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# Interleukin-6 and Granulocyte Colony-stimulating Factor Synergistically Increase Peripheral Blood Progenitor Cells in Myelosuppressive Mice

Hideki Suzuki,[1] Akira Okano,[1] Chieko Ejima,[1] Atsushi Konishi,[1] Yukio Akiyama,[1,3] Keiya Ozawa[2,4] and Shigetaka Asano[2]

[1]*Central Research Laboratories, Ajinomoto Co., Inc., 214 Maeda-cho, Totsuka-ku, Yokohama 244 and* [2]*Department of Hematology/Oncology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108*

**We previously reported a successful peripheral blood stem cell harvest by co-administration of recombinant human (rh) interleukin-6 (IL-6) and rh granulocyte colony-stimulating factor (G-CSF) in normal mice. In the present study, to evaluate further the utility of this observation for autologous peripheral blood stem cell transplantation, we examined the effects of rhIL-6 and rhG-CSF on peripheral blood granulocyte-macrophage colony-forming units (CFU-GM) in carboplatin (CBDCA)-induced and irradiation-induced myelosuppressive mouse models. After CBDCA administration, blood cell counts decreased to the nadir, and then recovered to a normal level. In this recovery phase, the peripheral CFU-GM level increased to 3.8-fold higher than the pretreatment level. Administration of rhIL-6 (10 μg/day) alone induced a 40-fold increase in peripheral CFU-GM from the normal level at day 14. In combination with rhG-CSF (0.35 μg/day), which alone induced a 74-fold increase, rhIL-6 synergistically increased the CFU-GM level by 1200-fold. In irradiated mice, similar results were observed. Administration of rhIL-6 at 3 and 10 μg/day significantly increased CFU-GM. Interestingly, in combination with rhG-CSF, a lower dose of rhIL-6 (1 μg/day) could induce CFU-GM increase. We also examined CFU-GM distribution in bone marrow, spleen and peripheral blood. Cytokine administration induced not only a change of CFU-GM distribution, but also an increase in total CFU-GM counts per mouse. These results suggest that co-administration of rhIL-6 and rhG-CSF may be useful for autologous peripheral blood stem cell transplantation.**

Key words: IL-6 — G-CSF — Myelosuppressive mice — CFU-GM — Autologous PBSCT

Autologous peripheral blood stem cell (PBSC) transplantation has been widely used for the treatment of leukemia, lymphoma and other solid tumors. Some reports have indicated that the number of PBSC increases transiently following chemotherapy and/or administration of cytokines such as recombinant human (rh) granulocyte colony-stimulating factor (G-CSF).[1-5] However, more effective methods to increase PBSC have been sought. We recently demonstrated that the co-administration in normal mice of rh interleukin (IL)-6 and rhG-CSF synergistically increases PBSC, which can rescue lethally irradiated mice.[6] This suggests the benefit of rhIL-6 and rhG-CSF for PBSC harvest. Although the effect of rhG-CSF has already been confirmed clinically,[1-4] the value of rhIL-6 for PBSC harvest following myelosuppressive treatment is not confirmed. In the present study, we examined the effects of rhIL-6 and rhG-CSF on hematopoietic progenitor cells (CFU-GM, granulocyte-macrophage colony-forming units) and evaluated their benefit for PBSC harvest in carboplatin (CBDCA)-induced and irradiation-induced myelosuppressive mouse models.

## MATERIALS AND METHODS

**Mice** Female C57BL/6 mice were purchased from Charles River Japan (Kanagawa). All mice were used between the ages of 8 to 10 weeks.

**Experimental protocol** In the CBDCA-induced myelosuppressive system, CBDCA (50 mg/kg/day; Paraplatin, Bristol-Myers Squibb, Tokyo) was administered by intravenous injection at days (−6), (−3), and (−1). At day 0, mini-osmotic pumps (Alzet, Palo Alto, CA; Model 2002) with a delivery rate of 0.5 μl/h for 14 days were filled with the desired cytokine solution and implanted subcutaneously on the dorsal side of the mice. Cytokines (rhIL-6, prepared in our laboratory as previously described[7]; rhG-CSF, lenograstim, Chugai Pharmaceutical, Tokyo) were diluted in phosphate-buffered saline containing 1% (v/v) syngeneic mouse serum. Each cytokine was used at the dose that was reported to be optimal (rhIL-6, 10 μg/day; rhG-CSF, 0.35 μg/day).[8,9] In control mice, human serum albumin (HSA) was administered instead of cytokines at 10 μg/day. In the irradiation-induced myelosuppressive system, mice were

[3] To whom correspondence should be addressed.

[4] Present address: Department of Molecular Biology, Institute of Hematology, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Tochigi 329-04.

irradiated with 4.0 Gy X-rays at day (−1). Cytokines were subcutaneously administered for 14 days (from day 0) as described above.

**Peripheral blood cell count** Peripheral blood was obtained by intracardiac puncture and cell counts were performed on a Sysmex K-1000 (Toa Medical Electronics Co., Hyogo).

**Preparation of cell suspensions** Blood from each group of 4 mice was obtained by intracardiac puncture and pooled. Blood samples were separated using Lympholyte-M (Cedarlane Laboratories Ltd., Ontario, Canada), and the interphase cells were harvested as peripheral blood mononuclear cells. Bone marrow cells were procured by flushing the femur with Iscove's modified Dulbecco's medium (Gibco BRL, Life Technologies, Inc., Grand Island, NY) containing 5% fetal bovine serum (Sanko Junyaku, Tokyo). Single cell suspensions of the spleen were prepared by mashing the organ.

**Assay for CFU-GM** CFU-GM was assayed using a conventional methylcellulose culture system as described previously.[10] In brief, cells were suspended in 1 ml of Iscove's modified Dulbecco's medium containing 0.8% (w/v) methylcellulose 4000 (Nacalai Tesque, Inc., Kyoto), 30% fetal bovine serum, 0.2 m*M* hemin (Sigma Chemical Co., St. Louis, MO), 1% deionized bovine serum albumin (Armour Pharmaceutical Co., Phoenix, AZ), 200 U/ml murine IL-3,[10] and 2 U/ml rh erythropoietin (epoetin beta, Chugai) and plated in 35-mm Petri dishes (Falcon 1008; Becton Dickinson Labware, Lincoln Park, NJ). Dishes were incubated at 37°C in humidified 5% $CO_2$/air. After 7 days of incubation, all granulocyte/macrophage colonies were scored as CFU-GM using an inverted microscope. After 15 days of incubation, all granulocyte/erythroid/macrophage/megakaryocyte colonies were scored as CFU-GEMM.

**Statistical analysis** Differences were evaluated using Student's *t* test, and *P* values of less than 0.05 were considered significant.

## RESULTS

**Synergistic increase in peripheral CFU-GM by rhIL-6 and rhG-CSF in CBDCA-administered mice** CBDCA was administered three times, followed by administration of rhIL-6 and/or rhG-CSF for 14 days. CBDCA administration caused the WBC, RBC and platelet counts to decrease to the nadir at day 7, and they subsequently recovered to the normal levels in HSA-administered mice (Fig. 1). During the hematopoietic recovery phase from the nadir, the peripheral CFU-GM increased markedly: 3.8-fold (140±45/ml blood) higher than the normal level (36±23/ml blood; Fig. 2). Co-administration synergistically increased the peripheral CFU-GM level as compared with administration of rhIL-6 alone or rhG-CSF alone. Administration of rhIL-6 alone showed a 40-fold increase in CFU-GM (1,400±58/ml blood) from the normal level at day 14. In combination with rhG-CSF, which alone induced a 74-fold increase (2,700±58/ml blood), rhIL-6 synergistically increased the CFU-GM level by 1200-fold (43,000±9,200/ml blood), and the level returned to normal after cessation. Similar syn-

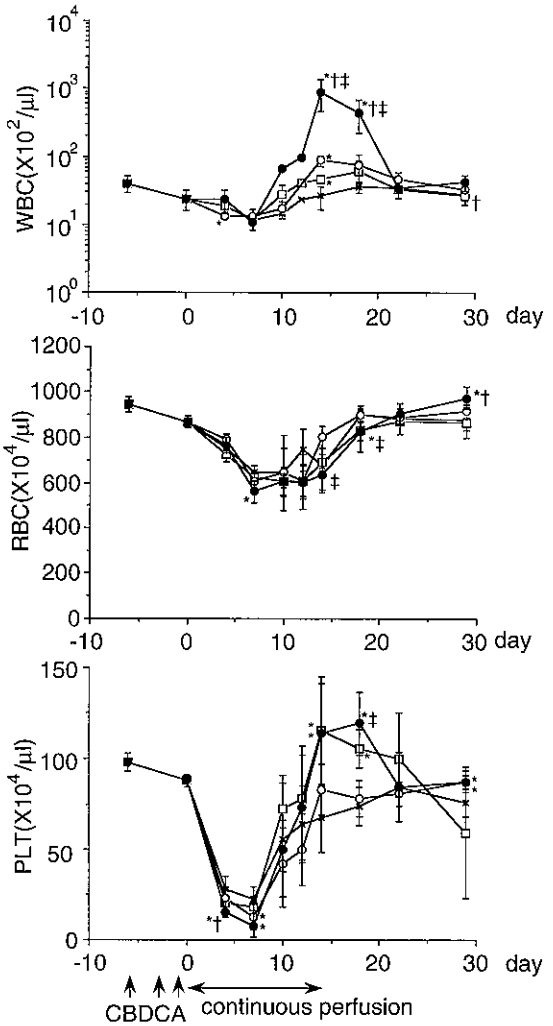


Fig. 1. Changes of blood cell counts by administration of rhIL-6 and/or rhG-CSF in CBDCA-administered mice. CBDCA (50 mg/kg/day) was administered at days (−6), (−3), and (−1), and rhIL-6 (10 μg/day) alone (□), rhG-CSF (0.35 μg/day) alone (○), or both rhIL-6 and rhG-CSF (●) were administered for 14 days, as indicated by arrows. HSA (10 μg/day) (×) was used as a control. Results represent mean±SD of 4 mice. Differences were evaluated using Student's *t* test compared with mice administered HSA (*), mice administered rhIL-6 alone (†), or mice administered rhG-CSF alone (‡), $P<0.05$.

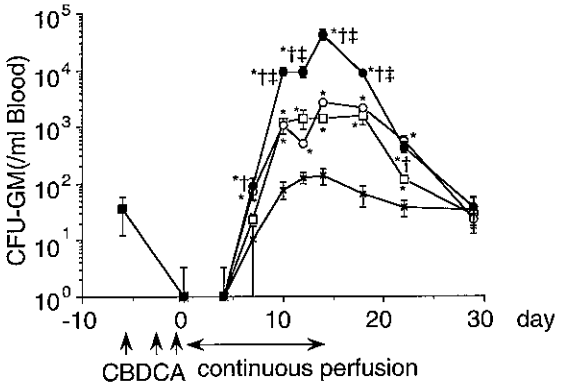


Fig. 2. Changes of peripheral CFU-GM by administration of rhIL-6 and/or rhG-CSF in CBDCA-administered mice. CBDCA (50 mg/kg/day) was administered at days (−6), (−3), and (−1), and rhIL-6 (10 μg/day) alone (□), rhG-CSF (0.35 μg/day) alone (○), or both rhIL-6 and rhG-CSF (●) were administered for 14 days, as indicated by arrows. HSA (10 μg/day) (×) was used as a control. Peripheral blood mononuclear cells were pooled (4 mice per treatment group) and assayed for CFU-GM in three dishes. Results represent mean±SD of CFU-GM per milliliter of blood. Differences were evaluated using Student's *t* test compared with mice administered HSA (*), mice administered rhIL-6 alone (†), or mice administered rhG-CSF alone (‡), $P<0.05$.

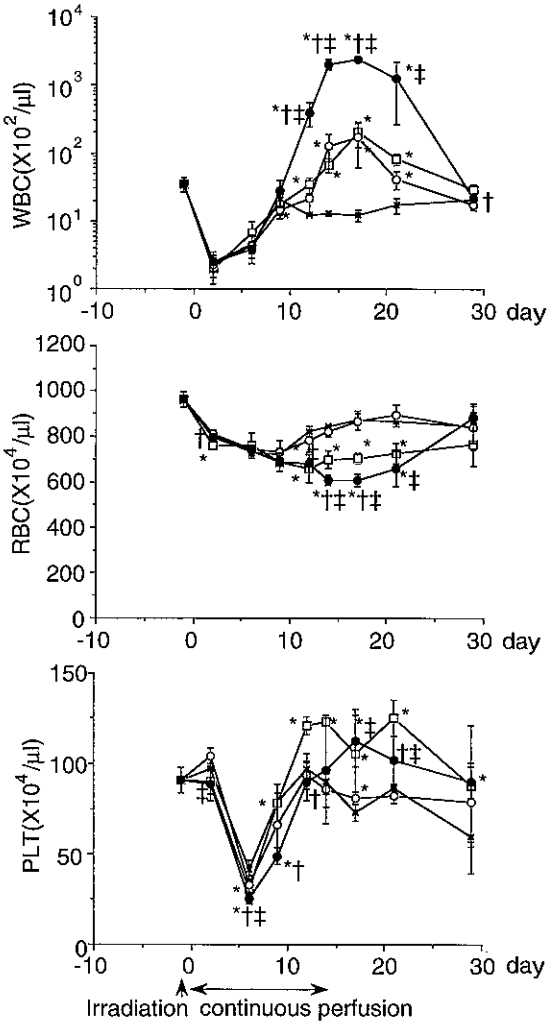


Fig. 3. Changes of blood cell counts by administration of rhIL-6 and/or rhG-CSF in irradiated mice. Mice were irradiated with 4.0 Gy of X-rays at day (−1), and rhIL-6 (10 μg/day) alone (□), rhG-CSF (0.35 μg/day) alone (○), or both rhIL-6 and rhG-CSF (●) were administered for 14 days, as indicated by arrows. HSA (10 μg/day) (×) was used as a control. Results represent mean±SD of 4 mice. Differences were evaluated using Student's *t* test compared with mice administered HSA (*), mice administered rhIL-6 alone (†), or mice administered rhG-CSF alone (‡), $P<0.05$.

ergistic increases were observed in peripheral CFU-GEMM (data not shown). Co-administration of rhIL-6 and rhG-CSF caused the WBC counts to recover synergistically and then to increase beyond the normal level (Fig. 1). In RBC recovery, there was no difference between any of the groups (Fig. 1). As regards platelet counts, administration of rhIL-6 alone or in combination with rhG-CSF stimulated recovery and increase from the nadir (Fig. 1).

**Synergistic increase in peripheral CFU-GM by rhIL-6 and rhG-CSF in irradiated mice** Mice were irradiated, followed by administration of rhIL-6 and/or rhG-CSF for 14 days. Compared with CBDCA-administered mice (the nadir of WBC was $12\pm1.8\times10^2/\mu l$ at day 7; Fig. 1), severe WBC depression was observed (the nadir of WBC was $2.5\pm0.6\times10^2/\mu l$ at day 2; Fig. 3). A higher peripheral CFU-GM rebound was thus seen (Fig. 4), and the maximum value increased to 8.6-fold (310±35/ml blood) higher than the normal level at day 9. In the case of co-administration of rhIL-6 and rhG-CSF, WBC counts and peripheral CFU-GM synergistically recovered and increased beyond the normal level. Administration of rhIL-6 alone induced a 94-fold increase in the peripheral CFU-GM level (3,400±460/ml blood) from the normal level, at day 14. In combination with rhG-CSF, which alone induced a 110-fold increase (4,000±590/ml blood), rhIL-6 synergistically increased the peripheral CFU-GM level by 1400-fold (49,000±20,000/ml blood). We next studied the effects of various doses of rhIL-6. Groups of 4 mice were irradiated, and 0.1 to 10 μg/day of rhIL-6 was administered alone or in combination with rhG-CSF for 14 days. Table I shows the peripheral CFU-GM level at day 14. Administration of rhIL-6

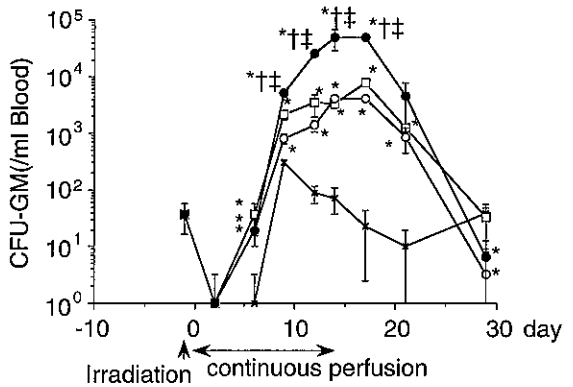


Fig. 4. Changes of peripheral CFU-GM by administration of rhIL-6 and/or rhG-CSF in irradiated mice. Mice were irradiated with 4.0 Gy of X-rays at day (−1), and rhIL-6 (10 μg/day) alone (□), rhG-CSF (0.35 μg/day) alone (○), or both rhIL-6 and rhG-CSF (●) were administered for 14 days, as indicated by arrows. HSA (10 μg/day) (×) was used as a control. Peripheral blood mononuclear cells were pooled (4 mice per treatment group) and assayed for CFU-GM in three dishes. Results represent mean±SD of CFU-GM per milliliter of blood. Differences were evaluated using Student's *t* test compared with mice administered HSA (*), mice administered rhIL-6 alone (†), or mice administered rhG-CSF alone (‡), $P<0.05$.

alone at 3 and 10 μg/day significantly increased CFU-GM. Interestingly, in combination with rhG-CSF, a lower dose of rhIL-6 (1 μg/day) could induce a CFU-GM increase.

**Change of CFU-GM distribution by co-administration of rhIL-6 and rhG-CSF** Using the CBDCA-induced and irradiation-induced myelosuppressive models, we examined CFU-GM distribution in bone marrow, spleen and peripheral blood at day 14 (Fig. 5). Administration of rhIL-6 and/or rhG-CSF induced not only a change of CFU-GM distribution, but also an increase in total CFU-GM counts per mouse. As described above, the peripheral CFU-GM level was strikingly increased by co-administration. The CFU-GM level in the spleen was also increased by administration of rhIL-6 alone or rhG-CSF alone, and increased more upon co-administration of the two. In contrast, the CFU-GM level in the bone marrow was increased only in mice administered rhIL-6 alone, and was not increased in mice administered rhG-CSF alone or in combination with rhIL-6. Similar results were observed in the irradiation-induced myelosuppressive model.

Table I. Dose-response Study of rhIL-6 Alone or in Combination with rhG-CSF in Irradiated Mice[a)]

| IL-6 (μg/day) | CFU-GM (/ml Blood) | |
|---|---|---|
| | (−) G-CSF | (+) G-CSF |
| 0 | 70±30 | 4,100±2,078 |
| 0.1 | 90±46 | 5,967±2,899 |
| 0.3 | 127±23 | 5,900±2,095 |
| 1 | 130±50 | 12,533±3,331‡ |
| 3 | 240±53* | 52,533±6,278‡ |
| 10 | 4,933±1,665* | 69,000±14,526‡ |

*a*) Mice were irradiated with 4.0 Gy of X-rays at day (−1), and rhIL-6 (0.1 to 10 μg/day) alone or in combination with rhG-CSF (0.35 μg/day) was administered for 14 days (from day 0). HSA (10 μg/day) was used as a control. At day 14, peripheral blood mononuclear cells were pooled (4 mice per treatment group) and assayed for CFU-GM in three dishes. Results represent mean±SD of CFU-GM per milliliter of blood. Differences were evaluated using Student's *t* test compared with mice administered HSA (*) or mice administered rhG-CSF alone (‡), $P<0.05$.

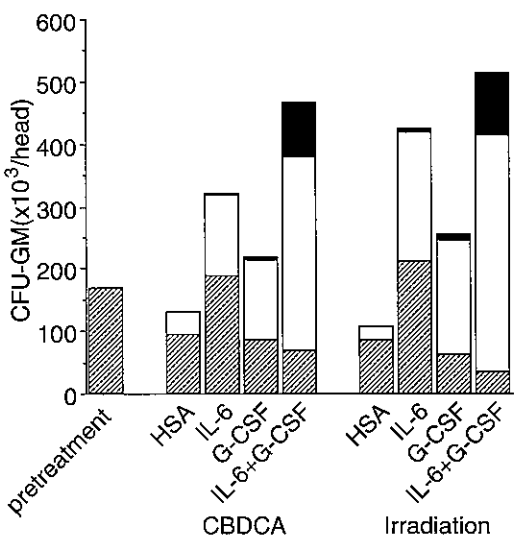


Fig. 5. Effects of rhIL-6 and rhG-CSF on CFU-GM distribution. CBDCA (50 mg/kg/day) was administered at days (−6), (−3), (−1), or X-rays (4.0 Gy) were applied at day (−1). RhIL-6 (10 μg/day) and/or rhG-CSF (0.35 μg/day) were administered for 14 days (from day 0). HSA (10 μg/day) was used as a control. At day 14, bone marrow, spleen and peripheral blood mononuclear cells were pooled (4 mice per treatment group) and assayed for CFU-GM in three dishes. Results represent mean CFU-GM in bone marrow (▨), spleen (□) and peripheral blood (■) per mouse. The number of CFU-GM in bone marrow per mouse is calculated based on one femur representing 5% of total bone marrow. The number of CFU-GM in peripheral blood is based on a blood volume of 2.0 ml per mouse.

## DISCUSSION

Co-administration of rhIL-6 and rhG-CSF has been suggested as a useful PBSC collection model.[6] However, in considering PBSC harvests following myelosuppressive treatment from patients themselves, we should take note of some differences between normal and myelosuppressed hematopoiesis. 1) Hematopoietic stem cells in bone marrow are decreased by cytotoxic chemotherapy and/or irradiation. 2) Levels of endogenous cytokines are elevated after myelosuppression.[11–14] In the present study, we demonstrated that co-administration of rhIL-6 and rhG-CSF synergistically recovered and increased peripheral CFU-GM in CBDCA-induced and irradiation-induced myelosuppressive mice.

During the hematopoietic recovery phase from the nadir, peripheral CFU-GM increased to 3.8- (CBDCA-administered mice) or 8.6-fold (irradiated mice) higher than the normal level, even in HSA-administered mice. We compared the maximum values of peripheral CFU-GM of the present study with those for normal mice administered rhIL-6 and/or rhG-CSF, which were previously reported.[6] Between the same cytokine-administered groups (for example; normal mice administered rhIL-6 alone and CBDCA-treated mice administered rhIL-6 alone), the CFU-GM level in the myelosuppressive mice reached 2.5- to 10-fold higher than the level in the normal mice. Many cytokines have recently been reported as inducers of PBSC: IL-1,[15] IL-2,[16] IL-3,[17–19] IL-7,[20] IL-8,[21] IL-11,[22] IL-12,[23] granulocyte-macrophage CSF,[17,24–27] stem cell factor,[28,29] and Flt-3 ligand.[30] These findings suggest that some of these factors may be produced endogenously during the recovery phase. In normal mice, 10 $\mu$g/day of rhIL-6 alone or in combination with rhG-CSF induced peripheral CFU-GM[6]; however, 1 $\mu$g/day of rhIL-6 had no effect on peripheral CFU-GM (unpublished data). In irradiated mice, administration of rhIL-6 alone at 3 and 10 $\mu$g/day significantly increased CFU-GM. Interestingly, in combination with rhG-CSF, a lower dose of rhIL-6 (1 $\mu$g/day) could induce CFU-GM increase. Further, 3 $\mu$g/day of rhIL-6 showed a synergistic effect, but 10 $\mu$g/day did not, resulting in a plateau in logarithmic analysis. These results suggest that co-administration of rhIL-6 and rhG-CSF might allow the use of lower doses of rhIL-6, especially in PBSC collection for autologous transplantation.

The reasons why chemotherapy induces an increase of PBSC are still not clear. Two possible mechanisms have been proposed: (a) endogenous cytokines stimulate proliferation of stem cells in bone marrow, and/or (b) they stimulate mobilization of stem cells from bone marrow to peripheral blood. In the present studies of CFU-GM distribution in bone marrow, spleen and peripheral blood, CFU-GM in bone marrow was increased only in mice administered rhIL-6 alone (Fig. 5). This result suggests that rhIL-6, compared to rhG-CSF, expands the stem cell pool in bone marrow, and that co-administration of both cytokines stimulates mobilization of increased stem cells from bone marrow to peripheral blood.

Although further studies are necessary before clinical use, co-administration of rhIL-6 and rhG-CSF may be useful not only for allogeneic PBSC transplantation, but also for autologous PBSC transplantation. A primate study on the safety and efficacy of co-administration of rhIL-6 and rhG-CSF is in progress.

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