Augmented anti-

Pseudomonas Activity

in Cyclophosphamide-Induced

Neutropenic Tumor-Bearing Mice Induced

by Granulocyte Colony-Stimulating Factor

in Combination with Protein-Bound Polysaccharide

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Protective effect of intraperitoneal (i.p.) injection of recombinant human granulocyte colony stimulating-factor (rhG-CSF) in combination with protein-bound polysaccharide (PSK) against Pseudomonas aeruginosa (E-2) was studied in cyclophosphamide (CPA)-induced neutropenic tumor-bearing mice. Meth A tumor (1 × 10^6) was inoculated subcutaneously (s.c.) ten days before infection, and CPA (200mg/kg) was i.p. injected four days before infection and G-CSF (500 µg/kg) was i.p. injected daily for four days before or after infection. PSK (500mg/kg) was orally administered daily for four days before infection or from the day of infection. Pseudomonas of 2.0 × 10^5 colony forming unit (cfu) was inoculated intravenously (i.v.) for the analysis of prophylactic effect of G-CSF, 2.0 × 10^4 cfu was inoculated i.v. for that of therapeutic effect, and the number of viable bacteria in liver was counted serially after infection. After infection with 2.0 × 10^5 cfu, all CPA-induced neutropenic mice without G-CSF died by two days after infection, however, 50% of G-CSF pretreated mice survived. Moreover, mice pretreated with G-CSF in combination with PSK showed significant suppression of bacterial growth in the liver compared with mice treated with G-CSF alone, and all survived. After infection with 2.0 × 10^4 cfu, mice post-treated with G-CSF in combination with PSK showed a prominent anti-pseudomonas effect with 70% survival and suppression of bacterial growth in the liver, in contrast to untreated controls which all died by 2 days after infection or mice treated only with G-CSF with 20% survival. For the analysis of the anti-pseudomonas mechanism, phagocytic activity and 2',7'-dichlorofluorescin (DCFH) oxidative activity, and Mac-1 positive neutrophils were measured. Both phagocytic and DCFH oxidative activities were alleviated to normal level by G-CSF or in combination with PSK, however, deferoxamine mesylate (DFM) treatment inhibited the improvement of DCFH oxidative activity. The percentage of Mac-1 positive neutrophils was alleviated to normal level by these treatments.

In conclusion, both a prophylactic and a therapeutic use of G-CSF greatly enhanced the host anti-pseudomonas activity in combination with PSK in CPA-induced neutropenic tumor-bearing mice, and it was suggested that the ferric ion would greatly contribute to this mechanism.


Keywords: G-CSF, PSK, pseudomonas, CPA, tumor-bearing

An intraperitoneal (i.p.) injection of cyclophosphamide (CPA) suppresses myelopoiesis and decreases the number of peripheral neutrophils to the minimum level at four days after injection). Systemic bacterial infection easily leads these neutropenic mice to death with a sublethal dose. Antibiotics are considered almost ineffective under these conditions. Granulocyte colony-stimulating factor (G-CSF) is one of the hematopoietic factors, and has the activities to promote proliferation and differentiation of granulocyte precursor cells. In vivo G-CSF studies revealed that recombinant G-CSF stimulated granulopoiesis and accelerated the recovery from neutropenia induced by cytotoxic drugs or irradiation in experimental animals, and clinical studies also showed the similar effects on humans. These reports mean that G-CSF enhances anti-microbial activity and decreases the lethal risk of infection during neutropenic period. As to the possible mechanism of anti-microbial activity, the restoration of phagocytosis and superoxide generation due to G-CSF was reported. Among various kinds of micro-organisms, pseudomonas is one of the most troublesome pathogens in immuno-compromised hosts and seems very difficult to control. As pseudomonas is eliminated mainly by neutrophils, this micro-organism is thought one of the most suitable bacteria for the study on anti-microbial activity of G-CSF.

In this paper, the authors presented one of the first reports on the therapeutic effect of G-CSF in combination with polysaccharide-K (PSK), known as one of biological response modifiers (BRMs), in CPA-induced tumor-bearing neutropenic mice and proved the mechanism of anti-microbial activity of peripheral neutrophils by deferoxamine mesylate (DFM).
treatment.

Materials and methods

Animals

Eight-week-old female BALB/c mice were purchased from Japan CLEA Co. Ltd. (Tokyo, Japan) and fed under a specific pathogen free (SPF) environment.

Tumor

Meth A tumor, a methylcholanthrene-induced fibrosarcoma, was subcutaneously (s.c.) inoculated to syngenic BALB/c mice with 1×10^6 cells in 0.1 ml phosphate buffered saline (PBS).

Drugs

Recombinant human G-CSF was kindly supplied by Sankyo Co. Ltd. (Tokyo, Japan). CPA (Endoxan®) was purchased from Shionogi Pharmaceutical Co. Ltd. (Osaka, Japan). PSK was a gift from Sankyo Co. Ltd. (Tokyo, Japan). Each drug was diluted with PBS at an appropriate concentration and used for the following experiments. 2',7'-dichlorofluorescin-diacetate (DCFH-DA) was purchased from Eastman Kodak Co. Ltd. (Rochester, NY). Deferoxamine mesylate (DFM) was purchased from Ciba-Geigy (Summit, New Jersey).

Bacteria

Pseudomonas aeruginosa (strain E-2) was sub-cultured on Brain Heart Infusion (BHI) Agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and then cultured in BHI broth (Nissui) overnight at 37°C. The 50% lethal dose (LD50) after i.v. infection for non-treated BALB/c mice was approximately 1×10^6.

Bacterial suspensions of appropriate dilutions were prepared with PBS. Mice were inoculated intravenously (i.v.) with 2.0×10^5 or 2.0×10^6 cfu in 0.2 ml PBS. The exact numbers of viable bacteria used in each experiment were determined by subculturing on BHI agar from 10-fold dilutions of the samples.

Mice Treatment

Each experiment was performed using 6 mice. Mice were s.c. inoculated 1×10^6 Meth A tumor cells in 0.1 ml PBS ten days before infection, then i.p. injected with CPA (200 mg/kg) in 0.5 ml PBS on four days before pseudomonas infection. For the analysis of G-CSF prophylactic effect, control mice were i.p. injected with 0.5 ml PBS daily from the day of infection for four days (group 1; CPA alone). Other mice were i.p. injected with G-CSF (500 μg/kg) daily from the day of infection for four days (group 2; G-CSF alone) or in combination with oral administration with PSK (500 mg/kg) daily from the day of infection (group 3; G-CSF+PSK). For the analysis of therapeutic effect of G-CSF, CPA-treated control mice were i.p. injected with 0.5 ml PBS daily from the day of infection for four days (group 4; CPA alone). Other mice were i.p. injected with G-CSF (500 μg/kg) daily from the day of infection for four days (group 5; G-CSF alone) or in combination with PSK treatment (group 6; G-CSF+PSK).

Mice were i.v. injected with 2.0×10^5 cfu Pseudomonas aeruginosa (E-2) for the assessment of the prophylactic effect of G-CSF and with 2.0×10^6 cfu bacteria for the therapeutic effect.

Survival after inoculation with pseudomonas aeruginosa

Ten mice in each group were observed for seven days after the treatments as related above.

Quantification of viable bacteria in the liver

At 6 hours, 1, 2, 3, 4 and 5 days after bacterial infection, mice were sacrificed and the livers were removed aseptically. The livers were homogenized and 10-fold diluted with PBS. Samples were plated on BH1 agar with a Spiral System (Gunze Sangyo, Tokyo, Japan) for colony count as described previously.

Phagocytic assay

Phagocytic activity of peripheral neutrophils was assayed by ingestion of opsonized zymosan. Neutrophils (2×10^6) were pre-incubated in 200 μl of RPMI 1640 medium (GIBCO, Grand Island, New York) containing 5% fetal calf serum (FCS; HyClone, Logan, Utah) for 30 min at 37°C. Then phagocytosis was initiated by adding 50 μl zymosan (Sigma, St. Louis, MO) solution (1-2×10^7 particles/ml). Phagocytosis was terminated by putting the reaction tubes into an ice-cold bath 30 min after stimulation. The smears of the neutrophils were made on the glass slides using a Cytospin and stained with Giemsa solution. The number of zymosan ingested by the neutrophils was scored per 100 neutrophils using a dissection microscope.

The results were expressed as percentages of the number of neutrophils ingesting more than four zymosan.

Dichlorofluorescin (DCFH) oxidation assay

H2O2 production of neutrophils was measured by the DCFH oxidation assay according to the method of Bass et al. with some modification. Briefly, 0.5 ml of...
heparinized peripheral blood, which was sampled at the next day of the last G-CSF i.p. injection, was 10-fold diluted with Dubbecco's PBS containing 5 mM glucose and 0.1% gelatin (PBS g). Then cells were preincubated for 15 min with 5 μM DCFH-DA in PBSg in water bath at 37°C. In case of DFM treatment, DFM (1 mg/ml) was added in the medium for the preincubation. After 15 min preincubation, neutrophils were washed twice with PBS to remove the untrapped dye and resuspended to 5 ml in PBS g. Then cells were stimulated with 100 ng/ml phorbol myristate acetate (PMA; Sigma). Thereafter at 15 min after PMA stimulation, 1 ml aliquots of the cells were sampled for analysis. After washed twice with PBS, the simultaneous lysis of red blood cells and partial fixation of white blood cells were performed using FACS Lysing Solution (Becton Dickinson Immunocytochemistry Systems, San Jose, CA). Subsequently cells were washed and analyzed by a fluorescence activated cell sorter (FACS; FACScan, Becton Dickinson Immunocytochemistry Systems, Mountain View, CA) using a 488 nm argon ion laser.

Data were acquired using selective gating to the area of neutrophils (forward light scatter and side scatter) and analyzed with Consort 30 software.

Flow cytometry analysis of Mac-1 positive neutrophils
Fifty μl whole peripheral blood cells were incubated for 30 min at 4°C with the primary monoclonal antibody: 5 μl FITC-conjugated Mac-1 (anti-C3 receptor antibody; CALTAG Laboratories, San Francisco, CA.). Each sample was hemolyzed with 1,000 μl of FACS lysing solution (Beckton Dickinson Immunocytochemistry Systems, San Jose, CA.), washed twice with 2 ml of 0.1% NaN3-PBS, suspended in 300 μl of 2% paraformaldehyde PBS, and analyzed on a FACScan instrument (Beckton Dickinson Instruments, Palo Alto, CA).

Neutrophil population was gated due to the scatter graph of forward and side scatter distribution and the data were analyzed with Consort 30 software. 2,000 events were analyzed in each sample and the positive cell percentage and the number of Mac-1 positive neutrophils was determined.

Statistics
The statistical significance of the data was determined by Student's t-test.

Results
Effects of G-CSF and PSK on the survival of CPA-induced neutropenic mice after pseudomonas infection.

For the analysis of the prophylactic or therapeutic effects of G-CSF and PSK treatment against infection with Pseudomonas aeruginosa, CPA-induced neutropenic tumor-bearing mice were i.v. challenged with 2.0×10⁷ or 2.0×10⁸ or viable bacteria. Figure 1A shows the survival rate of mice treated with G-CSF for the prophylactic use (group 1 to 3). All group 1 (CPA alone) mice died by two days after infection, however, 50% mice of group 2 (G-CSF) and all of group 3 (G-CSF+PSK) survived pseudomonas infection with 2.0×10⁷ cfu. The improvement of host antibacterial effect was obtained by prophylactic treatment with G-CSF alone, and prominent in combination with PSK.

Figure 1B shows the survival rate of mice treated with G-CSF for the therapeutic use (group 4 to 6). All mice of group 4 (CPA alone) and 80% mice of group 5 (G-CSF) mice died by three days after infection, however, 80% of group 6 (G-CSF+PSK) survived pseudomonas infection with 2.0×10⁸ cfu. Therapeutic treatment with G-CSF alone (group 5) showed 20% survival, though 50% survival was observed after infection with 10-fold number of bacteria in mice treated prophylactically with G-CSF alone (group 2). On the other hand, the therapeutic treatment with both G-CSF and PSK (group 6) showed an augmenting effect on anti-microbial activity with 70% survival, compared with G-CSF alone (group 5).

Numbers of viable bacteria in the liver
Figure 2A shows the change of the number of viable bacteria in the liver of mice treated with G-CSF for the prophylactic use. In group 1 (CPA alone), the number of bacteria progressively increased by 1 day after infection and all mice were dead until day 2. Group 2 (G-CSF) mice inhibited the bacterial growth in the early phase of infection, however, the number of viable bacteria in the liver kept increasing. Group 3 (G-CSF+PSK) mice showed the stronger anti-microbial activity than group 2 mice from two days after infection (p<0.05), and the number of viable bacteria in the liver were suppressed after infection.

Figure 2B shows the changes of the numbers of viable bacteria in the liver of the groups treated with G-CSF for the therapeutic use. In group 4 (CPA alone) and group 5 (G-CSF), mice could not suppress the bacterial growth and all mice of group 4 died by day 2 after infection. However, group 6 (G-CSF+PSK) mice showed a stronger anti-microbial activity than group 4 and 5 mice, and remarkably suppressed the bacterial growth by three days after infection.

These results showed that pretreatment with G-CSF...
Fig. 1 1A shows the survival curve of mice prophylactically treated with G-CSF pretreated mice after $2.0 \times 10^5$ cfu *Pseudomonas aeruginosa* i.v. infection, and 1B of mice therapeutically treated with G-CSF after $2.0 \times 10^4$ cfu *P. aeruginosa* infection.

Enhancing effects of G-CSF and PSK on phagocytic activity of neutrophils

Figure 3 shows the phagocytic activity of peripheral neutrophils of CPA-induced neutropenic tumor-bearing mice treated prophylactically with G-CSF (Fig.3A) and in those treated therapeutically with G-CSF (Fig.3B). Neutrophils of CPA-treated mice (group 1 and 4) showed depressed phagocytic activity compared with that of non-treated tumor-bearing mice. On the contrary, G-CSF administered mice (group 2, 3, 5 and 6) showed a restored phagocytic activity and more than half of neutrophils revealed positive phagocytosis compared with non-treated tumor-bearing mice (group 1 and 4 mice; p<0.05).

The phagocytic values in mice administered G-CSF and PSK together (group 3 and 6) showed significantly improved phagocytic activity compared with non-treated tumor-bearing mice (group 2 and 5).

Effects of G-CSF and PSK on DCFH oxidation activities

Figure 4 shows the DCFH oxidation activity of peripheral neutrophils of mice administered G-CSF for the prophylactic treatment (Fig.4A) and those for the therapeutic treatment (Fig.4B). CPA-induced neutropenic tumor-bearing mice (group 1) showed a depressed DCFH oxidation activity compared with that of non-treated tumor-bearing mice, however, G-CSF treated mice (group 2 and 3) showed a significantly improved oxidation activity compared with group 1 mice (p<0.05). The value of oxidation activity of mice treated with G-CSF and PSK together (group 3) was higher than that of mice treated with only G-CSF (group 2). Mice treated with G-CSF for the therapeutic treatment (group 5 and 6) showed a significantly elevated DCFH oxidation activity compared with those of group 4 mice. The value of oxidation activity of mice with therapeutic treatment of G-CSF and PSK together (group 6) was greater than that of mice treated with only G-CSF (group 5).

When DFM was added to the culture medium, DCFH oxidation activity of all the groups decreased and lower than non-treated tumor-bearing mice (Fig.4).

These data show the prominent effect of G-CSF on...
DCFH oxidation activity and the augmentation effect of PSK together with G-CSF. This oxidation activity improved by G-CSF or PSK was speculated to be due to ferric ion transport, which was inhibited by DFM.

**Percentages of Mac-1 positive neutrophils**

Figure 5 shows the percentages of Mac-1 positive neutrophils of mice administered G-CSF for the prophylactic treatment (Fig.5A) and those for the therapeutic treatment (Fig.5B). CPA-induced neutropenic tumor-bearing mice (group 1 and 4) showed nearly the same Mac-1 positive percentages as those of non-treated tumor-bearing mice.

However, G-CSF alone or in combination with PSK treated mice (group 2, 3, 5 and 6) showed significantly higher Mac-1 positive percentages compared with those of non-treated or CPA induced neutropenic tumor-bearing mice. Mice treated with G-CSF and PSK together (group 3 and 6) showed the highest Mac-1 positive percentages of all the group mice in these experiments.

**Discussion**

This paper is one of the first reports that proved the prophylactic and therapeutic effects of G-CSF against microbial infection in immuno-compromised tumor-bearing hosts. G-CSF is a bio-active glycoprotein and shown to be an effective agent to induce granulopoiesis in animals and also in humans, and the precise target cells of G-CSF were investigated. In ordinary cases of cancer therapy or bone marrow transplantation, prevention of patients during neutropenic period from lethal infection is critical and important. But it is difficult to manage these patients, to prolong the life time of them and to succeed in their therapy. Under such neutropenic conditions, antibiotics were almost ineffective in protection against infection. It is reported that CPA induced neutropenic mice well tolerated against various microbial infections of LD50 dose by prophylactic G-CSF effect. Some investigators succeeded in augmentation of anti-streptococcal activity by prophylactic use of rhG-CSF in combination with antibiotics, but failed in that only by therapeutic use of rhG-CSF. In this paper G-CSF is proved to be effective under such neutropenic circumstances induced by a chemotherapeutic agent such as CPA, not only by prophylactic use but also by therapeutic use.

The mechanism of G-CSF induced protection against infection was reported in vitro and in vivo. In in vitro studies, some investigators reported the improved bactericidal, but non-fungicidal, activity by G-CSF in the culture medium, the enhanced superoxide release, and the augmented chemotactic activity. In in vivo
Fig. 5A shows the percentages of Mac-1 positive neutrophils in tumor-bearing mice treated with CPA and prophylactically with G-CSF or PSK, or those in mice treated with CPA and therapeutically with G-CSF or PSK.

In some studies, some investigators showed the restorative activity of rhG-CSF on superoxide generation and phagocytic activity of peritoneal neutrophils in bone marrow-transplanted tumor-bearing mice after lethal irradiation. However, the reports regarding such improved functions of neutrophils by G-CSF are, however, still few. The authors reported the improvement of phagocytosis and DCFH oxidation activity, and the increased Mac-1 positive percentage of peripheral neutrophils in CPA induced neutropenic mice by treatment with G-CSF, and also revealed that DCFH oxidative activity was suppressed by DFM treatment. This improvement of phagocytosis and DCFH oxidation activity of peripheral neutrophils means nearly the same functional effects induced by G-CSF as previous reports and further strongly suggests the contribution of ferric ion to protection against microbial infection.

PSK is one of the protein-bound polysaccharides and known as one of BRMs in cancer therapy. It is also proved that its antimicrobial activities and various biological effects such as restoration of antibodies forming capacities, morphological change of macrophages, hematological differentiation and antimicrobial activity including the difference of infection routes have become increasingly clear. Intraperitoneal pretreatment with PSK was shown to improve O2 producing activity and chemiluminescence of host peritoneal macrophages, to alleviate the reduction in resistance to microbial infection in tumor bearing mice and to improve their survival rate. Induction of antimicrobial activity was shown to depend on molecular weight of PSK subfraction as shown by chemiluminescence. On its anti-tumor activities, it was reported in vitro and in vivo in animals and in humans. The mechanisms of restored and augmented anti-tumor activities by PSK was shown as tumor necrosis factor (TNF) induction, augmented lymphokine-activated killer (LAK) cell in vitro, and interleukin (IL)-8 induction and IL-2 production in vivo.

Our results showed the improvement of phagocytosis and H2O2 production by G-CSF or together with PSK in spite of CPA pretreatment. However, ferric ion chelator, DFM treatment, lowered the H2O2 production activity to the CPA treated control level, and this result leads to the explanation that ferric ion contributes to the improved antimicrobial activity induced by both PSK and G-CSF, therefore the Habor Weiss reaction, catalyzed by ferric ion. Both G-CSF and PSK partially consist of glycocalyx, and these portions are speculated to contribute to transportation of ferric ion into neutrophils and other cells and induce the improved protection against microbial infection.

These findings in this paper may propose the possibility that new therapy of the combined use of G-CSF and PSK will make it possible to increase the dose of CPA in the tumor therapy and shorten the interval of chemotherapy cycles in patients with reduced hematopoiesis.

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Effect of Perioperative Blood Transfusion on Prognosis of Gastric Cancer; Retrospective Evaluation Using the Proportional Hazard Model of Cox

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1973年に、Opitzらが腎移植において、手術前に輸血を行えば、移植腎の生着率が向上することを発表した。従来、免疫学の分野では輸血によりどのような現象が起こるか、