSF, rhGM-CSF, polymorphonuclear leukocytes, chemiluminescence

Introduction

Human granulocyte colony-stimulating factor (G-CSF) is a naturally occurring glycoprotein with an apparent molecular weight of 19,600. It is a member of a family of hemopoietic growth factors and regulates the proliferation and differentiation of granulocyte and macrophage precursor cells[1] A complementary DNA encoding human G-CSF has recently been cloned and expressed in *Escherichia coli* and Chinese hamster ovary cells, and large-scale production of recombinant human G-CSF (rhG-CSF) has been established[2,3]. In addition to its effect on granulopoietic cell growth and granulocyte production, rhG-CSF enhances a number of functional events in mature granulocytes, both *in vitro* and *in vivo*[4~8]. Although the priming effect of rhG-CSF on the PMNs of healthy adults has been established, the effect on PMN functions in patients with underlying diseases has not been fully elucidated. In this study, we examined the effect of rhG-CSF on the chemiluminescence (CL) response of human polymorphonuclear leukocytes (PMNs) obtained from healthy young adults, and compared it with that of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF). We also investigated the priming effect of rhG-CSF on the CL response of PMNs in compromised hosts, including patients with lung cancer and elderly persons, whose PMNs functions had been reported to be impaired[9,10], and in patients with acute bacterial infections in whom the CL response of PMNs was enhanced[11].

Materials and Methods

Subject population

Our subjects were twenty healthy volunteers (13 males, 7 females, mean age 30.8±4.3 years, range 23—36), twelve patients with lung cancer (9 males, 3 females, mean age 67.3±10.6 years,

range 49—80), twelve patients with acute bacterial infections (11 males, 1 females, mean age 66.1± 11.4 years, range 42—80) and twelve elderly persons (5 males, 7 females, median age 75.2±4.8 years, range 68—84) without specific underlying diseases except for hypertension and arteriosclerotic disease.

Patients with lung cancer: All patients were staged clinically according the New International Staging for Lung Cancer. Clinical staging for cancer revealed 1 patient with Stage IIIa, 2 with Stage IIIb, and 9 with Stage IV cancers. The histologic diagnosis was as follows: adenocarcinoma 6, squamous cell carcinoma 4, and small cell carcinoma 2. Those with acute inflammatory disorders were excluded from our study.

Patients with acute bacterial infection: Their classification was as follows; six with pneumonia, 1 with lung abscess, 1 with sepsis, 2 with sepsis suspected, 1 with peritonitis, 1 with ileus pulus infection. The causative organisms were identified in 3 patients. *Staphylococcus aureus* was isolated in a patient with sepsis and *Pseudomonas aeruginosa* was isolated from sputum in 2 patients with pneumonia. Eleven patients had mild or moderate underling diseases; Three with cerebral infarction, 5 with lung cancer, 1 with congestive heart failure, 1 with colon cancer and 1 with ileus.

Chemicals and media

Dulbecco's modified Eagle's medium (MEM, Nissui Co., Tokyo) containing 25 mM HEPES and L-glutamine 0.3 g/L, pH 7.4, was used to the dilute blood samples. Luminol (Tokyo Kasei Kogyo Co., Tokyo) and zymosan (Sigma Chemical Co.) were dissolved in phosphate-buffered saline (PBS) in concentrations of 20 μg/ml and 25 mg/ml, respectively. Recombinant human granulocyte colony-stimulating factor (rhG-CSF) produced by *Escherichia coli* (apparent molecular weight 18,800 and specific activity 1×10^8 U/mg protein) was kindly provided by Kirin Brewery Co., Ltd. (Tokyo, Japan) and Sankyo Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) produced by *Escherichia coli* (molecular weight 22,000 and specific activity 5×10^7 U/mg protein) was kindly provided by Hoechst Japan Ltd. (Tokyo, Japan). Endotoxin judged by *Limulus* amebocyte assay was not detectable in the rhG-CSF and rhGM-CSF preparations.

Isolation of human PMNs

Freshly drawn heparinized blood (10 U/ml) from healthy human donors and patients with lung cancer, bacterial infections, and elderly subjects was collected in sterile plastic tubes and sedimented with 4.5% Dextran solution for 40 minutes, following which the leukocyte-rich plasma was centrifuged at 400×g across a Ficoll-paque gradient for 30 minutes. The remaining pellets in the sediment were treated sequentially with hypotonic (0.2%) and hypertonic (1.6%) saline to lyse the erythrocytes. Cells were washed twice with MEM and adjusted to a final concentration of 1×10^7 PMNs/ml in MEM.

Measurement of chemiluminescence

The production of reactive oxygen species by PMNs was measured by luminol-dependent CL assay. The reaction mixture contained 0.1 ml of PMN suspension (5×10^5 cells), 0.1 ml of solution containing various concentrations of CSF (0.5—100 ng of rhG-CSF or 2—50 ng of rhGM-CSF) or 0.1 ml of 0.9% saline as the control; and 20 μl luminol solution. The final volume of each mixture was adjusted to 1 ml with MEM. After preincubation with various concentrations of rhG-CSF, rhGM-CSF or 0.9% saline for 10 minutes at 37°C, 20 μl (500 μg) non-opsonized zymosan was added to the mixtures and CL was measured continuously for 20 minutes with a six-channel Biolumat LB 9505 (Berthold Co., Germany). The chemiluminescence index (CL-index) for each drug studied was determined. This was defined as the mean difference between drug-treated CL and untreated CL (control). The index was calculated by dividing the integral CL of drug-exposed PMNs by that of untreated PMNs.

Statistics

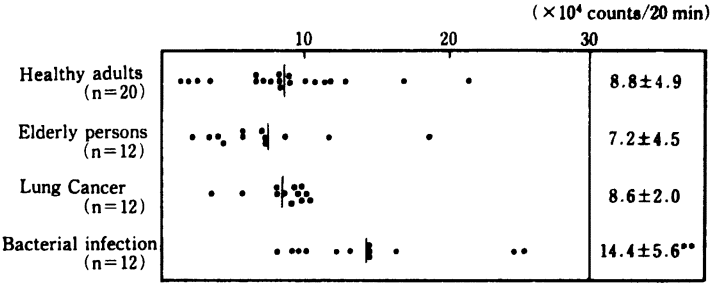
Date are shown as mean ±SD, and each experiment was conducted in duplicate. Statistical analysis was performed using Student's t-test.

Results

The CL responses of PMNs in elderly persons and

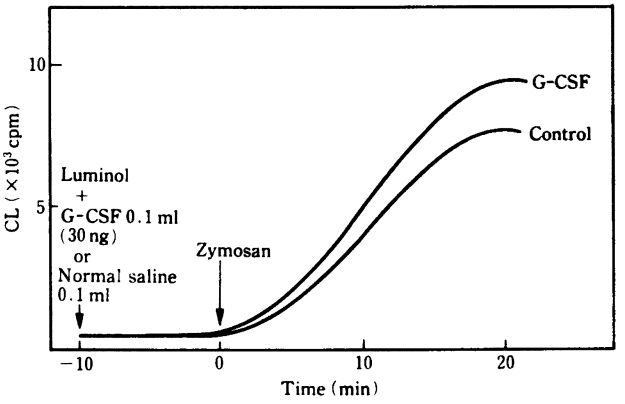
the patients with lung cancer or acute bacterial infection were investigated and compared with those of PMNs from healthy adults. The CL response of PMNs in patients with bacterial infections was greater than that of the PMNs of healthy adults, while this response tended to be lower than in healthy adults in patients with lung cancer and elderly persons (Fig. 1).

Fig. 2 shows a typical pattern of the priming effect of rhG-CSF on the CL response of PMNs to stimulation with non-opsonized zymosan in healthy adults. rhG-CSF alone did not induce a CL response in PMNs. About a 1.25 times increase in the integral value for the CL response of PMNs, compared with the response in untreated PMNs, was achieved after incubating 30 ng/ml of rhG-CSF and PMNs at 37°C for 10 minutes, followed by stimulation with zymosan. Fig. 3 shows that the priming effect of rhG-CSF on the CL response of PMNs in healthy adults was significant beginning at a concentration of 4 ng/ml, with the optimal concentration being 30 ng/ml, and the CL response gradually decreasing at the higher concentrations. Fig. 4 shows the priming effect of rhG-CSF and rhGM-CSF on the CL respo-



Data are shown as integral CL counts for 20 minutes (means and SDs).
**Significantly different ($p<0.01$) from the values in healthy adults.
CL: chemiluminescence, PMNs: polymorphonuclear leukocytes.

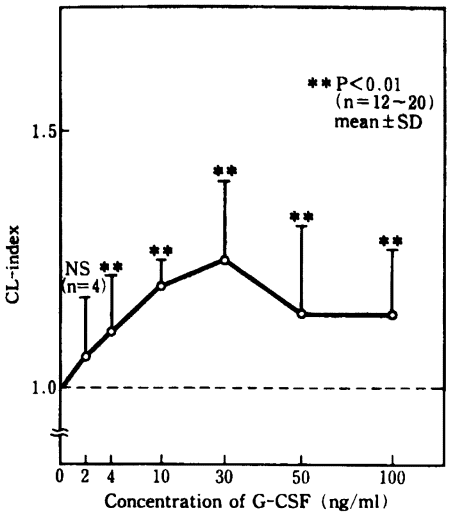
Fig. 1. CL values of PMNs stimulated with non-opsonized zymosan in elderly persons, patients with lung cancer and acute bacterial infection compared with those of healthy adults.



PMNs (5×10^5/ml) were incubated with rhG-CSF (30 ng/ml) or normal saline (control) for 10 minutes in the presence of luminol; then PMNs CL were measured by stimulation with non-opsonized zymosan.
rhG-CSF: recombinant human granulocyte colony-stimulating factor.

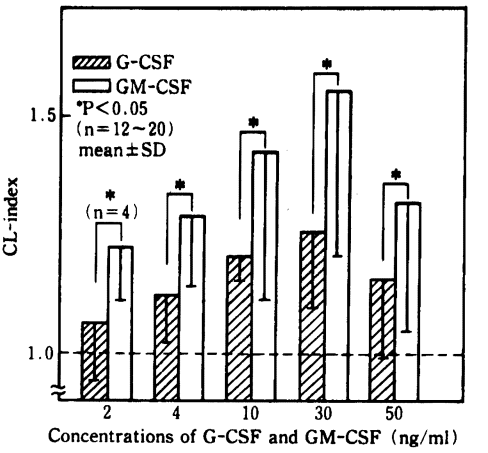
Fig. 2. Typical pattern of the priming effect of rhG-CSF on the CL response of PMNs in a healthy adult to stimulation with non-opsonized zymosan.

nse of PMNs from healthy adults to stimulation with zymosan. rhGM-CSF had a stronger effect than rhG-CSF at all levels studied. The priming effect of rhG-CSF on the CL response of PMNs from patients with lung cancer and from elderly persons was investigated and compared with that of PMNs from healthy adults. The priming effect of



The CL-index was determined as described in Materials and Methods. Student's *t*-test for paired data, comparison between rhG-CSF-treated and untreated PMNs (control) under identical incubation conditions.

Fig. 3. Priming effect of various concentrations of rhG-CSF on the CL response of PMNs in healthy adults to stimulation with non-opsonized zymosan.



Student's *t*-test for paired data, comparison between rhG-CSF-treated and rhGM-CSF-treated PMNs under identical incubation conditions.
rhGM-CSF: recombinant human granulocyte-macrophage colony-stimulating factor.

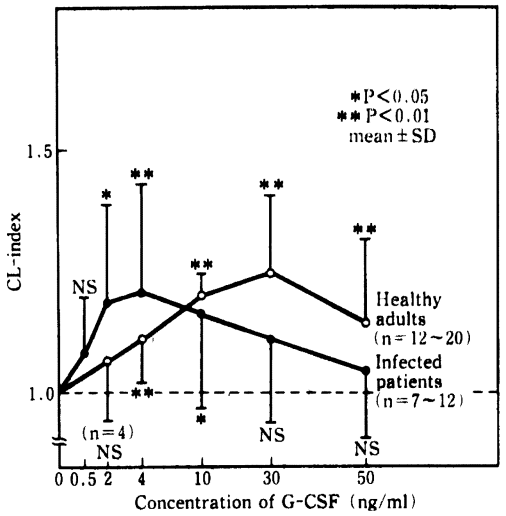
Fig. 4. Priming effect of rhG-CSF and rhGM-CSF on the CL response of PMNs in healthy adults to stimulation with non-opsonized zymosan.

Table 1. Effect of rhG-CSF on the CL response of PMNs in healthy adults, in elderly persons, and in patients with lung cancer and acute bacterial infections

	Concentration of G-CSF (ng/ml)			
	4	10	30	50
Healthy adults (n=20)	1.13±0.08**	1.23±0.12**	1.25±0.14**	1.16±0.15**
Elderly persons (n=12)	1.10±0.13*	1.17±0.25*	1.20±0.10**	1.07±0.10
Lung Cancer (n=12)	1.22±0.21**	1.31±0.41*	1.34±0.35*	1.30±0.29*
Bacterial infection (n=12)	1.24±0.25**	1.20±0.22**	1.11±0.19	1.03±0.14

§ (between Healthy adults and Elderly persons) and § (between Lung Cancer and Bacterial infection)

Data are shown as the CL-index (mean±SD of n experiments).
The CL-index was determined as described in Materials and Methods *p<0.05, **p<0.01, Student's *t*-test for paired data, comparison between the rhG-CSF-treated and untreated PMNs (control), under identical incubation conditions.
§ p<0.05, Student's *t*-test for paired data, comparison between the CL-index in healthy adults, in elderly persons, and in patients with lung cancer and acute bacterial infections, at the same concentrations of rhG-CSF.



Student's *t*-test for paired data, comparison between rhG-CSF-treated and untreated PMNs (control).

Fig. 5. Priming effects of various concentrations of rhG-CSF on the CL response of PMNs in healthy adults and patients with acute bacterial infections to stimulation with non-opsonized zymosan.

rhG-CSF in these compromised hosts was almost the same as in healthy adults (Table 1). The priming effect of rhG-CSF was also compared in PMNs from healthy adults and in PMNs from patients with bacterial infections, in whom the CL response of PMNs was enhanced. Fig. 5 shows that in these patients a significant effect was noted starting at from 2 ng/ml; this was lower than the minimal level (4 ng/ml) for the priming effect in healthy adults, the optimal level being 4 ng/ml, as opposed to 30 ng/ml in healthy adults, and the CL response tended to decrease at higher levels.

Discussion

Cytokines such as G-CSF and GM-CSF have been reported to promote *in vitro* PMN functions including phagocytosis, arachidonic acid release, and superoxide anion production, in response to a stimulus[4~8]. In this study using a luminol-dependent CL method, we examined the influence of rhG-CSF and rhGM-CSF on the potential of the PMNs of healthy adults to produce reactive oxygen species by following stimulation with non-opsonized zymosan. We found that rhGM-CSF exhibited a stronger priming effect on the CL response than rhG-CSF. Yuo et al. determined the production of superoxide anions induced by N-formyl-methionyl-leucyl-phenylanine (FMLP) using the ferricytochrome *c* reduction test and reported that the activity of rhGM-CSF in promoting the production of superoxide anions (priming effect) was higher than that of rhG-CSF[6]. This is in agreement with our own results. Although the priming effect of rhG-CSF on PMNs from healthy adults has been studied, the effect on PMN function in patients with underlying diseases has been less extensively investigated. Our investigation of the priming effect of rhG-CSF on the CL response of PMNs in compromised hosts including patients with lung cancer and elderly persons, whose PMNs CL responses are slightly depressed, revealed that significant enhancement of the CL response was achieved after PMNs were preincubated with rhG-CSF at a concentration of more than 4 ng/ml for 10 minutes, followed by stimulation with non-opsonized zymosan. Although, there have been some reports of the priming effect of rhG-CSF on the function of PMNs obtained from compromised hosts, restoration of the reduced superoxide production was observed in studies performed in patients with myelodysplastic syndrome and lymphoma[12,13].

However, to our knowledge, no previous investigators have reported the effect of rhG-CSF on the function of activated PMNs obtained from patients with infections. Our investigation of the priming effect of rhG-CSF on the CL response of PMNs from patients with bacterial infections revealed results different from those in healthy adults. In the case of patients with infections, a significant effect was observed starting at 2 ng/ml, which is lower than the minimum concentration (4 ng/ml) required for the priming effect in healthy adults. The optimal level of rhG-CSF for the CL response in the patients with infections was 4 ng/ml as opposed to 30 ng/ml in healthy adults. Although the mechanism for underlying the differences in the reactivity of rhG-CSF to the CL response of PMNs associated with infections is unknown, prior priming of PMNs with rhG-CSF, which is increased in blood due to infection[14] may be one explanation for the diffe-

rence. Interaction with such cytokines as GM-CSF, tumor necrosis factor, interleukin-1 (IL-1), and IL-8, which activate PMNs in the presence of bacterial infection, as well as interaction with bacterial components including lipopolysaccharide (LPS), may also be responsible[15]. Furthermore, *in vitro* studies may be required to elucidate changes that occur in the priming effect of rhG-CSF on the CL response of PMNs when rhG-CSF is combined with other cytokines or LPS.

The bactericidal activity of PMNs has been shown to be reduced in patients with lung cancer[9] and in elderly patients[10] and, this is thought to be a cause of the high incidence of infection in such patients. The present study, using a CL method, showed that rhG-CSF enhanced the function of PMNs in producing reactive oxygen species in patients with lung cancer and elderly persons. Hence, the clinical employment of rhG-CSF may be of value as adjuvant therapy in the treatment and prevention of serious infections in these patients when granulocytopenia is present, not only to induce granulocyte proliferation but also to activate granulocyte bactericidal function.

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遺伝子組み換え型ヒト顆粒球コロニー刺激因子のヒト好中球機能におよぼす効果

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遺伝子組み換え型ヒト顆粒球コロニー刺激因子（rhG-CSF）のヒト好中球機能におよぼす効果を chemiluminescence（CL）法を用いて *in vitro* で検討した。rhG-CSF を健康成人より得た好中球と 37°C で 10 分間保温した後に，非オプソニン化 zymosan で刺激すると，rhG-CSF 4 ng/ml 以上の濃度で好中球の CL 反応が未処理のものに比較して有意に増強した。この rhG-CSF の CL 反応におよぼす priming 効果は遺伝子組み換え型ヒト顆粒球・マクロファージーコロニー刺激因子（rhGM-CSF）に比較して弱かった。rhG-CSF は肺癌患者や高齢者の好中球に対しても健康成人の場合とほぼ同等の priming 効果を認めた。一方，細菌感染症患者の活性化した好中球に対する priming 効果は，健康成人の場合と異なり，低濃度で強く，高濃度で弱い傾向がみられた。

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