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Effects of the *in vivo* Administration of Recombinant Human Granulocyte Colony-stimulating Factor Following Cytotoxic Chemotherapy on Granulocytic Precursors in Patients with Malignant Lymphoma[1]

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We examined the effects of the *in vivo* administration of recombinant granulocyte colony-stimulating factor (rhG-CSF) on granulocytic precursors in the bone marrow of 4 patients with malignant lymphoma who received chemotherapy. Patients were treated with rhG-CSF at doses of 100–800 $\mu g/m^2$/day intravenously for 14 days only in the first course of chemotherapy (G-CSF course) followed by the second course of chemotherapy without rhG-CSF which was used as a control course. In the G-CSF course, white blood cell counts (WBCs) demonstrated a biphasic response consisting of a first peak observed within a few days after the initiation of rhG-CSF administration, and a second peak observed on the last day of rhG-CSF injection or the day after. In the second peak, the incidence of granulocyte-macrophage colony-forming units (CFU-GM) in mononucleated bone marrow cells did not change significantly after treatment with rhG-CSF as compared with a control. However, since the number of nucleated cells in the bone marrow increased, the absolute number of CFU-GM in the bone marrow increased. The number of mature and immature granulocytes in the bone marrow increased. These findings suggest that G-CSF stimulates the proliferation and differentiation of granulocytic precursors in the bone marrow in granulocytopenic patients who received cytotoxic drugs and causes mature granulocytes to be released from the bone marrow.

Key words: Recombinant granulocyte colony-stimulating factor — Granulocyte-macrophage colony-forming units — Malignant lymphoma

Granulocyte colony-stimulating factor (G-CSF) is one of the colony-stimulating factors (CSFs) which support the proliferation and differentiation of hematopoietic precursors *in vitro*.[1,2] Recently, human G-CSF was purified and molecularly cloned,[3,4] and now, recombinant human G-CSF (rhG-CSF) has become available for clinical use.

Clinical effects of the *in vivo* administration of human macrophage colony-stimulating factor (M-CSF)[5] and granulocyte-macrophage colony-stimulating factor (GM-CSF)[6–8] have been reported previously. Phase I/II studies showed that rhG-CSF reduced the period of neutropenia following cytotoxic chemotherapy and accelerated the recovery from neutropenia.[10–13] GM-CSF and G-CSF are remarkably effective in increasing peripheral neutrophils.

Socinski *et al.*[14] reported that GM-CSF produced an increase in peripheral blood granulocyte-macrophage colony-forming units (CFU-GM), but had no effect on bone marrow (BM) CFU-GM. Aglietta *et al.*[15] demonstrated that GM-CSF increased the percentage of BM S phase CFU-GM and erythroid burst-forming units (BFU-E). Recently, Bronchud *et al.*[11] reported that G-CSF stimulated the proliferation and differentiation of neutrophil precursors in the bone marrow in patients with small cell lung cancer during the phase I part of the phase I/II study. Dührsen *et al.*[16] also reported, as the result of the phase I clinical trial, that the absolute number of circulating progenitor cells of trilineages increased and the frequency of progenitor cells in the bone marrow was variable after treatment with G-CSF in cancer patients. However, it is not well understood whether the *in vivo* administration of rhG-CSF is effective on granulocytic precursors in the bone marrow after exposure to cytotoxic drugs. We investigated changes in BM granulocytes and CFU-GM after the administration of rhG-CSF in patients with malignant lymphoma who received two courses of the same chemotherapy. The rhG-CSF was administered only during the first course. The second course was used as a control course.

[1] This study was performed as a part of a phase II group study in Japan chaired by Professor F. Takaku, University of Tokyo, with the support of Kirin Brewery Co.

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[5] Abbreviations: rhG-CSF, recombinant human granulocyte colony-stimulating factor; CFU-GM, granulocyte-macrophage colony-forming units.

MATERIALS AND METHODS

Patients Four patients (2 males, 2 females, ranging in age between 44 and 77) were admitted to our hospital with non-Hodgkin's lymphoma which was classified histologically according to the standards of the Lymphoma Study Group (LSG) in Japan. Ann Arbor staging standards were applied in determining the clinical stage. The bone marrow was involved in cases 1–3 (Table I). Bone marrow infiltration of lymphoma cells was found to be only 0.4% on the bone marrow aspiration smear in case 1. Lymphoma cells were not detectable on bone marrow smears in cases 2 and 3. However focal infiltration was recognized histologically in bone marrow biopsy specimens in cases 1–3. We assessed that the influence of infiltration on hematopoiesis was minimal because complete blood cell counts (CBCs) were within normal limits and the hemopoiesis of trilineages in the bone marrow was maintained at diagnosis in each case. Informed consents were obtained from all patients prior to the study.

Protocol Each patient underwent two identical courses of chemotherapy, including prednisolone as shown in Table I. Combinations of cytotoxic drugs were varied in each case. In the first course (G-CSF course) for each patient, rhG-CSF was administered for 14 days after chemotherapy had been finished. The second course was a control course consisting of only chemotherapy, not followed by rhG-CSF (Fig. 1). RhG-CSF (KRN 8601, specific activity 1×10^8 unit/mg, supplied by Kirin Brewery Co., Ltd., Tokyo) was given at doses of 100, 200, 400, and 800 $\mu g/m^2$/day, via intravenous drip infusion, for 30 min. There were 3 days between the end of the chemotherapy and the initiation of the G-CSF adiministration. CBCs and differential counts of leukocytes were monitored every 2–3 days. Bone marrow aspirations were performed 3 times during the 2 courses as follows: (1) a bone marrow examination was performed just before the initial administration of rhG-CSF in the G-CSF course (BM-BEFORE). (2) a bone marrow examination was carried out on the day after the last day of rhG-CSF injection during the G-CSF course (BM-AFTER). (3) a bone marrow examination was performed on the day corresponding to the day of BM-AFTER in the control course (BM-CONTROL). Nucleated cell counts and dif-

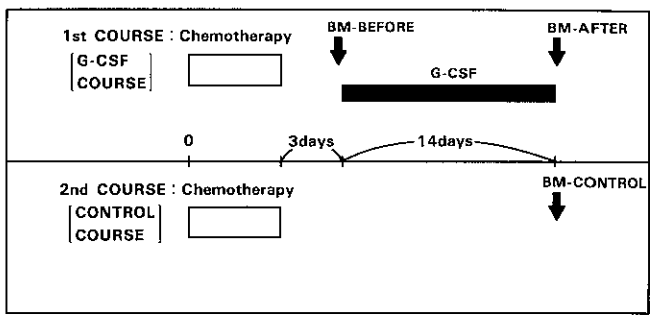

Fig. 1. Protocol. The 1st course was the G-CSF course consisting of chemotherapy and rhG-CSF injection; the 2nd course was the control course consisting of only chemotherapy; bone marrow aspirations were performed just before the first administration of rhG-CSF (BM-BEFORE), on the day after the last administration of rhG-CSF (BM-AFTER), and on the day corresponding to the day of BM-AFTER in the control course (BM-CONTROL).

Table I. Profiles of Patients with Malignant Lymphoma

Case	Name	Age/Sex	Diagnosis	Stage	BM involvement	Chemotherapy	G-CSF Dose ($\mu g/m^2$)	G-CSF Duration (days)
1	S.K.	44/F	NHL (d, m)	IVA	+	IFM, VP16 MIT, PSL	100	14
2	K.Y.	64/M	IBL-T	IVB	+	CPM, ADR VCR, PSL	200	10+3
3	T.T.	62/M	IBL-T	IVA	+	CPM, ADR VCR, PSL	400	14
4	C.Y.	77/F	NHL (d, l)	IIIB	−	IFM, ACM VCR, PSL MTX	800	14

BM involvement, bone marrow involvement, NHL(d, m), non-Hodgkin's lymphoma (diffuse, medium cell sized); NHL(d, l), non-Hodgkin's lymphoma (diffuse, large cell sized); IBL-T, immunoblastic lymphoadenopathy like T-cell lymphoma; IFM, ifosfamide; VP-16, etoposide; MIT, mitoxanthrone; PSL, prednisolone; CPM, cyclophosphamide; ADR, adriamycin; VCR, vincristine; MTX, methotrexate.

ferential counts of bone marrow aspirates were counted at each time.

Colony assay for CFU-GM Colony assays for CFU-GM were performed by methylcellulose culture. Briefly, mononuclear cells (MNCs) were prepared by Ficoll-Metrizoate (Lymphoprep, Nyegaad, Oslo) density gradient centrifugation of bone marrow cells obtained from patients. MNCs were washed 3 times with Iscove's modified Dulbecco's medium (IMDM, GIBCO Laboratories, Grand Island, New York) before culture. One ml of IMDM consisting of 5×10^4 MNCs, 1.2% methylcellulose (Fisher Scientific, Norcross, Georgia), 30% fetal calf serum (FCS, Flow Laboratories, North Ryde, N.S.W. Australia), 1% deionized bovine serum albumin (Sigma Chemical Company, St. Louis, Missouri), and 2×10^{-5} *M* 2-mercaptoethanol (Sigma Chemical Company), with or without 20 ng of rhG-CSF (specific activity 1×10^8 unit/mg, Chugai Pharmaceutical Company, Tokyo) was cultured in 35 mm non-tissue culture dishes (Falcon, Oxnard, California). Five percent phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM)[17] was added to the methylcellulose medium instead of G-CSF, because PHA-LCM could support all kinds of myeloid colonies. The incubation was continued at 37°C in a humidified atmosphere of 5% CO_2 for 14 days. The numbers of colonies and clusters were counted on days 7 and 14 using an inverted microscope. Aggregations containing 40 or more cells were defined as colonies, and those containing 8 to 40 cells were defined as clusters. On day 14, all granulocyte-macrophage colonies were collected from the semisolid media and suspended in 0.2 ml of IMDM containing 30% FCS. The morphology of the cells in the GM colonies was examined in Cytospin (Shandon Southern, Sewickley, Pennsylvania) preparations after May-Grünwald-Giemsa staining.

RESULTS

Time courses of peripheral white blood cell counts The pattern of WBCs during the G-CSF courses was significantly different from that of the control courses (Fig. 2). In the control courses, WBCs decreased gradually after chemotherapy commenced, and subsequently increased to the initial levels. On the other hand, two peaks were observed in the G-CSF courses. The first peak appeared within a few days after the initiation of rhG-CSF. The levels of the first peak were different in each case. The second peak was observed on the last day of rhG-CSF administration or the day after. WBCs always decreased to the base level within a few days after the termination of rhG-CSF injection. Differential counts of leukocytes revealed that only neutrophils increased in response to the administration of rhG-CSF. There was no

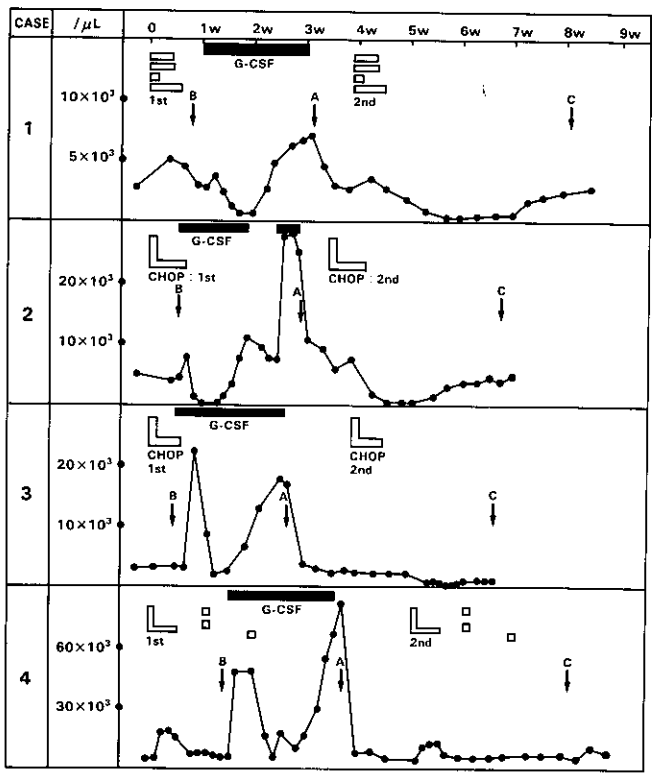

Fig. 2. White blood cell counts in cases 1–4. 1st, first chemotherapy; 2nd, second chemotherapy; B, on the day when bone marrow aspirations were carried out just before the administration of rhG-CSF; A, on the day after cessation of rhG-CSF; C, on the day when control bone marrow aspirations were performed. In case 1: B=d7, A=d22 in the first course, C=d30 in the second course. In case 2: B=d4, A=d21 (1st), C=d21 (2nd). In case 3: B=d4, A=d19 (1st), C=d19 (2nd). In case 4: B=d10, A=d24 (1st), C=d22 (2nd).

significant difference in the number of reticulocytes and platelets between the two courses. The absolute number of lymphocytes or monocytes did not increase beyond a normal range after the administration of rhG-CSF (data not shown).

Bone marrow analysis Bone marrow aspiration revealed that nucleated cell counts and myeloid/erythroid (M/E) ratios were varied in the BM-BEFORE of each case (Fig. 3). The nucleated cell count in case 4 was within the normal range, whereas the nucleated cell counts in the other cases decreased right after chemotherapy, and in particular, the bone marrow from case 2 showed hypocellularity. After chemotherapy, M/E ratios exceeded the normal value in all cases. M/E ratios were extremely high in cases 2, 3 and 4. Nucleated cell counts were higherin the BM-AFTER than in the BM-CONTROL in all cases except case 1. In case 1, the nucleated cell count was higher in the BM-CONTROL

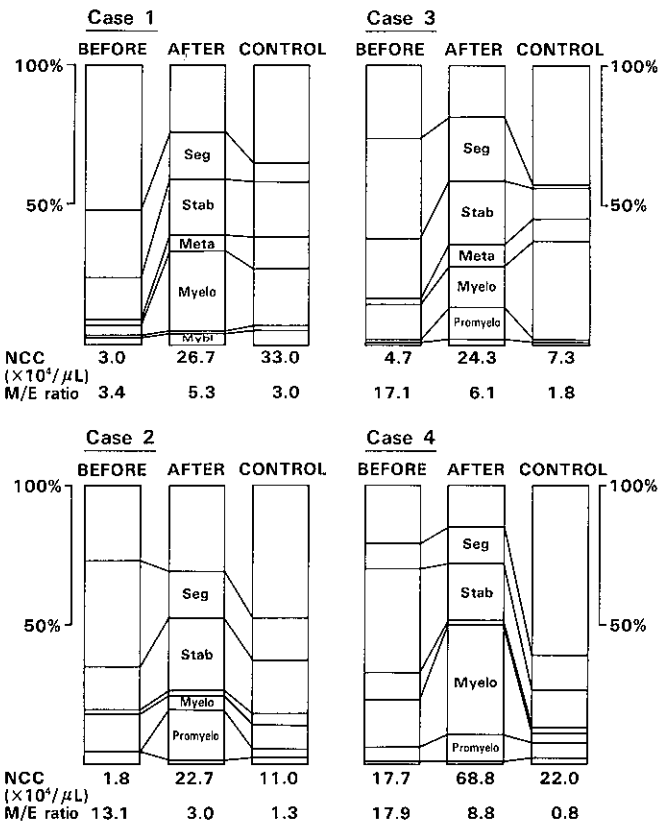

Fig. 3. Results of bone marrow analyses. Comparison of nucleated cell counts (NCCs), M/E ratios, and differential counts of bone marrow examinations performed three times for each case. BEFORE, just before the first administration of rhG-CSF; AFTER, after the last administration of rhG-CSF; CONTROL, in a control course.

because the sampling of the BM-CONTROL was delayed until WBCs recovered. Differential counts of BM smear samples revealed that percentages of mature and immature granulocytes were higher in the BM-AFTER than in the BM-CONTROL (Fig. 3). Percentages of mature granulocytes (stab form and segmented) were higher after treatment with rhG-CSF than in controls in all cases. Those of promyelocytes increased after rhG-CSF treatment in cases 2, 3, and 4, and those of myelocytes increased in cases 1 and 4.

Effects of the *in vivo* administration of rhG-CSF on CFU-GM in the bone marrow We counted the number of colonies on day 14 of culture, and that of clusters on day 7. This convention was adopted because mapping studies demonstrated that the peak of colony formation was observed from days 10 to 14, while the peak of cluster formation was observed from days 5 to 8 (data not shown).

Table II (left side) shows the results of colony formation of CFU-GM using bone marrow aspirates from BM-BEFORE, BM-AFTER, or BM-CONTROL. There were no significant changes in the number of CFU-GM/5×10^4 MNCs on day 14 between BM-AFTER and BM-CONTROL regardless of the use of G-CSF or PHA-LCM for colony assay. The time courses of colony or cluster formation and the size of colonies were similar between BM-AFTER and BM-CONTROL.

Table II (right side) shows the results of cluster formation on day 7. The number of clusters was varied in each case after the treatment of rhG-CSF. Morphological analysis indicated that cells in the colonies or clusters, formed in the presence of G-CSF, were immature or mature granulocytes. However, we used the term CFU-GM, instead of granulocyte colony-forming units

Table II. Results of Colony Assay

Case	CSF	GM-colonies			GM-clusters		
		Before	After	Control	Before	After	Control
1	G-SF	0	20±2	19±2	0	56±18	12±1
	PHA-LCM	77±3	21±1	17±1	200	100±15	34±6
2	G-CSF	*	15±8	16±7	*	84±5	7±9
	PHA-LCM	*	16±7	51±4	*	279±35	321±11
3	G-CSF	14±3	10±1	13±2	18±7	52±9	87±5
	PHA-LCM	15±1	9±3	22±2	19±5	61±7	67±3
4	G-CSF	20	10±2	10±0	86	48±6	61±6
	PHA-LCM	15±4	17±1	16±2	55±5	39±3	69±3

GM-colonies and GM-clusters/5×10^4 mononuclear cells (MNCs). Results are expressed as mean±range. The number of GM-colonies was counted on day 14 of culture. The number of GM-clusters was counted on day 7.
* A sufficient number of MNCs for culture was not obtained from specimens of bone marrow aspirations due to hypocellularity.

(CFU-G), because it was difficult to distinguish macrophages generated from a colony-forming cell from those existing in the background of colonies. No spontaneous GM colony formed in the absence of G-CSF or PHA-LCM was observed before or after the administration of rh-G-CSF in any case.

DISCUSSION

In this study, we examined changes in granulocytes and their precursors after the administration of rhG-CSF following cytotoxic chemotherapy in patients with malignant lymphoma. Three of 4 patients presented a minimal infiltration of lymphoma cells to the bone marrow. The rhG-CSF was effective on granulocytes and their progenitors in all patients even if there was bone marrow involvement. In these patients, a biphasic response of WBC to the administration of rhG-CSF after chemotherapy was observed. This observation is consistent with the finding reported by Gabrilove *et al.*[13] The first peak which was seen within a few days after the initiation of rhG-CSF injection was considered to be due to the release of mature granulocytes from the bone marrow and marginal pool. We found a tendency that when large numbers of granulocytes were reserved in the bone marrow, the first peak became higher. For example, in case 4, the nucleated cell count was kept at a normal level and the M/E ratio was extremely high due to chemotherapy, which resulted in a high first peak. In contrast, in case 1, due to a low nucleated cell count accompanied with a not so high M/E ratio, the first peak was low. In case 2, the absolute number of granulocytes may not have been large enough to respond to G-CSF in the bone marrow (Fig. 3). The rhG-CSF, then, causes mature granulocytes to be released from the bone marrow.

After a period of myelosuppression, WBCs recovered gradually, and increased in number to produce a second peak on the last day of G-CSF injection or the day after. It is likely that the second peak resulted from the increase in the number of CFU-GM in the bone marrow, the differentiation of granulocytes and the release of mature granulocytes from the bone marrow in response to rhG-CSF. Our data indicated no difference in the incidence of bone marrow CFU-GM/5×10^4 MNCs between the BM-AFTER and BM-CONTROL. These results were consistent with those on GM-CSF reported by Socinski *et al.*[14] and those on G-CSF reported by Bronchud *et al.*[11] However, the absolute number of CFU-GM in the bone marrow was estimated to increase in response to rhG-CSF, because the bone marrow cellularity increased.

Although differential counts of bone marrow smears might change as myelosuppression recovered, there were increased levels of promyelocytes and/or myelocytes and a large number of mature granulocytes in the bone marrow after the administration of rhG-CSF. There was a small increase in WBCs during chemotherapy in all control courses except for case 3, as well as in G-CSF courses. This increase may be mainly due to the effect of prednisolone on leukocyte mobilization from their pool.

In this study, it is not clear whether the increase in granulocytic precursors was only a direct effect of rhG-CSF or whether there was some other indirect effect of rhG-CSF. Similar to the findings *in vitro,* the continuous presence of rhG-CSF seemed necessary to increase or maintain peripheral WBCs, as demonstrated in case 2. Discontinuation of the rhG-CSF administration caused WBC counts to decrease immediately to the base level.

In conclusion, we have shown that the *in vivo* administration of rhG-CSF stimulates the proliferation and differentiation of bone marrow granulocytes and their precursors in a recovery phase from granulocytopenia following cytotoxic chemotherapy.

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