Jpn. J. Cancer Res. 83, 746–753, July 1992

Granulocyte-colony-stimulating Factor Enhances the Circulating Hematopoietic Progenitors in Lung Cancer Patients Treated with Cisplatin-containing Regimens

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A phase II study examining the effects of human recombinant granulocyte-colony-stimulating factor (G-CSF) on the growth of colony-forming unit-granulocyte macrophage (CFU-GM) in the bone marrow and in the peripheral blood was performed in lung cancer patients treated with cisplatin-containing regimens. Treatment with G-CSF following chemotherapy significantly increased the absolute granulocyte count. No significant effect of G-CSF on either the platelet or the red blood cell count was observed. Treatment with G-CSF did not affect the CFU-GM levels in the bone marrow, but did have a significant effect on peripheral blood CFU-GM levels 14 days after initiation of chemotherapy ($P<0.05$). Four patients demonstrated a rebound increase in the level of peripheral blood CFU-GM during the first course of chemotherapy without G-CSF. In contrast, eight patients displayed increase in peripheral blood CFU-GM levels during the second course of chemotherapy with G-CSF treatment. These findings demonstrate that G-CSF is a potent stimulator of granulocyte proliferation as well as a potent agent for promoting transport of hematopoietic progenitors from the bone marrow into the peripheral blood.

Key words: Chemotherapy — Lung cancer — Hematopoietic progenitor — Granulocyte-colony-stimulating factor

We and others have previously shown that patients with lung cancer displayed a "rebound overshoot" phenomenon of the circulating level of peripheral blood hematopoietic progenitor cells (PBHPs).[1,2] These PBHPs may be useful as an alternative source for bone marrow stem cells in lung cancer patients treated with marrow-ablative chemotherapy. Early hematopoietic reconstitution with autotransplantation of PBHPs following myeloablative chemotherapy was observed in one patient with lung cancer.[3] However, this phenomenon appeared to be dependent upon the specific chemotherapeutic regimen used.[2,4] It was observed most frequently in patients who had received combination chemotherapy with cisplatin (CDDP) and etoposide (VP-16). In contrast, it was not well developed in patients receiving chemotherapy with either CDDP, mitomycin (MMC), and vindesine (VDS), or CDDP and MMC, or CDDP alone. Although the reason for this rebound effect remains unclear, the dose, timing, and schedule of drugs administered may be important factors in determining this process.

Recently, several human myeloid colony-stimulating factors (CSFs) have been cloned and sequenced, and their biological activities have been shown to be equivalent to those of their natural counterparts.[5,6] Granulocyte-colony-stimulating factor (G-CSF) has growth and differentiation effects on a population of late progenitor cells in the granulocyte lineage. Granulocyte macrophage (GM)-CSF interacts with cells of an earlier lineage than does G-CSF and is reported to stimulate erythroid and mixed colony formation as well.[7–12] Furthermore, megakaryocytes have been shown to respond to GM-CSF.[13] Interleukin-3 (IL-3) can support the growth of cells from relatively early pluripotent progenitors to mature cells of several lineages. PBHPs are considered to be more primitive than their counterparts in the bone marrow,[14–17] and they have been described in patients with lung cancer as well as in those with leukemia, multiple myeloma, and lymphoma.[18]

The *in vivo* effects of GM-CSF and G-CSF in humans have been previously reported. Phase I and II studies have shown that treatment with G-CSF both reduced the period of neutropenia following cytoreductive chemotherapy and accelerated the recovery from neutropenia.[19,20] Both GM-CSF and G-CSF treatments were also reported to enhance the production of PBHPs when given alone.[21–25] However, it remains unclear whether G-CSF is effective in increasing the levels of PBHPs when used in conjunction with cisplatin-containing regimens for lung cancer. In this study, we report that human recombinant G-CSF can accelerate the recovery from neutropenia, increase the frequency of the "rebound overshoot" phenomenon of PBHPs, and accelerate the time at which the phenomenon occurs following cytoreductive chemotherapy in lung cancer patients as well.

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PATIENTS AND METHODS

Patient Eleven patients with histologically proven lung cancer were entered into the study. Patients were started on the protocol after undergoing the following studies: chest X-ray examination; fiberoptic bronchoscopy with cytologic washing, brushing, and biopsy; bone marrow aspiration and biopsy; bone scan; brain CT scan; and abdominal ultrasound. None of the patients had received previous chemotherapy and/or radiotherapy in the six weeks prior to initiation of chemotherapy. All patients gave informed consent to participate in the study.

Protocol of chemotherapy in combination with G-CSF and timing of sample aspiration for colony-forming unit-granulocyte macrophage (CFU-GM) measurement The details of the second course of chemotherapy with G-CSF and the timing of sample aspiration are shown in Fig. 1. The chemotherapy regimen consisted of CDDP (80 mg/m^2, day 1) and VP-16 (75 mg/m^2/day, days 1–5) for 4 patients and CDDP (80 mg/m^2, day 1), MMC (8 mg/m^2, day 1), and VDS (3 mg/m^2, days 1 and 8) for 7 patients. CDDP was administered with 2000 ml of Ringer's lactate solution and 1000 ml of saline containing mannitol, metoclopramide, dexamethasone and furosemide for 13 h. Each cycle was repeated every 4–5 weeks depending on recovery from myelosuppression induced by the first course of chemotherapy. Five patients were treated every 5 weeks, and three patients were treated every 4 weeks in the study. Human recombinant G-CSF, 75 μg/day (specific activity, 1×10^8 unit/mg; Kirin Brewery Co., Ltd., Tokyo) was administered subcutaneously on days 4–17 during the second course of chemotherapy. Bone marrow specimens were obtained from the anterior iliac crest before and on days 14 and 28 after initiation of chemotherapy. Blood samples were obtained by venipuncture on days 4, 7, 14, 28 and 35 after initiation of chemotherapy. The schedule for the first course of chemotherapy without G-CSF and the timing of sample collection were identical to those shown in Fig. 1 except G-CSF was not administered.

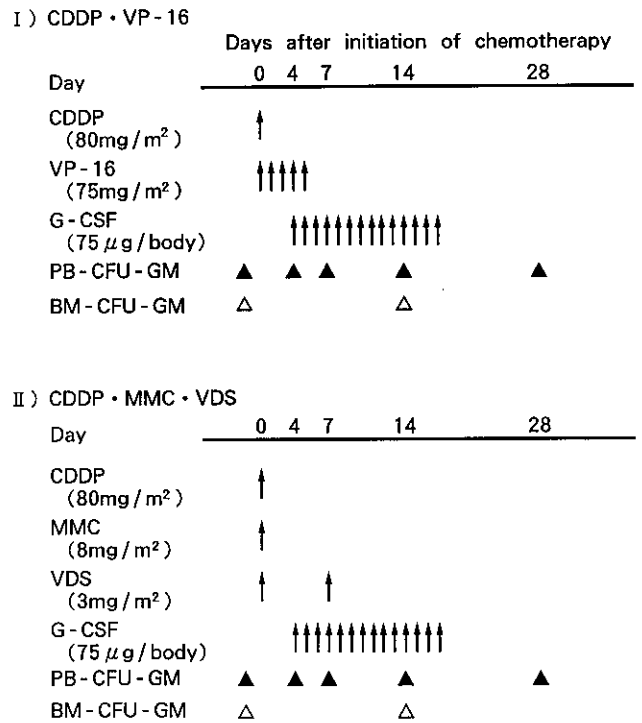

Fig. 1. Chemotherapy regimen (second course) in combination with G-CSF and timing of sample aspiration for CFU-GM measurement in peripheral blood (PB) and in bone marrow (BM). The first course was identical to the second course except that G-CSF was not administered. The intervals between the first and second courses of chemotherapy were 4 to 5 weeks, depending on the recovery from myelosuppression induced by the first course of chemotherapy.

Measurement of CFU-GM The bone marrow aspirate was diluted 5-fold with Dulbecco's minimum essential medium (DMEM), and blood was diluted 1:1 with calcium- and magnesium-free phosphate-buffered saline (PBS). Mononuclear cells were separated by centrifugation using Lymphocyte Separation Medium (LSM; Organon Teknika Co., Durham). For the CFU-GM assay, the mononuclear cells were plated in 35 mm Petri dishes DMEM supplemented with 0.8% methylcellulose, 20% fetal bovine serum (FBS), 1% bovine serum albumin (BSA), and with either 100 U/ml of recombinant human GM-CSF (Genetics Inst., Cambridge), 100 U/ml of recombinant IL-3 (Genetics Inst.), or 1 ng/ml of recombinant G-CSF (Kyowa Co., Tokyo) at 5×10^5 (for peripheral blood) or 1×10^5 (for bone marrow) per plate and cultured in a humidified incubator at 37°C and 5% CO_2. Duplicate cultures were set up, and colonies (>40 cells) were scored under an inverted microscope after incubation for 14 days. The number of CFU-GM was calculated as total number of colonies per ml of blood or per ml of bone marrow specimen.

RESULTS

Eleven patients with lung cancer were enrolled in the study. One patient was not evaluable because of an inability properly to collect blood samples. The characteristics of each patient are presented in Table I. In eight of ten patients, the levels of CFU-GM were measured serially. The kinetics of leukocytes following the G-CSF treatment was remarkably different compared with the case of chemotherapy alone (Fig. 2). The results of the first course of chemotherapy with either CDDP and VP-

Table I. Characteristics of Patients

Eligible cases	11
Evaluable cases	10[a]
Median age (range)	58.5 (46–70)
Sex	
Male	8
Female	2
Histology	
Squamous cell carcinoma	3
Adenocarcinoma	3
Small cell carcinoma	2
Others	2
Clinical stage	
IIIa	4
IIIb	2
IV	4
Chemotherapy	
CDDP+VP-16	4
CDDP+MMC+VDS	6
Assay of CFU-GM	8[b]

a) Ten patients were treated with the first course (without G-CSF) and the second course (with G-CSF) of chemotherapy and the effects of G-CSF on routine hematological examinations were evaluated.

b) CFU-GM assays in bone marrow and in peripheral blood from eight of ten patients were performed serially during the first and second courses of chemotherapy.

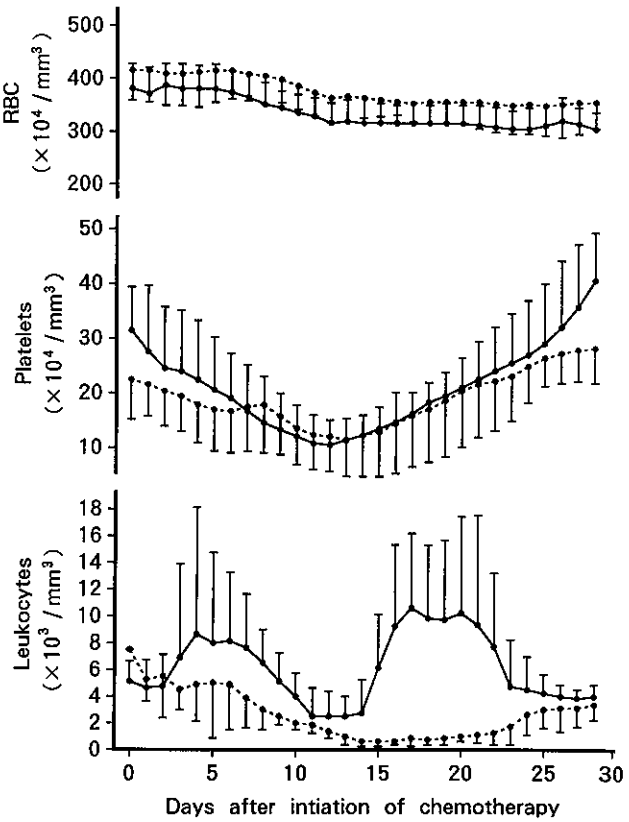

Fig. 2. Effect of G-CSF on the leukocyte, platelet, and RBC counts in ten patients treated with chemotherapy. Control courses (first course; ●····●) include six courses of CDDP, MMC, and VDS and four courses of CDDP and VP-16 without G-CSF in ten patients. During the second course (●—●), G-CSF (75 μg/body/day, days 4–17) was administered along with chemotherapy. Vertical bars indicate the standard error.

16 (4 patients) or CDDP, MMC, and VDS (6 patients) are presented as broken lines. In the first course (chemotherapy alone), the leukocyte count reached a nadir on day 16 and gradually recovered to the pretreatment level on day 28 after initiation of chemotherapy. In contrast the leukocyte count significantly increased following G-CSF treatment (solid line). The first increase was observed one day after initiation of G-CSF (day 5), while the second increase was observed one day after cessation of G-CSF (day 18). The leukocyte count decreased within 4–5 days following cessation of G-CSF. There was no significant effect of G-CSF on either the platelet count or the red blood cell (RBC) count.

The white blood cell differential count from a representative patient is shown in Fig. 3. Only the levels of neutrophils were increased following the administration of G-CSF. The absolute number of lymphocytes or monocytes did not increase significantly. The kinetics of the leukocyte response following a subcutaneous single injection of G-CSF from a representative patient is presented in Fig. 4. The leukocyte count initially decreased within 1 h of G-CSF treatment but then increased, reaching a peak 8 h later. The peak leukocyte count remained stable for the next 24 h.

When mononuclear cells were stimulated with GM-CSF *in vitro*, the average number±standard error of CFU-GM per milliliter of bone marrow in 10 patients with lung cancer was 5074±2413 before chemotherapy, while that of peripheral blood was 12.2±2.8. There was a considerable variation in the CFU-GM levels among individuals before initiation of chemotherapy (data not shown). Two of 10 patient samples were not evaluable for sequential analysis of CFU-GM as one refused bone marrow aspiration, and an error in the CFU-GM assay occurred in the second case. The effects of *in vivo* G-CSF on hematopoietic progenitors of bone marrow and peripheral blood were evaluated for the remaining 8 lung cancer patients. Fig. 5 shows the *in vivo* effect of G-CSF on CFU-GM from the bone marrow in patients treated with myelosuppressive chemotherapy. After administration of chemotherapeutic agents, the levels of CFU-GM

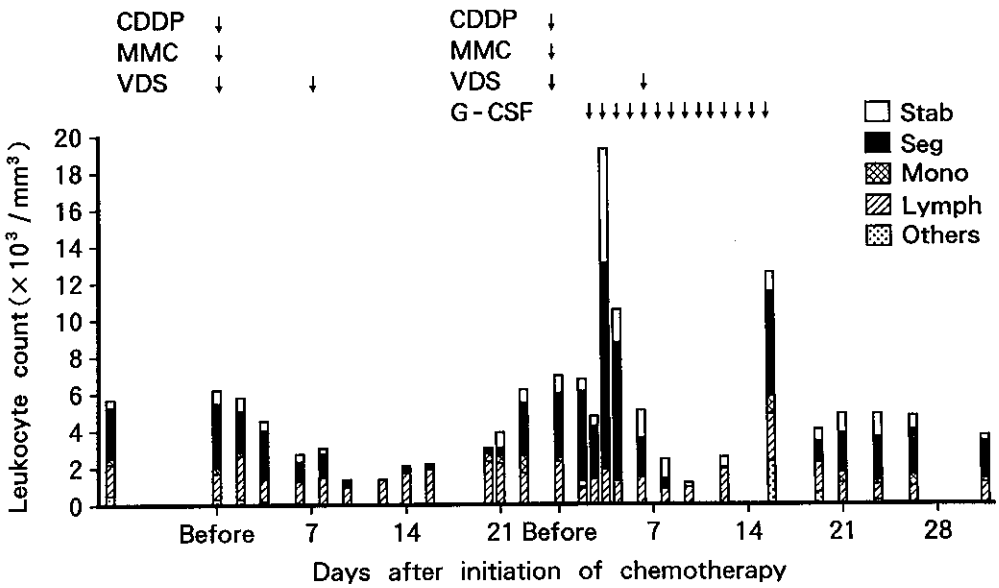

Fig. 3. Effect of G-CSF on the differential count of leukocytes in a representative patient treated with chemotherapy. The chemotherapy regimen of CDDP (80 mg/m^2, day 1), MMC (8 mg/m^2, day 1), and VDS (3 mg/m^2, days 1 and 8) was administered to the patient twice every 4 weeks. During the second course, G-CSF (75 μg/body/day, days 4–17) was administered along with chemotherapy.

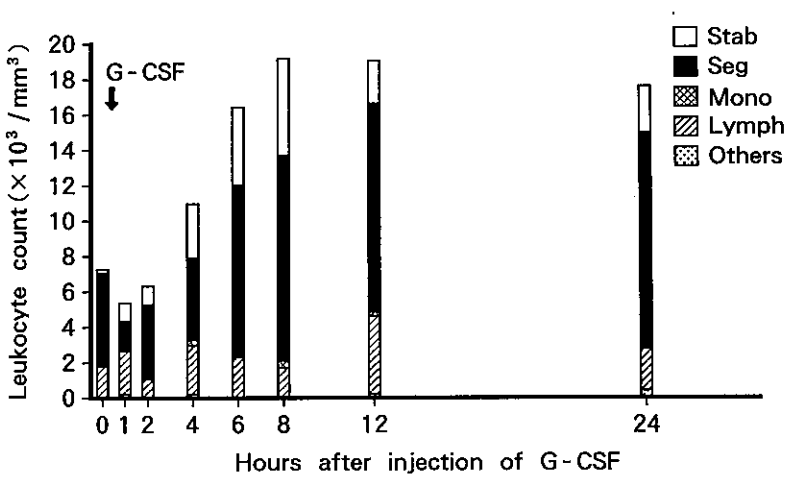

Fig. 4. Effect of a single subcutaneous injection of G-CSF (75 μg/body) on the differential count of leukocytes in a representative patient. The patient had received chemotherapy consisting of CDDP (80 mg/m^2, day 1) and VP-16 (75 mg/m^2, days 1–5) twice. This figure shows the short-term effect of G-CSF on peripheral blood leukocytes on day 4 of the second course of chemotherapy when the administration of G-CSF was initiated.

in the bone marrow decreased markedly 14 days after the initiation of chemotherapy, and they had partially recovered but had not reached the prechemotherapy level when the second course of chemotherapy was started. In the second course of chemotherapy with G-CSF, there was no effect on the CFU-GM in the bone marrow compared with that in the first course of chemotherapy without G-CSF. Fig. 6 shows the *in vivo* effect of G-CSF on CFU-GM from the peripheral blood in patients treated with chemotherapy. During drug administration, the levels of CFU-GM in the peripheral blood were markedly decreased 4 days after, remained low for the next 2 weeks and then displayed a "rebound overshoot" phenomenon 28 days after initiation of the first course of chemotherapy. When the second course started, high levels of CFU-GM in the peripheral blood persisted, but they decreased markedly again 4 days after the initiation of the second course of chemotherapy. Fourteen days of

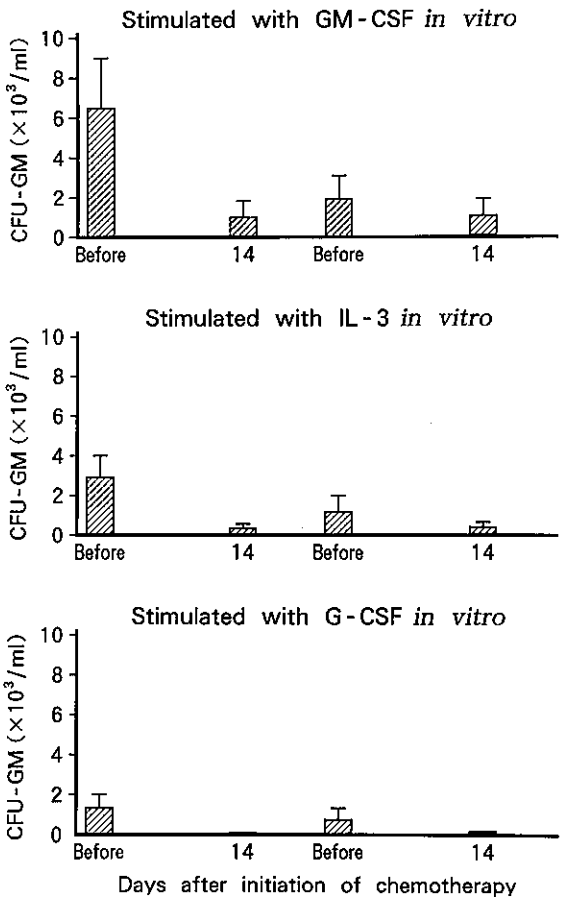

Fig. 5. The kinetics of the CFU-GM response in bone marrow following chemotherapy in the absence or presence of G-CSF in eight patients. The evaluation of bone marrow CFU-GM was done sequentially in four patients treated with CDDP and VP-16 and in four patients treated with CDDP, MMC, and VDS. The details of each chemotherapy regimen are shown in Fig. 1. The first "Before" relates to the level of CFU-GM before the first course of chemotherapy without G-CSF, and the second "Before" relates to the level of CFU-GM just prior to the second course of chemotherapy with G-CSF (28–35 days after initiation of the first chemotherapy). The level of CFU-GM was calculated as the total number of colonies per ml of bone marrow samples in the presence of GM-CSF, IL-3, or G-CSF. Vertical bars indicate the standard error of eight different experiments done in duplicate.

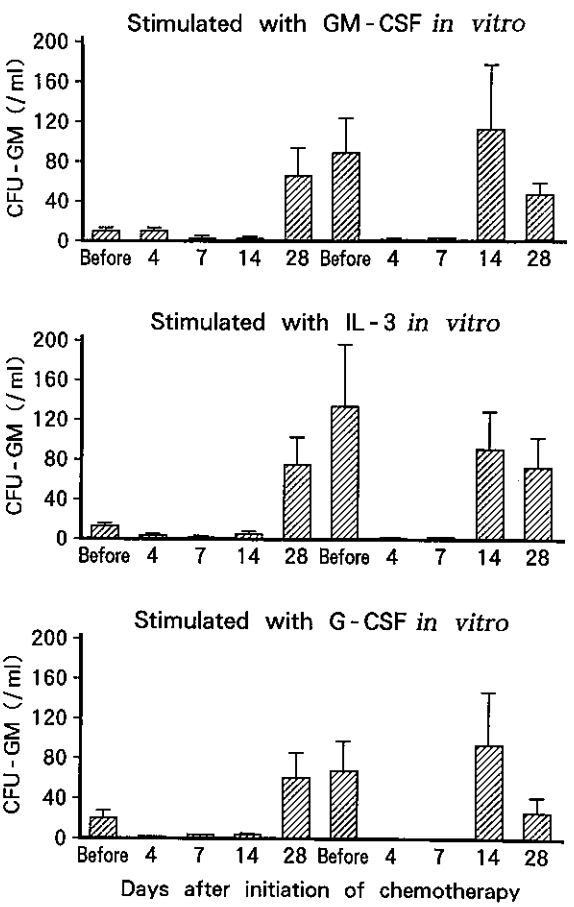

Fig. 6. The kinetics of the CFU-GM response in peripheral blood following chemotherapy in the absence or presence of G-CSF in eight patients. The evaluation of peripheral blood CFU-GM was done sequentially in four patients treated with CDDP and VP-16 and in four patients treated with CDDP, MMC, and VDS. The details of each chemotherapy regimen are shown in Fig. 1. The first "Before" relates to the level of CFU-GM before the first course of chemotherapy without G-CSF, and the second "Before" relates to the level of CFU-GM just prior to the second course of chemotherapy with G-CSF (28–35 days after initiation of the first chemotherapy). The level of CFU-GM was calculated as the total number of colonies per ml of peripheral blood samples in the presence of GM-CSF, IL-3, or G-CSF. Vertical bars indicate the standard error of eight different experiments done in duplicate.

daily administration of G-CSF started 4 days after the initiation of chemotherapy. The levels of CFU-GM in the peripheral blood remained low 7 days after, showed a marked increase 14 days after and then decreased 28 days after the initiation of the second course of chemotherapy. Significant differences of CFU-GM between the first and the second course were seen "Before" ($P<0.05$) and 14 days after the initiation of chemotherapy ($P<0.05$). When the rebound overshoot phenomenon in the blood was defined as a minimum of six-fold increase in the progenitor level over that before chemotherapy, four overshoots (50%) in the first course without G-CSF

Table II. The Frequency of G-CSF-Induced "Rebound Overshoot" Phenomenon of CFU-GM Levels in Peripheral Blood Following Chemotherapy

Chemotherapy regimen	G-CSF (−)	G-CSF (+)
CDDP+VP-16	4/4 (100%)	4/4 (100%)
CDDP+MMC+VDS	0/4 (0%)	4/4 (100%)
Total	4/8 (50%)	8/8 (100%)

The "rebound overshoot" phenomenon in the blood was defined as at least a six-fold increase in the CFU-GM level over that determined prior to chemotherapy.

administration, and eight overshoots (100%) in the second course with G-CSF administration were indicated (Table II).

The toxicities of G-CSF were generally acceptable. Two patients developed mild lumbosacral back discomfort which was controlled with indomethacin on days 13 and 14 after initiation of G-CSF treatment. One patient developed moderate fever on day 7 after initiation of G-CSF treatment.

DISCUSSION

In this study, we examined the *in vivo* effect of G-CSF on hematopoietic progenitor cells as well as on mature blood cells in the peripheral blood and in the bone marrow of lung cancer patients treated with chemotherapy. As has been previously reported by a number of investigators, treatment with G-CSF increases the leukocyte count, specifically granulocytes, and accelerates the recovery from the leukopenia induced by chemotherapy. We have shown that following the initiation of G-CSF administration via the subcutaneous route, peripheral blood leukocytes initially decreased but then increased rapidly over the next 24 h. The early granulocytopenia has previously been reported,[26] and appears to result from an increased adherence of leukocytes to the endothelium. The subsequent granulocytosis appears to result from the release of granulocytes from the marginal pool, such as postcapillary venules. This is in contrast to G-CSF-stimulated granulopoiesis, since the latter requires a longer period of time to induce proliferation and differentiation of hematopoietic stem cells.[27] The second episode of granulocytosis appears to be induced by G-CSF 14 days after initiation of treatment. Our findings indicate that G-CSF was able both to decrease the nadir induced by chemotherapy and to shorten the duration of leukopenia.

Next, we have examined the levels of CFU-GM stimulated *in vitro* by IL-3, GM-CSF, and G-CSF in the bone marrow and in the peripheral blood in order to evaluate the reserve capacity of hematopoiesis. Although the physiological significance of hematopoietic progenitors in the peripheral blood has not yet been clarified, they are thought to be counterparts of hematopoietic progenitors in the bone marrow and indicate bone marrow function.[14-17] Chemotherapy significantly suppressed CFU-GM in both bone marrow and peripheral blood. The suppression of CFU-GM preceded cytopenia of mature granulocytes. The "rebound overshoot" phenomenon of CFU-GM in the peripheral blood occurred at 21–28 days after the initiation of chemotherapy, when the recovery of leukocytopenia occurred, or at the time when the second course of chemotherapy was initiated. On the other hand, no overshoot in the bone marrow appeared at the time when peripheral blood progenitor overshoot was evident. Namely the dissociation of CFU-GM kinetics between bone marrow and peripheral blood was shown during the recovery phase of hemocytopenia induced by chemotherapy.

When G-CSF was administered in combination with anticancer agents at the second course of chemotherapy, earlier recovery and overshoot of CFU-GM in the peripheral blood were noteworthy compared with those during the first course of chemotherapy; however, G-CSF did not affect the level of CFU-GM in the bone marrow. Namely, G-CSF seems to release CFU-GM from the bone marrow to the peripheral blood and seems to play an important role in the differentiation of hematopoietic progenitors to mature granulocytes *in vivo*. Another useful aspect of G-CSF in addition to leukocytosis, is that G-CSF could clinically induce transport of hematopoietic progenitor cells from the bone marrow to the peripheral blood. Recently, much attention has been focused on the PBHPs as an alternative to bone marrow transplantation following ablative chemotherapy.[28-34] The advantages of PBHPs are that they can be obtained without the use of anesthesia and without the discomfort involved in multiple bone marrow aspiration. Moreover, it is conceivable that PBHPs might be less likely to be contaminated with tumor cells. Harvesting of PBHPs is usually performed 3–4 weeks following conventional chemotherapy when the rebound increase in progenitor cells occurs in the peripheral blood. However, these increases depend on the specific chemotherapeutic regimen used. Namely, the regimen of CDDP and VP-16 appears to induce this "rebound overshoot" phenomenon most effectively, in contrast to the regimen of CDDP, VDS, and MMC.[4] When each of these regimens was combined with G-CSF treatment, all patients entered into this study displayed the "rebound overshoot" of progenitor cells in the peripheral blood. We have also established the time when the "rebound overshoot" was evident (i.e., 10 days after initiation of G-CSF treatment) in lung cancer patients treated with cisplatin-containing regimens and G-CSF. These findings suggest

that G-CSF is useful not only in reversing leukocytopenia but also in stimulating the release of progenitor cells from the bone marrow into the peripheral blood.

ACKNOWLEDGMENTS

The authors thank the following physicians for providing materials and clinical information; Drs. K. Asada, S. Ishikawa, W. Ichikawa, A. Okubo, E. Orino, T. Shinohara, A. Shinohara, K. Tani, H. Takehara, T. Takahashi, E. Takeuchi, Y. Nakamura, Y. Nishioka, M. Bando, K. Hiramatsu, K. Maniwa, K. Mizuno, S. Yoshida, A. Yamamoto, and K. Yoneda. The authors are also grateful to Mr. and Mrs. Duht, and Dr. E. Chew for many valuable suggestions, to Kirin Brewery Co. and Kyowahakko Co. for providing recombinant human G-CSF, and to the Genetics Institute for providing recombinant human IL-3 and GM-CSF. This work was supported in part by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science and Culture, Japan.

(Received January 14, 1992/Accepted April 30, 1992)

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