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Leucostim, A Human Granulocyte-Colony Stimulating Factor, Facilitates Granulopoiesis After Bone Marrow Transplantation In Mice

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ABSTRACT – *In vivo* administration of Leucostim, a human recombinant granulocyte colony-stimulating factor (G-CSF), was evaluated for the effects on survival, hematologic recovery, and colony forming unit-spleen (CFU-s) in murine bone marrow transplantation (BMT) model. Sublethally irradiated (9 Gy) mice received bone marrow cells from untreated mice, and then were treated with G-CSF subcutaneously at doses of 2.5, 5, or 10 μg/kg or vehicle solution (control) for 14 days from one day after BMT. There was no effect of irradiation and BMT on mortality. The repeated subcutaneous injections of Leucostim for 14 days post-BMT significantly facilitated hematologic recovery compared with vehicle control in a dose-dependent manner. Moreover, mice treated with Leucostim had significantly increased numbers of CFU-s colonies on day 10 post-BMT. These results suggest that Leucostim, a new G-CSF, has beneficial effects on hematologic reconstitution after BMT.

Key words □ Leucostim, G-CSF, Bone marrow transplantation, Irradiation, Mice

The prognosis of patients undergoing bone marrow transplantation (BMT) is critically dependent on the reconstitution of the hematopoietic system. The recovery of immunity after marrow transplantation is a complicated procedure dependent on pre- and post-transplant factors (Lum, 1990; Lenarski, 1993). Bone marrow transplantation therapy involves ablation of the existing immunohematopoietic marrow elements. This intensive therapy creates a prolonged period of immune deficiency. Hematopoietic growth factors are now clinically used in an effort to speed up the recovery of the hematopoietic system, decrease infectious complications (Advani *et al.*, 1992), shorten the length of hospitalization (Linch *et al.*, 1993) and decrease costs (Brandt *et al.*, 1988).

Granulocyte colony-stimulating factor (G-CSF) is one of the hematopoietic growth factors that regulates the proliferation and differentiation of bone marrow progenitor cell populations (Clark and Kamen, 1987; Griffin, 1988). The rationale for the use of G-CSF in the treatment of cancer patients is that 1) chemotherapy or radiotherapy usually causes neutropenia, which results in patients' morbidity and mortality due to bacterial and fungal infection, 2) frequently, the dose of chemotherapy must be reduced due to the myelosuppressive toxicity of anticancer agents, which in turn is thought to impair the antitumor response of effective therapeutic regimens.

Leucostim® is a recently developed recombinant human G-CSF. Its favorable effect on the recovery of granulopoiesis was reported in chemotherapy-induced neutropenic mice (Park *et al.*, 1994). This study was designed to examine the effect of Leucostim on the number of peripheral leukocytes after BMT in sublethally irradiated mice.

MATERIALS AND METHODS

Test material

Leucostim® (code name : DA-3030, lot CTS-9701) was obtained from the Research Laboratories of Dong-A Pharmaceutical Company (Kyunggido, Korea). Leucostim was produced by recombination technology. In brief, after fermentation of the *E. coli* containing human G-CSF cDNA, the cell was disrupted by freezing and thawing with liquid nitrogen. The inclusion body containing G-CSF was separated and solubilized. After removing the host-derived proteins by precipitation, the supernatant was purified by chromatography to obtain recombinant human G-CSF. As a reference, Grasin® (filgrastim, Jeil Pharm., Korea) was used.

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For administration, Leucostim was dissolved in buffer solution (pH 4) containing 10 mM sodium acetate, 5% mannitol, and 0.004% tween 80.

Mice

All experimental procedures were performed in accordance with the institutional guideline "Procedure for animal care and experiment (SOP-ANC)" of Dong-A Pharmaceutical Company. Six-week old male C3H/He mice (B & K, USA) weighing 22 to 25 g, were used after 7-day acclimation. They were housed in standard polycarbonate cages under controlled temperature (23-25 °C) and light (lights on from 08:00 to 20:00). Food (Cheiljedang, Korea) and water were freely available during the experiment.

Bone marrow transplantation

Recipient C3H/He mice were exposed to ^{137}Cs irradiation source using CLINAC 2100C/D (6Mev, Varian Co., CA, USA). The mice received 9 Gy of total irradiation (0.9 Gy/min). Mice then received 2×10^6 syngeneic bone marrow cells (BMC) intravenously.

Test procedure

After BMT, the recipient mice were administered subcutaneously with Leucostim at doses of 2.5, 5, or 10 μg/head daily for 14 days. The vehicle solution for Leucostim or Grasin (5 μg/head) was used under the same conditions in control groups. Untreated C3H/He mice (n=8) were also employed as a control. For the analysis of peripheral blood (PB), blood was collected from the orbital sinus of the mice on days -1, 1, 7, 10, 14, 18, and 21 after BMT. Total number of leukocytes was counted with an automatic cell counter (Minos Vet, Roche). Colony forming unit-spleen (CFU-s) cells were also counted on day 10 after BMT as described by McCulloch *et al.* (1970). Briefly, ten days after irradiation and BMT, the mice were sacrificed by ether anesthesia, and then spleens were removed. After fixation with Bouin's solution for 5 days, the number of CFU-s colonies forming on the spleen surface was counted using a dissecting microscope (SZH, Olympus, Japan). At least 8 animals were used for each test condition.

Statistical analysis

Data were analyzed by ANOVA and Dunnett's test, where a $P<0.01$ value was considered to be significant. Sigmastat® was used for the analysis. Data were presented as the mean and S.E.M.

RESULTS

Effect of G-CSF on peripheral leukocyte count after syngeneic BMT

Fig. 1. shows the effect of G-CSF on the number of peripheral leukocyte in mice after whole-body irradiation (^{137}Cs; 9 Gy) and subsequent syngeneic BMT. Twenty-four hours after BMT, mice were given an injection of G-CSF once a day for 14 days. A marked reduction in the total number of leukocytes was observed 1 day after irradiation, and the nadir was observed on day 7 (7 days after irradiation) in all irradiated mice. Subsequently, the number of leukocytes increased gradually and reached normal range within 21 days in control irradiated mice. However, repeated subcutaneous administration with G-CSF dose-dependently accelerated the recovery of the total number of leukocytes. Middle (5 μg/head/day) and high dose (10 μg/head/day) Leucostim significantly increased the total number of leukocytes on days 10, 14 and 18 when compared with that of control irradiated group ($p<0.001$). In these groups, the number of leukocytes peaked on day 14, which was significantly higher than that in untreated normal group ($p<0.001$) and then gradually decreased to normal range. During the experiment, the number of total leukocytes in untreated control mice were within the normal range (4.9 to 5.9×10^3/

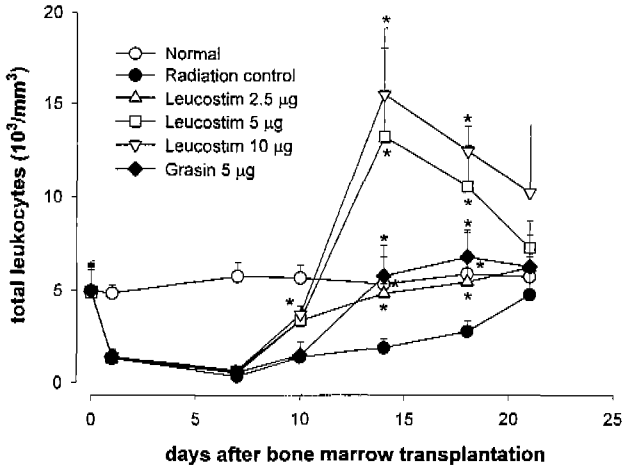

Fig. 1. Effect of Leucostim on the recovery of peripheral leukocyte counts in mice after bone marrow transplantation. Male C3H/He mice received 9 Gy whole body irradiation and 2×10^6 syngeneic bone marrow cells. Leucostim at doses of 0 (●), 2.5 μg (△), 5 μg (□) or 10 μg (▽), or Grasin at a dose of 5 μg (◆) was subcutaneously administered for 14 days. Each value is the mean ± S.E. (n=8). The hematopoietic reconstitution was more rapid in Leucostim- or Grasin-treated mice than in control irradiated mice. *Significantly different from control irradiated group at $p<0.001$.

Table I. Effect of Leucostim on CFU-s formation in mice after bone marrow transplantation

Group	Untreated control	Irradiated control	Leucostim 2.5 μg	Leucostim 5 μg	Leucostim 10 μg	Grasin 5 μg
No. of CFU-s	0.0	15.5 ± 2.9	32.8 ± 3.2*	37.0 ± 4.6*	42.7 ± 3.6*	29.7 ± 5.7*

After whole body irradiation (9 Gy) and bone marrow transplantation (2×10^6 syngeneic bone marrow cells, iv), Leucostim or Grasin was injected subcutaneously for 10 consecutive days. The number of CFU-s colonies on the surface of spleen was counted 10 days after marrow transplantation. Each value represents mean ± S.E.
*Significantly different from control irradiated group ($p<0.001$).

mm^3). Grasin (5 μg/head/day) and low dose (2.5 μg/head/day) of Leucostim elicited a similar recovery pattern in the number of total leukocytes, which returned to normal level on day 14.

Effect of G-CSF on CFU-s colonization after syngeneic BMT

Table I shows the effect of G-CSF on the number of CFU-s colonies in mice receiving WBI (^{137}Cs; 9 Gy) and syngeneic BMT (2×10^6 cells, iv). On day 10, the mice from control irradiated group showed 15.5 ± 2.88 CFU-s colonies. All the animals treated with Leucostim for 10 consecutive days showed a significant increase in the number of CFU-s colonies in a dose-dependent manner ($p<0.001$). The mice treated with Grasin (5 μg/head/day) under the same condition also showed a significant increase in the number of CFU-s colony when compared with that of control irradiated animals ($p<0.001$).

DISCUSSION

Bone marrow transplantation (BMT) is a procedure, in which the patient receives healthy bone marrow harvested from a genetically-matched donor or the patient's own marrow. Candidates for BMT include patients with severe aplastic anemia, specific immune deficiency states, certain leukemias, resistant forms of lymphoma, and advanced or resistant solid tumors. Between 1985 and 1990 the annual volume of autologous BMT in the United States rose by more than 600 percent, from less than 1,000 transplants per year to more than 6,000 (Rowlings *et al.*, 1999).

Following the BMT procedure, patients have reduced white blood cell counts, which puts them at a great risk for infectious diseases, including pneumonia and septicemia. In order to reduce the period showing low leukocyte counts and the infection rate, the use of hematopoietic growth factors, such as granulocyte colony-stimulating factor (G-CSF) are now on increase (Souetre *et al.*, 1996; Moreau *et al.*, 1994).

Leucostim is a recombinant human G-CSF recently developed by Dong-A Pharmaceutical Company. We previously observed that the recovery of leukopenia in mice receiving myeloablating chemotherapy could be facilitated by treatment with Leucostim (Park *et al.*, 1994). The present study was conducted to investigate the effect of Leucostim on the hematopoietic recovery after BMT in mice. The leukocyte-increasing effect of G-CSF was considered to reflect the increase in neutrophil counts and neutrophil granulopoiesis (Okabe *et al.*, 1990). Namely, no significant changes in other blood cells, such as erythrocyte, lymphocyte or monocyte counts were observed during the treatment of G-CSF, although neutrophil counts showed a significant increase. In this regard, total leukocyte counts instead of differential blood cell counts have been measured as an index of pharmacological effect of G-CSF. In this study, treatment with G-CSFs (both Leucostim and Grasin) did not prevent the leukopenia determined 7 days after irradiation (Fig. 1). However, the recovery to the normal values of total leukocytes, monitored from 10 days after irradiation, was more rapid in G-CSF-treated mice than in untreated animals. This result indicates that it takes about 10 days to observe the apparent increase in peripheral leukocytes by repeated subcutaneous injection with G-CSF in the present test condition. Because the number of CFU-s colonies, examined 10 days after BMT, was significantly increased by G-CSF dose-dependently, it is believed that the leukocyte-increasing effect of G-CSF is due to its action on pluripotent stem cell, such as CFU-s cell.

Interestingly, the reconstitution of peripheral leukocytes was more rapid in Leucostim (5 μg/day)-treated mice than Grasin (5 μg/day)-treated animals. The statistical difference was confirmed on days 10 and 14 after BMT (data not shown). This phenomenon was also reported in chemotherapy-induced leukopenia models (Park *et al.*, 1994). These results suggest that biological activity of Leucostim is superior to that of Grasin, another version of rhG-CSF.

In conclusion, our data demonstrate that treatment with Leucostim can accelerate hematopoietic recovery in patients receiving bone marrow transplantation.

REFERENCES

Advani, R., Chao, N. J., Horning, S. J., Blume, K. G., Ahn, D. K., Lamborn, K. R., Fleming, N. C., Bonnem, E. M. and Greenberg, P. L. (1992). Granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjunct to autologous hemopoietic stem cell transplantation for lymphoma. *Ann. Intern. Med.*, **116**, 183-189.

Brandt, S. J., Peters, W. P., Atwater, S. D., Kurtzberg, J., Borowitz, M. J., Jones, R. B., Schrall, E. J., Bast, R. C., Gilbert, C. J. and Oette, D. H. (1988). Effect of recombinant human granulocyte-macrophage colony-stimulating factor on hematopoietic reconstitution after high-dose chemotherapy and autologous bone marrow transplantation. *N. Engl. J. Med.*, **318**, 869-874.

Clark, S. C. and Kamen, R. (1987). The human hematopoietic colony-stimulating factors. *Science*, **236**(4806), 1229-1237.

Griffin, J. D. (1988). Clinical applications of colony-stimulating factors. *Oncology(Huntingt.)*, **2**(1), 15-23.

Lenarski, C. (1993). Mechanisms in immune recovery after bone marrow transplantation. *Am. J. Pediatr. Hematol. Oncol.*, **15**, 49-55.

Linch, D. C., Scarffe, H., Proctor, S., Chopra, R., Taylor, P. R. A., Morgenstern, G., Cunningham, D., Burnett, A. K., Cawley, J. C., Franklin, I. M., Bell, A. J., Lister, T. A., Marcus, R. E., Newland, A. C., Parker, A. C. and Yver, A. (1993). Randomised vehicle-controlled dose-finding study of glycosylated recombinant human granulocyte colony-stimulating factor after bone marrow transplantation. *Bone Marrow Transplant.*, **11**, 307-311.

Lum, L. G. (1990). Immune recovery after bone marrow transplantation. *Bone Marrow Transplant.*, **4**, 659-675.

McCulloch, E. A., Thompson, M. W., Siminovitch, L. and Till, J. E. (1970). Effects of bacterial endotoxin on hemopoietic colony-forming cells in the spleens of normal mice and mice of genotype S1-S1d. *Cell Tissue Kinet.*, **3**(1), 47-54.

Moreau, P., Le Tortorec, S., Mahe, B., Legros, L., Milpied, N. and Harousseau, J. L. (1994). Administration of granulocyte colony-stimulating factor from day 7 after autologous bone marrow transplantation: effects on neutropenia and duration of hospitalization. *Nouv. Rev. Fr. Hematol. (Germany)*, **36**(6), 455-458.

Okabe, M., Asano, M., Kuga, T., Komatsu, Y., Yamasaki, M., Yokoo, Y., Itoh, S., Morimoto, M. and Oka, T. (1990) In vitro and in vivo hematopoietic effect of mutant human granulocyte colony-stimulating factor. *Blood*, **75**(9), 1788-1793.

Park, J. B., Ryu, B. K., Kang, S. H., Kim, W. B. and Yang, J. (1994). Recovery effect of DA-3030, a recombinant human granulocyte colony-stimulating factor, on neutropenia induced by anticancer agent in mice. *Korean J. Biol. Response Mod.*, **4**(2), 307-318.

Rowlings, P. A., Williams, S. F., Antman, K. H., Fields, K. K., Fay, J. W., Reed, E., Pelz, C. J., Klein, J. P., Sobocinski, K. A., Kennedy, M. J., Freytes, C. O., McCarthy, P. L. Jr., Herzig, R. H., Stadtmauer, E. A., Lazarus, H. M., Pecora, A. L., Bitran, J. D., Wolff, S. N., Gale, R. P., Armitage, J. O., Vaughan, W. P., Spitzer, G. and Horowitz, M. M. (1999). Factors correlated with progression-free survival after high-dose chemotherapy and hematopoietic stem cell transplantation for metastatic breast cancer. *JAMA (United States)*, **282**(14), 1335-1343.

Souetre, E., Qing, W. and Penelaud, P. F. (1996). Economic analysis of the use of recombinant human granulocyte colony stimulating factor in autologous bone marrow transplantation. *Eur. J. Cancer (England)*, **32A**(7), 1162-1165.