MPXI and Early Neutrophilia: New Potential Therapeutic Biomarkers for Recombinant Human Granulocyte Colony-Stimulating Factor

Navapun Charuruks,^{1*} Narin Voravud,² and Virote Sriuranpong,²

¹Department of Laboratory Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

²Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

We evaluated the effect of recombinant human granulocyte colony-stimulating factor (rhG-CSF) given after myelosuppressive chemotherapy in 15 cancer patients. No severe neutropenia (absolute neutrophil count, ANC < $0.5 \times 10^3/\mu\text{L}$) was noticed in 10 rhG-CSF primary prophylactic patients, but was noticed in two of five rhG-CSF secondary prophylactic patients. Neutrophilia characterized by shift to the left occurred within 24 hours after starting rhG-CSF prophylaxis. Thereafter, conversion to normal level occurred within 24 hours. The peak of neutrophilia occurred earlier in the primary group than in the secondary prophylactic group. The detection of myeloperoxidase (MPO) using flow cytochemistry blood autoanalyzer (Technicon^R H*1) was evalu-

ated as mean peroxidase index (MPXI). Leukocyte alkaline phosphatase (LAP) using the method of Kaplow (*Am J Clin Pathol* 39:439–449, 1963) was recorded as LAP score. There was a statistically significant elevation of MPXI in the primary group over the secondary prophylactic patients. The LAP activity was in normal range. There was a slightly decreased red blood cell (RBC) count, hemoglobin (Hb), and platelet count. In conclusion, rhG-CSF induced neutrophilia with efficient enzymatic activity. These findings demonstrate the value of rhG-CSF in patients receiving chemotherapy. MPXI and early neutrophilia may serve as a potential biomarker of therapeutic efficacy of rhG-CSF. *J. Clin. Lab. Anal.* 12:41–46, 1998. © 1998 Wiley-Liss, Inc.

Key words: MPXI; neutrophilia; therapeutic biomarkers; rhG-CSFprophylaxis; chemotherapy

INTRODUCTION: TRACKING NEUTROPHENIA

Recently, considerable progress has been made in the management of cancer, including the use of new antineoplastic agents and regimens. The development of intensive antitumor regimens aimed at potential cure of some malignancies has led to severe and prolonged chemotherapy-induced cytopenia, especially neutropenia. The risk of infection following chemotherapy-induced neutropenia increases proportionally to the degree and duration of neutropenia (1). If the nadir absolute neutrophil count (ANC) falls below 1,000, 500, and $100 \times 10^3/\mu\text{L}$, the frequency of life-threatening infection rises steeply from 10% to 19% and 28%, respectively. Patients with severe neutropenia persisting for > 3 weeks had a 60% chance of developing severe infection. In spite of intensive antibiotic therapy, which is also associated with morbidity and mortality, death attributable to infection ranges from 5–20% (2,3). Another significant problem due to severe neutropenia has been the delay in chemotherapy treatment and of its consequential inefficacious chemotherapy cycle (4).

Specific cytokines regulating hematopoietic activity have been identified, purified, and cloned during the past 20 years.

Recombinant DNA technology enabled a production of recombinant human granulocyte colony-stimulating factor (rhG-CSF), which has been shown to accelerate the production of neutrophils by increasing the number of neutrophil progenitor cells and by shortening their normal maturation time from 5 days to 1 day (5). Therefore, the use of rhG-CSF may offer clinicians the opportunity to reduce the risk of neutropenia and enable delivery of efficacious regimens without the need for dose modification due to myelotoxicity.

To evaluate the effectiveness of rhG-CSF in patients receiving myelosuppressive chemotherapy, we conducted an investigation of the effect of rhG-CSF on both quantitative and qualitative changes of peripheral neutrophils.

*Correspondence to: Dr. Navapun Charuruks, Department of Laboratory Medicine, Faculty of Medicine, Chulalongkorn University Hospital, Rama IV Rd., Pathumwan, Bangkok 10330, Thailand.

Received 1 May 1997; Accepted 19 June 1997

MATERIALS AND METHODS

We studied 15 patients receiving 5–22 courses (average, 18 courses) of myelosuppressive chemotherapy without previous rhG-CSF administration of Chulalongkorn Hospital. Ten patients received rhG-CSF as a primary prophylaxis, and another 5 patients received rhG-CSF as a secondary prophylaxis. Primary prophylaxis was defined as the use of rhG-CSF for amelioration of chemotherapy-induced neutropenia, and secondary prophylaxis was defined as the prevention of a second episodes of febrile neutropenia ($ANC < 0.5 \times 10^3/\mu L$ with fever) in patients demonstrating severe myelosuppressive and/or infection with previous cycles of chemotherapy (6). The rhG-CSF was administered subcutaneously once daily 24 hours after completion of the last dose of chemotherapy. Glycosylated rhG-CSF was given as a 2 $\mu g/kg/day$ and nonglycosylated rhG-CSF at 5 $\mu g/kg/day$. Each patient received only the same type of rhG-CSF for the whole study. The treatment was continued until absolute nadir granulocyte count (ANC) $> 10 \times 10^3/\mu L$ and beyond the granulocyte nadir period. The details are shown in Table 1. The first peripheral blood sample was drawn before rhG-CSF prophylaxis, and the second sample was collected 24 hours after rhG-CSF administration. The following samples were collected daily for 12 consecutive days. All samples were kept in ethylenediaminetetraacetic acid (EDTA) and heparin tubes and immediately sent to the laboratory for analysis.

All EDTA blood samples were analyzed by Technicon^R (H*1 (H*1, Technicon Instrument Corp., Tarrytown, NY) for a complete blood count (CBC), white blood cell (WBC), and differential count on whole blood samples using both cytochemistry

and light scattering (laser) technology. Twenty-five useful parameters obtained by the automated blood analyser, Technicon^R H*1, have been previously studied for precision and accuracy (7). The absolute numbers and percentages of leukocytes reported by the instrument were concentrated for quantitative analysis of neutrophils and the other leukocytes.

Myeloperoxidase (MPO) activity is necessary for antimicrobial function of neutrophils (8,9). The mean peroxidase activity is expressed by the Technicon^R H*1 as the mean peroxidase index (MPXI) (10,11) and could thus be used to study the effect of rhG-CSF on MPXI on our 15 patients as a qualitative indicator of neutrophil function. The MPXI also was previously found to correlate with benzidine dihydrochloride cytochemical staining (12). All EDTA blood samples were also examined using the Wright's stain. The percentage means of promyelocytes, myelocytes, metamyelocytes, and band were evaluated as immature forms and segmented neutrophils was considered as mature forms.

To evaluate leukocyte alkaline phosphatase (LAP) activity, blood smears were made from heparin samples using the method of Kaplow (13). Three hundred neutrophils from each sample were scored according to the intensity of staining and the number of blue granules in the cytoplasm. Individuals cells were rated as: 0 = negative, no granules; 1 = positive but very few blue granules; 2 = positive with few to moderate number of granules; 3 = strongly positive with numerous granules; 4 = very strongly positive with cytoplasm crowded with granules. The score of an individual smear consisted of the sum of scores of 100 consecutive neutrophils.

We also evaluated the red blood cell (RBC) count, hemoglobin (Hb), and platelet counts generated from Technicon^R H*1 to complete the hematologic evaluation of rhG-CSF on erythrocytes and thrombocytes.

Reference values from a previous study of 1,005 healthy Thai subjects in 1994 (14) was use for data analysis (Table 2). Statistical analysis was performed by using the unpaired Student's t-test, and P value < 0.05 was considered statistically significant.

RESULTS

Evaluation of Quantitative Changes of Neutrophils

Table 3 presents changes in hematologic values from Technicon^R H*1 before, during, and after rhG-CSF administration in primary and secondary prophylactic patients. There were increments of WBC and ANC from all phases with rhG-CSF application, especially in secondary prophylactic patients where statistical significant evaluations were observed. There were slight changes in lymphocytes, monocytes, and basophils in most of the cases. However, there were slight lymphocytosis, monocytosis, and basophilia in some cases. Figures 1 and 2 show the peripheral WBC and ANC changes by mean ± 1 SD beginning from before rhG-CSF prophylaxis through the course of rhG-CSF prophylaxis in primary

TABLE 1. Patient Characteristics

Characteristics	Prophylactic type	
	Primary	Secondary
Number	10 cases	5 cases
Age: range (mean)	42–77 (57.5) years	54–74 (63.0) years
Sex: male: female	7:3	3:2
Diagnosis		
Small cell lung cancer	4 cases	2 cases
Non-small cell lung cancer	2 cases	2 cases
Oat cell cancer	1 case	–
Liposarcoma	1 case	–
Adenocarcinoma (breast cancer)	1 case	–
Unknown primary origin	1 case	1 case
Chemotherapy ^a		
CP, VP-16	6 cases	2 cases
IFX, VP-16	1 case	–
DDP, IFX, VP-16	1 case	–
DDP, VP-16	1 case	–
Paclitaxel	2 cases	2 cases
rhG-CSF		
Nonglycosylated rhG-CSF	6 cases	3 cases
Glycosylated rhG-CSF	4 cases	2 cases
Duration of administration	3–9 (6.3) days	7–10 (8.6) days

^aCP, carboplatin, IFX, ifosfamide, DDP, cisplatin, VP-16, etoposide.

TABLE 2. Manufactural Reference Ranges in 1,005 Healthy Thai Subjects^a

Parameters	Reference ranges			
	Krailadsiri et al. (1994)		Manufacturer	
	Absolute number ($\times 10^9/L$)	Percent (%)	Absolute number ($\times 10^9/L$)	Percent (%)
WBC	3.6–9.8	–	4.8–10.8	–
Neutrophil	1.31–6.75	36.4–68.9	1.92–7.99	40.0–47.0
Lymphocyte	0.73–1.47	20.4–49.0	0.91–5.18	19.0–48.0
Monocyte	0.09–0.08	2.5–8.1	0.16–0.97	3.4–9.0
Eosinophil	0.00–0.82	0–8.4	0.00–0.7	0–0.7
Basophil	0.00–0.24	0–2.5	0.00–0.16	0–1.5
Large unstained cell (LUC)	0.00–0.93	0–9.5	0.00–1.03	0–4.0
Mean peroxidase index (MPXI)	(–11)–2	–	(–10)–10	–

^aModified from Krailadsiri et al. 1994.

(n = 10) and secondary (n = 5) prophylactic patients, respectively. Figures 3 and 4 show the morphological analysis of neutrophils with rhG-CSF administration in primary and secondary prophylactic patients, respectively. We found that promyelocytes are the earliest myeloid cells seen in PBS during rhG-CSF prophylaxis. Thereafter, the percentage of myeloid cells were converted to normal level of the PBS milieu. We also found that in primary prophylactic patients, the mean peak of neutrophilia occurred on the fourth day, whereas in the secondary prophylactic patients, the mean peak of neutrophilia occurred on the ninth day. The ratio of mature VS

immature forms was noted to be higher in the primary prophylactic group.

Analysis of MPO and LAP Activity: Qualitative Study of Neutrophils

There was a significant increase of MPXI in the primary prophylactic group with rhG-CSF administration. In the secondary prophylactic group, there was a slight increase during the study period (Table 3, Fig. 5). There were statistically significant differences observed between primary and secondary prophylactic patients from day 3 to day 8.

TABLE 3. Hematologic Change in rhG-CSF Prophylactic Patients Receiving Myelosuppressive Chemotherapy

Parameters	Before rhG-CSF Rx mean ($\pm$ SD)	During and after rhG-CSF Rx mean ($\pm$ SD)
Primary prophylaxis (n = 10, duration of rhG-CSF administration = 5.2 ± 3.3 days)		
RBC ($\times 10^{12}/L$)	4.03 (± 0.44)	3.78 (± 0.61)
Hb (g/dL)	11.88 (± 1.28)	10.77 (± 1.13)
Platelet count ($\times 10^9/L$)	247 (± 127)	173 (± 75)
WBC ($\times 10^9/L$)	7.32 (± 2.56)	9.69 (± 5.86)
Neutrophil ($\times 10^9/L$)	5.86 (± 2.30)	8.05 (± 5.45)
Lymphocyte ($\times 10^9/L$)	1.06 (± 0.63)	1.17 (± 0.89)
Monocyte ($\times 10^9/L$)	0.15 (± 0.11)	0.19 (± 0.10)
Eosinophil ($\times 10^9/L$)	0.12 (± 0.05)	0.15 (± 0.08)
Basophil ($\times 10^9/L$)	0.02 (± 0.02)	0.04 (± 0.03)
Large unstained cell, LUC ($\times 10^9/L$)	0.11 (± 0.09)	0.09 (± 0.05)
Meann peroxidase index, MPXI	1.1 (± 2.8)	6.5 (± 6.8)*
Secondary prophylaxis (n = 5, duration of rhG-CSF administration = 7.8 ± 1.4 days)		
RBC ($\times 10^{12}/L$)	3.82 (± 0.48)	3.10 (± 0.78)
Hb (g/dL)	11.90 (± 1.43)	9.35 (± 0.98)
Platelet count (($\times 10^9/L$)	147 (± 78)	81.77 (± 49)
WBC ($\times 10^9/L$)	4.47 (± 1.92)	10.82 (± 13.23)
Neutrophil ($\times 10^9/L$)	2.76 (± 2.30)	8.76 (± 12.32)
Lymphocyte ($\times 10^9/L$)	1.29 (± 0.78)	1.18 (± 0.65)
Monocyte ($\times 10^9/L$)	0.14 (± 0.07)	0.61 (± 0.31)
Eosinophil ($\times 10^9/L$)	0.12 (± 0.05)	0.18 (± 0.16)
Basophil ($\times 10^9/L$)	0.04 (± 0.03)	0.04 (± 0.02)
Large unstained cell, LUC ($\times 10^9/L$)	0.12 (± 0.07)	0.05 (± 0.03)
Mean peroxidase index, MPXI	–2.9 (± 6.9)	–1.7 (± 3.9)

*Statistic significance, $P < 0.05$.

Fig. 1. WBC changes in rhG-CSF primary and secondary prophylactic patients.

LAP activity increased slightly during rhG-CSF primary prophylaxis. The LAP activity was normal, with a median of 41 (range 23–81).

Effect of rhG-CSF on RBC and Platelets

The effects of rhG-CSF on RBC, Hb, and platelet counts in patients receiving myelosuppressive chemotherapy are shown in Table 3 and Figure 6. The results demonstrated no significant change on RBC number in primary and secondary prophylactic patients. There was a slight decrease in Hb levels. There was no significant change in platelet number. However, the decrement of platelets were noticed from the beginning through the course of the prophylaxis treatment.

DISCUSSION

Administration of rhG-CSF produced an increased neutrophil count in peripheral blood. In some patients there was > 10-fold increase of neutrophils, whereas there was little or no increase in the other white cells. This demonstrated the speci-

Fig. 2. ANC changes in rhG-CSF primary and secondary prophylactic patients..

Fig. 3. ANC in mature and immature form in rhG-CSF primary prophylactic patients.

ficity of rhG-CSF in stimulation of the proliferative and functional activation aspects of the neutrophil lineage. Souza et al. (15) and Welte et al. (16) observed a selectively stimulated neutrophil production. The objective of rhG-CSF prophylactic therapy is to prevent severe neutropenia and infection. However, there was little lymphocytosis, monocytosis, and basophilia seen in some cases. Previous studies (17–18) reported that the production of lymphocytosis in vivo was largely unaffected by the administration of rhG-CSF in humans and mice. However, lymphocytosis has been reported in cats treated with rhG-CSF (17–19). It is possible that these effects are due to a direct stimulatory effect of rhG-CSF on lymphoid precursor cells; however, there have been reports of indirect stimulation of pre-B cells by rhG-CSF in combination with other growth factors (20). Interestingly, the kinetics of monocyte production are also affected during rhG-CSF administration in mice (21). It is noteworthy that Mecal (22) showed rhG-CSF to be a growth stimulus for

Fig. 4. ANC in mature and immature form in rhG-CSF secondary prophylactic patients.

Fig. 5. MPXI changes in rhG-CSF primary and secondary prophylactic patients.

macrophage progenitors, indicating that this effect may well be due to direct stimulation on monocyte growth. For basophilia, the mechanism may not involve rhG-CSF, and we recommend further studies to evaluate this mechanism.

This study has shown that although rhG-CSF stimulates proliferation and maturation of granulocytes up to 15-fold, the MPXI will still be in the normal ranges according to the manufacturer reference (normal range of MPXI is -10 – 10). However, according to Krailadsiri (14), the MPXI is considered as elevated compared to normal in Thai people. This indicates the effectiveness of rhG-CSF in improving MPO activity. However, the MPXI activity is enhanced significantly after primary rhG-CSF prophylaxis. We also found statistically significant differences between the primary and the secondary prophylactic patients. Interestingly, the neutrophils produced during the proliferative

response to rhG-CSF prophylaxis appeared to possess enhancing function. It seemed that the significant enhanced MPXI could be used as a potential therapeutic biomarker for predicting a satisfactory response in the patients.

According to the result of PBS from Wright's stain, we found that promyelocytes are the earliest myeloid cells seen during rhG-CSF prophylaxis. The percentage mean showed high percentages of PMNs and bands with some metamyelocytes, myelocytes, and promyelocytes. They were converted to the normal composition of PBS milieu after 24 hours of rhG-CSF prophylaxis. This demonstrates the specificity of rhG-CSF to stimulate proliferation and differentiation of granulocyte. The stimulation of immature cells were observed with the most immature form being the promyelocytes. A study of cell kinetic in bone marrow should give more information and complete the kinetics for human granulopoiesis and the other hemopoiesis following rhG-CSF prophylaxis. This demonstrated the earlier response of rhG-CSF in primary prophylactic patients. The peak of neutrophilia and the ratio of matured and immatured forms may be used as the therapeutic markers as well as MPXI.

For LAP activity, the result showed normal LAP activity. Teshima et al. (23) and Sato (24) demonstrated enhancement of LAP activity with G-CSF in a dose-dependent manner. However, the assay for LAP activity in these studies were quantitated and expressed as U, with 1 U representing the amount of enzyme activity that generated 1.0 nmol of p-nitrophenol/30 min (25), which differed from our study using Kaplow's method (13). The pattern of hematologic change in this condition may mimic chronic myelocytic leukemia (CML) and leukemoid reaction. It differed clearly from CML, since LAP activity in CML is lower than normal, but LAP activity could not differentiate it from leukemoid reaction. However, Tsushita et al. (26) studied the effects of rhG-CSF in CML and demonstrated the induction of LAP gene expression in response to G-CSF stimulation.

From our study, erythrocytes slightly decreased in both number and hemoglobin (Hb) during the first few days of rhG-CSF prophylaxis, as did the platelet number. The antineoplastic agents directly suppressed the bone marrow causing anemia and thrombocytopenia, and frequently accompanied by neutropenia. Thrombocytopenia usually occurs within 7–10 days (27). The side effect of myelosuppressive agents has more effects on platelets than on RBC, because the life span of RBC (120 days) is longer than that of platelets (10 days) (28). Erythropoiesis is suppressed in mice following prolonged treatment with rhG-CSF (18). In the first few days of treatment (4–5 days), the deficiency is seen only in marrow differential counts and radioactive iron incorporation into the bone marrow, but this short fall of RBC production is offset by the increased erythropoietic activity noted in the spleen (18,29). In splenectomized mice, however, protracted administration of rhG-CSF resulted in anemia. This anemia was present in splenectomized animals not only after doses of 250 $\mu\text{g/kg/day}$, but also in mice treated with 10 $\mu\text{g/kg/day}$, a dose

Fig. 6. RBC, Hb, and platelet count in rhG-CSF prophylactic patients.

used in routine clinical use. However, the mouse differs in important areas in hemopoiesis from humans: (1) extramedullary hemopoiesis is rare, although splenomegaly has been reported in patients with congenital agranulocytosis receiving rhG-CSF for up to 13 months (30), and (2) in humans there are restricted skeletal sites of red cell production.

There were no individual reports where investigators found that thrombocytopenia was definitely related to rhG-CSF therapy. It might be due to the fact that patients treated with chemotherapy and rhG-CSF received almost twice as many chemotherapy cycles. We conclude that rhG-CSF does not appear to have an adverse effect on RBC and platelets in most clinical settings.

In summary, this study demonstrated that rhG-CSF caused neutrophilia with improved enzymatic activity without any problem of anemia or thrombocytopenia. However, RBC, Hb, and platelets were suppressed by antineoplastic agents. Interestingly, our study suggested that the values of MPXI and early evidence of neutrophilia following rhG-CSF prophylaxis could be used as a potential prognostic marker to predict rhG-CSF response in patients. This suggest MPXI may be used as a response marker in patients who receive chemotherapy for the first time. This will not only help the clinician in optimal therapeutic design but also assist with cost benefit analysis of rhG-CSF prophylaxis.

ACKNOWLEDGMENTS

We thank the patients for their patience and cooperation. We also thank the laboratory staffs for their laboratory assistance.

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