Inductive Potential of Recombinant Human Granulocyte Colony-Stimulating Factor to Mature Neutrophils from X-Irradiated Human Peripheral Blood Hematopoietic Progenitor Cells

Takeo Katsumori, Hironori Yoshino, Masako Hayashi, Kenji Takaashi, and Ikuo Kashiwakura*

Department of Radiological Life Sciences, Hirosaki University Graduate School of Health Sciences; 66–1 Hon-cho, Hirosaki, Aomori 036–8564, Japan.

Received July 22, 2009; accepted August 14, 2009; published online August 24, 2009

Recombinant human granulocyte colony-stimulating factor (rhG-CSF) has been used for treatment of neutropenia. Filgrastim, Nartograstim, and Lenograstim are clinically available in Japan. However, the differences in potential benefit for radiation-induced disorder between these types of rhG-CSFs remain unknown. Therefore, the effects of three different types of rhG-CSFs on granulocyte progenitor cells and expansion of neutrophils from nonirradiated or 2 Gy X-irradiated human CD34+ hematopoietic progenitor cells were examined. For analysis of granulocyte colony-forming units (CFU-G) and a surviving fraction of CFU-G, nonirradiated or X-irradiated CD34+ cells were cultured in methylcellulose containing rhG-CSF. These cells were cultured in serum-free medium supplemented with rhG-CSF, and the expansion and characteristics of neutrophils were analyzed. All three types of rhG-CSFs increased the number of CFU-G in a dose-dependent manner; however, Lenograstim is superior to others because of CFU-G-derived colony formation at relatively low doses. The surviving fraction of CFU-G was independent of the types of rhG-CSFs. Expansion of neutrophils by rhG-CSF was largely attenuated by X-irradiation, though no significant difference in neutrophil number was observed between the three types of rhG-CSFs under both nonirradiation and X-irradiation conditions. In terms of functional characteristics of neutrophils, Lenograstim-induced neutrophils produced high levels of reactive oxygen species compared to Filgrastim, when rhG-CSF was applied to nonirradiated CD34+ cells. In conclusion, different types of rhG-CSFs lead to different effects when rhG-CSF is applied to nonirradiated CD34+ cells, though Filgrastim, Nartograstim, and Lenograstim show equal effects on X-irradiated CD34+ cells.

Key words recombinant human granulocyte colony-stimulating factor; CD34+ cell; neutrophil; X-irradiation

Neutrophils are the most abundant type of white blood cells and are indispensable for the immune system. They have multiple functions, such as phagocytosis and reactive oxygen species (ROS) production, for killing invading bacteria and providing the first line of defense of the innate immune system. Therefore, neutropenia derived from hematopoietic injury after chemotherapy or radiotherapy of patients with malignancy is a serious problem and cause of infectious diseases. Granulocyte colony-stimulating factor (G-CSF) is an effective agent for promoting neutrophil recovery and is therefore generally used for treatment of neutropenia. Furthermore, G-CSF showed high efficiency in cases of accidental irradiation, though recombinant human G-CSFs (rhG-CSF) have not been approved for treatment of radiation-induced neutropenia in Japan. G-CSF mainly acts on granulocyte progenitor cells, enabling their proliferation, mobilization into peripheral blood, and differentiation into neutrophils. It also induces the function and mobilization of neutrophils into organization. Three types of rhG-CSFs, Filgrastim, Lenograstim, and Nartograstim, are clinically available in Japan. Filgrastim and Nartograstim consist of 175 amino acid residues and are produced in culture from Escherichia coli, and Lenograstim consists of 174 amino acid residues and is produced in culture from Chinese hamster ovary cells. Furthermore, Lenograstim is the only glycosylated rhG-CSF, and Nartograstim possesses certain mutated N-terminal amino acids. There are some differences in chemical structure and physicochemical properties between the three types of rhG-CSFs. The pharmacodynamics of these three types have been reported to be identical in a monkey model, in spite of some small differences in the pharmacokinetics. However, little is known about whether treatment efficacy of rhG-CSF on chemotherapy-induced neutropenia depends on the type of rhG-CSF used. Furthermore, the differences in potential benefit for radiation-induced disorders between these three types of rhG-CSFs remain unknown.

In this study, we examined the effects of three different types of rhG-CSFs on granulocyte progenitor cells and expansion of neutrophils from nonirradiated or X-irradiated human CD34+ hematopoietic progenitor cells in vitro.

MATERIALS AND METHODS

Reagents Filgrastim and Lenograstim were purchased from Kirin Brewery Co., Ltd. (Tokyo, Japan) and Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Nartograstim was kindly provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Recombinant human thrombopoietin (TPO) and human stem cell factor (SCF) were purchased from Biosource (Tokyo, Japan), and N-formyl-Met-Leu-Phe (fMLP) was purchased from Sigma-Aldrich, Inc. (Tokyo, Japan). The EasySep™ human CD34 selection kit was purchased from Stem Cell Technologies (Vancouver, Canada). Luminol and lucigenin were purchased from Nacalai Tesque, Inc. (Tokyo, Japan).

Collection and Purification of CD34+ Cells from Normal Human Peripheral Blood Mononuclear Cells This study was approved by the Committee of Medical Ethics of Hirosaki University School of Medicine (Hirosaki, Japan). After obtaining informed consent from all the normal human blood donors, peripheral blood was collected, from which the
buffy coat was prepared and supplied to our research team by the Aomori Red Cross Blood Center (Aomori, Japan). Light-density mononuclear cells were separated from the buffy coat by centrifugation for 30 min at 400 × g on a cushion of Lymphosepar I (1.077 g/ml; Immuno-Biological Laboratories Co., Ltd., Takasaki, Japan). After centrifugation, these cells were washed twice with phosphate-buffered saline containing 5 mM ethylenediaminetetraacetic acid, treated with Türk solution, and counted using an automated hematology analyzer (Sysmex SF-3000; Toa Medical Electronics Co., Ltd., Kobe, Japan). CD34+ cells were purified from these cells according to the manufacturer’s instructions. The EasySep® human CD34 selection kit was used for positive selection of CD34+ cells. At the end of the procedure, the obtained cells were counted by the trypan blue dye exclusion method, and CD34+ cell purity was measured by a flow cytometer (EPICS® XL, Beckman-Coulter, Fullerton, CA, U.S.A.). The purity of CD34+ cells used in this study was 50—80%.

**In Vitro Irradiation of Peripheral Blood CD34+ Cells**

The X-ray irradiation (150 kVp, 20 mA, 0.5 mm Al, and 0.3 mm Cu filter) was performed using an X-ray generator (MBR-1520R; Hitachi Medical Corporation, Tokyo, Japan) at a distance of 45 cm from the focus at a dose rate of 80.0—90.0 cGy/min.

**Granulocyte Colony-Forming Unit (CFU-G) Analysis**

CFU-G was assayed using methylcellulose culture, as described previously, with minor modifications.12) Two experiments were performed in this study. In the first experiment to investigate a dose-dependent effect of rhG-CSF, CD34+ cells (1 × 10^6 cells) were suspended in 1 ml methylcellulose medium (Methocult H4230, Stem Cell Technologies Inc.) supplemented with rhG-CSF (0.1, 0.5, 1.0, 10, 50, 500 ng/ml). This mixture was transferred into 24-well cell culture plates (0.3 ml/well) and incubated at 37 °C for 14 d in a humidified atmosphere containing 5% CO₂. Colonies consisting of more than 50 cells were counted using an inversion microscope. In the second experiment to measure a surviving fraction of CFU-G, CD34+ cells (cell concentration, 1 × 10^3 to 1 × 10^4 cells/ml) after irradiation (0—4 Gy) were cultured in methylcellulose medium supplemented with rhG-CSF (100 ng/ml) and analyzed in the same manner as mentioned above.

**Serum-Free Liquid Culture**

Nonirradiated (1 × 10^4 cells/ml) or 2 Gy-irradiated CD34+ cells (1 × 10^5 cells/ml) were cultured in serum-free Iscove’s modified Dulbecco’s medium (Gibco BRL, Grand Island, U.S.A.) supplemented with BIT9500 (a serum substitute for serum-free culture; Kobe, Japan). CD34+ cells were assayed in methylcellulose culture, and rhG-CSF was added to the serum-free liquid cultures supplemented with Filgrastim, Lenograstim, or Nartograstim. All three types of rhG-CSFs increased the number of CFU-G in cultures induced from non-irradiated CD34+ cells by Filgrastim treatment.

**Phagocytosis Assay**

Neutrophils expanded from non-irradiated CD34+ cells in serum-free liquid cultures supplemented with rhG-CSF (100 ng/ml) were harvested, and the number of viable cells was determined by the trypan blue exclusion assay. The Vybrant phagocytosis assay kit (Invitrogen, Inc.) was used to determine the phagocytic activity of neutrophils. Neutrophils were incubated with fluorescent-labeled E. coli K-12 BioParticles (Bioparticle) in a final concentration of 1.0 mg/ml for 10 min at 37 °C. After incubation, the phagocytic activity of neutrophils was stopped by addition of trypan blue solution, and BioParticle-positive neutrophils were counted using a fluorescence microscope (Olympus, Tokyo, Japan). Neutrophils placed on ice were prepared as a control.

**Measurement of ROS Production**

Neutrophils expanded from nonirradiated or X-irradiated CD34+ cells in the serum-free liquid cultures supplemented with rhG-CSF (100 ng/ml) were harvested, and the number of viable cells was determined by the trypan blue exclusion assay. The ROS production ability of neutrophils was assayed using luminol and lucigenin. Luminometry detection measures intracellular ROS kinetic production and extracellular ROS detection using luminal and lucigenin, respectively.13) Luminol can detect hydrogen peroxide. Neutrophils suspended in Hanks balanced salt solution (HBSS) containing 150 μM luminol were protected from light with aluminum foil and incubated for 5 min at 37 °C. After incubation, 1 μM fMLP was added at once, and chemiluminescence was measured for 15 min/sample using a luminometer (LB9507; Berthold Technologies, Tokyo, Japan). Lucigenin can detect superoxide anion (O₂•−). Neutrophils were suspended in HBSS containing 150 μM lucigenin and assayed by the same procedure performed in luminol. ROS production were shown as the relative value against the chemiluminescence value obtained in neutrophils induced from non-irradiated CD34+ cells by Filgrastim treatment.

**Statistical Analysis**

Data are expressed as mean values±standard deviation (S.D.). The significance of difference to each experimental group was performed by multiple comparison procedures in a one-way ANOVA model. A p-value <0.05 was considered to be statistically significant. Statistical analysis was performed using Excel 2003 software (Microsoft, U.S.A.) with Statcel 2 add-in software.14)

**RESULTS**

**Effect of Three Different Types of rhG-CSFs on CFU-G Growth**

The number of CFU-G derived from CD34+ cells was measured using methylcellulose culture supplemented with Filgrastim, Lenograstim, or Nartograstim. All three types of rhG-CSFs increased the number of CFU-G in a dose-dependent manner (Fig. 1). At doses of 0.5 and

---

**Phagocytosis Assay**

Neutrophils expanded from non-irradiated CD34+ cells in serum-free liquid cultures supplemented with rhG-CSF (100 ng/ml) were harvested, and the number of viable cells was determined by the trypan blue exclusion assay. The Vybrant phagocytosis assay kit (Invitrogen, Inc.) was used to determine the phagocytic activity of neutrophils. Neutrophils were incubated with fluorescent-labeled E. coli K-12 BioParticles (Bioparticle) in a final concentration of 1.0 mg/ml for 10 min at 37 °C. After incubation, the phagocytic activity of neutrophils was stopped by addition of trypan blue solution, and BioParticle-positive neutrophils were counted using a fluorescence microscope (Olympus, Tokyo, Japan). Neutrophils placed on ice were prepared as a control.

**Measurement of ROS Production**

Neutrophils expanded from nonirradiated or X-irradiated CD34+ cells in the serum-free liquid cultures supplemented with rhG-CSF (100 ng/ml) were harvested, and the number of viable cells was determined by the trypan blue exclusion assay. The ROS production ability of neutrophils was assayed using luminol and lucigenin. Luminometry detection measures intracellular ROS kinetic production and extracellular ROS detection using luminal and lucigenin, respectively. Luminol can detect hydrogen peroxide. Neutrophils suspended in Hanks balanced salt solution (HBSS) containing 150 μM luminol were protected from light with aluminum foil and incubated for 5 min at 37 °C. After incubation, 1 μM fMLP was added at once, and chemiluminescence was measured for 15 min/sample using a luminometer (LB9507; Berthold Technologies, Tokyo, Japan). Lucigenin can detect superoxide anion (O₂•−). Neutrophils were suspended in HBSS containing 150 μM lucigenin and assayed by the same procedure performed in luminol. ROS production were shown as the relative value against the chemiluminescence value obtained in neutrophils induced from non-irradiated CD34+ cells by Filgrastim treatment.

**Statistical Analysis**

Data are expressed as mean values±standard deviation (S.D.). The significance of difference to each experimental group was performed by multiple comparison procedures in a one-way ANOVA model. A p-value <0.05 was considered to be statistically significant. Statistical analysis was performed using Excel 2003 software (Microsoft, U.S.A.) with Statcel 2 add-in software.

**RESULTS**

**Effect of Three Different Types of rhG-CSFs on CFU-G Growth**

The number of CFU-G derived from CD34+ cells was measured using methylcellulose culture supplemented with Filgrastim, Lenograstim, or Nartograstim. All three types of rhG-CSFs increased the number of CFU-G in a dose-dependent manner (Fig. 1). At doses of 0.5 and
1.0 ng/ml, Nartogristim and Lenograstim resulted in more CFU-G in comparison to Filgrastim treatment (Fig. 1). Furthermore, at a dose of 1.0 ng/ml, the number of CFU-G by Lenograstim treatment was significantly higher than that by Nartogristim. In brief, Lenograstim is superior to the other types of rhG-CSFs because of CFU-G colony formation at relatively low doses. The following experiments were performed at a concentration of 100 ng/ml rhG-CSFs because the effects of rhG-CSFs on CFU-G reached a plateau at 50 ng/ml (Fig. 1).

In order to investigate whether recovery of CFU-G colony formation after X-irradiation depends on the type of rhG-CSF, X-irradiated CD34+ cells were cultured in methylcellulose medium supplemented with Filgrastim, Nartogristim, or Lenograstim (100 ng/ml). As a result, no significant difference in the surviving fraction of CFU-G was observed between the three types of rhG-CSFs (Fig. 2).

**Expansion of Mature Neutrophils from Nonirradiated or X-Irradiated CD34+ Cells**

Nonirradiated or 2 Gy-irradiated CD34+ cells were cultured in serum-free liquid culture medium supplemented with a combination of TPO, SCF, and each type of rhG-CSF. The generated cells were harvested after 14 d of culture. In order to determine the proportion of mature cells among the harvested cells, CD13, CD14, and CD15 expression was analyzed by flow cytometry (Fig. 3). The expression of CD13 and CD15, a marker antigen of the myeloid lineage and marker antigens of the leukocyte lineage, was increased from the initial input value (14%) to the range of 47—50% in the culture of nonirradiated and irradiated cells. Expression of CD14 and CD15, marker antigens of the leukocyte lineage, was increased from the initial value (13%) to the range of 38—51% in both cultures. These results indicate that half of the cells generated in the culture became mature neutrophils. However, no significant difference was observed between the culture of nonirradiated and irradiated cells. Furthermore, no significant difference was observed between the three types of rhG-CSFs tested (data not shown). Findings of an automated hematology analyzer showed that the nonirradiated CD34+ cells were differentiated into mature neutrophils and that the number of mature neutrophils increased from 100- to 150-fold compared to the initial CD34+ cell number (Fig. 4A). However, the number of mature neutrophils derived from 2 Gy-irradiated CD34+ cells was increased 3-fold compared to the initial number (Fig. 4B), thus indicating that expansion of mature neutrophils from CD34+ cells is largely attenuated by X-irradiation. In both conditions of nonirradiation and 2 Gy-irradiation, Nar-
Tograstim tended to produce greater expansion of mature neutrophils from CD34+/H11001 cells, although there was no statistical difference between the three types of rhG-CSFs.

**Function of Mature Neutrophils Derived from Nonirradiated or X-Irradiated CD34⁺ Cells** In order to investigate whether functional activities of mature neutrophils induced from CD34⁺ cells depend on the type of rhG-CSF, phagocytic activity and ROS production ability were examined. The phagocytic activity of mature neutrophils from nonirradiated CD34⁺ cells was analyzed using the Vybrant phagocytosis assay kit. Each rhG-CSF-induced neutrophil absorbed fluorescein-labeled BioParticles (Fig. 5). Although there was no statistically significant difference between the three types of rhG-CSFs, mature neutrophils induced by Lenograstim treatment showed comparatively higher phagocytic activity than those induced by Filgrastim and Nartograstim treatment.

The ROS production ability of mature neutrophils was assayed using luminol and lucigenin. When mature neutrophils were induced from nonirradiated CD34⁺ cells, no significant difference in the ROS production ability was observed between the three types of rhG-CSFs in the test using luminol (Fig. 6A). However, in the test using lucigenin, Lenograstim-induced mature neutrophils produced high levels of ROS compared to Filgrastim-induced ones (Fig. 6B). On the other hand, the neutrophils induced from 2 Gy-irradiated CD34⁺ cells produced same or higher levels of ROS in comparison to the neutrophils from nonirradiated CD34⁺ cells. In both tests using luminol and lucigenin, no significant difference was observed among the three types of rhG-CSFs when mature neutrophils were induced from 2 Gy-irradiated CD34⁺ cells (Figs. 6A, B).

**DISCUSSION**

Three types of rhG-CSFs, Filgrastim, Lenograstim, and Nartograstim, are clinically available in Japan. Although many reports compare and analyze these three types of rhG-CSFs, it remains unknown whether recovery from hematopoietic injuries by ionizing radiation depends on the type of rhG-CSF. In this study, we investigated the effects of these three different types of rhG-CSFs on human granulocyte progenitor cells and expansion of neutrophils from nonirradiated or X-irradiated human CD34⁺ hematopoietic progenitor cells. Guidelines of rhG-CSF provided by the Japan Society of Clinical Oncology and American Society of Clinical Oncology state an administration dose of 2 and 5 μg/kg/d, respectively. Tanaka et al. intravenously or subcutaneously administrated 1.5 or 5 μg/kg rhG-CSF (Filgrastim, Lenograstim, and Nartograstim) to normal cynomolgus monkeys and compared the pharmacokinetics and pharmacological effects of the three rhG-CSFs. In terms of pharmacokinetics, clearance of Lenograstim was greater, and AUC (the
area under the blood concentration time curve) and MRT (mean residence time) of Lenograstim were smaller than those of the other two types; however, there was no significant difference in pharmacodynamics. This observation may imply that the effective concentration of rhG-CSF in blood depends on the type. Our present study (Fig. 1) and Nissen’s result support this theory to some extent, because Lenograstim showed greater potency in in vitro neutrophil progenitor colony formation. However, expansion of more mature neutrophils from CD34+ cells by Lenograstim is identical to that of the other types (Fig. 4A). Carlsson et al. reported that the absolute neutrophil number produced by Lenograstim treatment was lower than that produced by Filgrastim treatment during administered to patients with severe congenital neutropenia. Therefore, although Lenograstim has greater ability to stimulate granulocyte progenitor cells, it may be inferior to the others in terms of induction of mature neutrophils.

Glycosylation is the difference between Lenograstim and the others. In brief, Lenograstim is a glycosylated rhG-CSF, while the others are nonglycosylated rhG-CSFs. Some reports suggest that glycosylation confers functional differences on G-CSF; for example, taxis ability is significantly altered while the others are nonglycosylated rhG-CSFs. Some results showed that ROS production by Lenograstim-induced mature neutrophils is higher than that by Filgrastim-induced mature neutrophils. Contrary to our expectation, no significant difference in survival of neutrophil progenitors and expansion of mature neutrophils after X-irradiation was observed among the three types of rhG-CSFs. Furthermore, the ROS production ability of mature neutrophils that expanded from X-irradiated CD34+ cells is same or higher than that of neutrophils from nonirradiated CD34+ cells and also independent of the type of rhG-CSFs (Fig. 6). Therefore, although further functional examination, such as taxis ability, is needed, the effect of these three different types of rhG-CSFs on X-irradiated CD34+ cells is identical.

Berger et al. reported that based on experience in radiation oncology, hematology, limited radiation accident experience, and market availability, rhG-CSF is recommended for radiation-induced neutropenia or aplasia. Furthermore, although not approved by the U.S. Food and Drug Administration and the Japan’s Ministry of Health, Labor and Welfare for treatment of radiation-induced neutropenia, the rationale for use of CSFs is clear. rhG-CSF decreased the period of neutropenia in the limited number of radiation accident victims studied and was also able to activate or prime neutrophils to enhance their function. Hirama et al. suggested that using G-CSF or other growth factors that can mobilize myeloid cells is an important therapeutic option for treating acute radiation syndrome. The present study demonstrated that the types of rhG-CSFs produced different effects when rhG-CSF was applied to nonirradiated CD34+ cells but not to X-irradiated CD34+ cells. However, we have to pay attention to the low levels of G-CSF after administration in vivo. Thus, we conclude that Filgrastim, Nartograsit, and Lenograstim have equal effects on X-irradiated CD34+ cells in vitro.

Acknowledgments This study was supported by a Grant for Co-medical Education Program in Radiation Emergency Medicine by the Ministry of Education, Culture, Sports, Science and Technology of Japan. This study was also supported by a Grant for Hirosaki University Institutional Research (2008).

REFERENCES