Increase in Total Blood Leukocyte Count Following Intranasal Administration of Recombinant Human Granulocyte Colony-Stimulating Factor (rhG-CSF) in Rabbits with Cyclophosphamide-Induced Leukopenia

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We investigated the effects of intranasal (i.n.) administration of recombinant human granulocyte colony-stimulating factors (rhG-CSF) on the total count of leukocytes in peripheral blood (total blood leucocyte count) of rabbits with leukopenia who received cyclophosphamide (CPA). When CPA (30 mg/kg per d) was administered intravenously, the total blood leukocyte count decreased to levels below 5000/µl approximately 4d after the initiation of CPA multiple dosing. The decreased level of the total blood leukocyte count was maintained throughout the period of CPA dosing. RhG-CSF was given once a day for 3d in CPA-treated rabbits via i.n. administration of aqueous preparations containing rhG-CSF with or without α-cyclodextrin (α-CyD). The total blood leukocyte count increased from levels below 5000/µl to the normal physiological level following i.n. administration of rhG-CSF preparation and reduced the period of leukopenia induced by CPA. The coadministration of rhG-CSF and α-CyD was more effective in increasing the total blood leukocyte count. It is suggested that i.n. administration of rhG-CSF is promising for reducing the risk of cytotoxic chemotherapy (CPA)-induced leukopenia as an adverse side effect.

Key words granulocyte colony-stimulating factor; intranasal administration; leukopenia; cyclophosphamide-treated rabbit; α-cyclodextrin; blood leukocyte count

Human granulocyte colony-stimulating factor (hG-CSF) has recently been biologically characterized and purified, and molecularly cloned as a recombinant protein.2,3) (rhG-CSF). This granulopoietic regulator is capable of inducing the formation of granulocytic colonies from committed precursor cells.4) RhG-CSF is clinically administered parenterally for the treatment of neutropenia induced by chemotherapy or irradiation. To lessen the inconvenience associated with the parenteral administration of rhG-CSF in patients, attempts to achieve acceptable alternative methods via various administration routes have been studied.5~8)

In our previous paper,9) we demonstrated that the intranasal (i.n.) administration of rhG-CSF and cyclodextrin (CyD) such as α- or β-CyD, results in a considerable increase in the nasal absorption of rhG-CSF and in the total count of leukocytes in peripheral blood (total blood leukocyte count) in rabbits. Furthermore, we found that the amount of rhG-CSF administered influences its in vivo pharmacodynamic effects (leukopoietic effect) and pharmacokinetic parameters in normal rabbits.10 These findings led us to investigate whether the leukopoietic effect of rhG-CSF administered intranasally might occur in experimental animals with leukopenia induced by chemotherapy. To elucidate the effectiveness of nasal rhG-CSF delivery, it is very important to evaluate the leukopoietic effect of rhG-CSF following i.n. administration in the leukopenic animals.

In this report, we investigated the accelerated recovery of total blood leukocyte count after i.n. administration of rhG-CSF preparations in rabbits treated with cyclophosphamide (CPA), which belongs to the group of pharmacological agents named alkylating agents, to evaluate the possibility of future applications of the nasal rhG-CSF delivery system.

MATERIALS AND METHODS

Materials A lyophilized rhG-CSF (filgrastim, Kirin Brewery Co., Tokyo, Japan), which consists of 175 amino acids without O-glycoside (molecular weight of 18600 daltons), was used. CPA (Endoxan®, for injection) was purchased from Shionogi Pharmaceutical Co., Osaka, Japan. α-CyD was obtained from Nihon Shokuhin Kako Co., Tokyo, Japan. All other reagents used were of analytical grade.

Preparation for i.n. Administration The aqueous preparations (freshly prepared for nasal administration were made by dissolving appropriate amounts of rhG-CSF with or without α-CyD in a 10 mM acetate buffer solution at pH 4.0.1) The placebo formulations contained the same amount of α-CyD in acetate buffer solution without rhG-CSF.

Animal Experiments Male rabbits (Japan White) weighing 3.0 to 3.5 kg were used in this study. They had free access to water and food and were housed individually in cages under environmentally controlled conditions (23 ± 1°C, 55% relative humidity, 12-h on/off light/dark cycle). Experimental designs of rhG-CSF administration and determination of the total blood leukocyte count are summarized in Tables 1 and 2, respectively.

Concerning leukopenia induced by CPA in experimental animals, only a few studies of leukopenia have been carried out in rabbits. To confirm the CPA dose required to induce leukopenia, rabbits were intravenously (i.v.) administered various doses (Table 1) of CPA in the
Table 1. Experimental Design of i.n. Administration of rhG-CSF in Rabbits with Leukopenia Induced by CPA

<table>
<thead>
<tr>
<th>Objective</th>
<th>Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA (i.v.) (mg/kg/d)</td>
</tr>
<tr>
<td>1) Induction of leukopenia</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td>2) Effect of rhG-CSF administration with α-CyD</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>or without α-CyD</td>
<td>30</td>
</tr>
<tr>
<td>3) Difference in initiation of rhG-CSF administration:</td>
<td>30</td>
</tr>
<tr>
<td>6d after initiation of CPA injection</td>
<td>30</td>
</tr>
<tr>
<td>14d after initiation of CPA injection</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2. Experimental Design of Determinations of Total Blood Leukocyte Count and Serum G-CSF Concentration in Rabbits with Leukopenia Induced by CPA

<table>
<thead>
<tr>
<th>Objective</th>
<th>Determination time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total blood leukocyte count</td>
</tr>
<tr>
<td>1) Induction of leukopenia</td>
<td>Once a day, at noon every day</td>
</tr>
<tr>
<td>2) Effect of rhG-CSF administration with α-CyD</td>
<td>At 0, 6, 12 and 24 h after rhG-CSF administration</td>
</tr>
<tr>
<td>or without α-CyD</td>
<td></td>
</tr>
<tr>
<td>3) Difference in initiation of rhG-CSF administration:</td>
<td>The same time intervals mentioned above (2)</td>
</tr>
<tr>
<td>6d after initiation of CPA injection</td>
<td></td>
</tr>
<tr>
<td>14d after initiation of CPA injection</td>
<td></td>
</tr>
</tbody>
</table>

preliminary tests. It has been reported that the normal physiological level of leukocyte count in blood is between 5200 and 12000/μl in rabbits. Therefore, we determined that leukopenia was induced when the total blood leukocyte count decreased to a level below 5000/μl in rabbits receiving CPA. Leukopenia was not induced in rabbits that received a CPA dose of 10 mg per kg body weight once a day (10 mg/kg/d) for one week. On the other hand, some rabbits died following multiple CPA dosing of 60 mg/kg/d. Therefore, we chose a dose of 30 mg/kg/d for multiple CPA dosing to induce leukopenia. All rabbits received multiple dosings of CPA (30 mg/kg/d) during the experimental period.

I.n. administration of rhG-CSF in CPA-treated rabbits was started at the 6th or 14th day after the initiation of i.v. administration of CPA (Table 1). The method of i.n. administration in rabbits reported by Watanabe et al. was applied. Briefly, rabbits were subjected to an overnight fast with only tap water available. On the day of the experiment, their heads were held in a vertical position for ease of administration. RhG-CSF test solution, with the dosage volume calibrated to 50 μl per kg body weight, was administered into one nostril with a micropipette (Eppendorf®). Immediately after the i.n. administration of drugs, rabbits were placed in a supine position for 2 min; then they were secured in a crouching posture during the blood sampling experiments. Two-milliliter aliquots of blood samples were taken from the auricular vein by a syringe with or without edetate disodium (EDTA-2Na) at predetermined time intervals (Table 2). The serum was separated by centrifugation and stored at −30 °C until the G-CSF assays.

**Determination of Total Leukocyte Count in Blood and Serum G-CSF Concentration**

For the determination of the total leukocyte count, blood samples were diluted with a balanced electrolyte solution (Isoton®-II, Nikkaki, Tokyo, Japan) containing potassium cyanide as a hemolyzing agent (Zap-Oglobin®-II, Nikkaki, Tokyo, Japan). Then the total blood leukocyte count was measured by the Coulter Counter (Model ZM, Nikkaki, Tokyo, Japan). The G-CSF concentration in serum was determined by the enzyme immunoassay reported by Tanaka and Tokiwa.

**Data Analysis**

Pharmacokinetic parameters such as the peak serum concentration (Cmax) and the time to reach peak serum G-CSF concentration (tmax) were determined from the individual serum G-CSF concentration–time curve. The area under the serum G-CSF concentration–time curve from 0 to 24 h after i.n. administration (AUC0–24) was calculated using the trapezoidal rule.

Statistical analysis of the results was conducted by the one-way analysis of variance and the Dunnett's test. A significant difference was estimated using p=0.05 as the minimal level of significance.

**RESULTS AND DISCUSSION**

**Total Blood Leukocyte Count and Serum G-CSF Concentration Following i.n. Administration of rhG-CSF in CPA-Treated Rabbits**

To investigate the effects of rhG-CSF on the total blood leukocyte count in rabbits with leukopenia induced by CPA, rabbits received multiple doses of CPA intravenously prior to the i.n. administration of rhG-CSF. As described in the experimental section, CPA at a dose lower than 10 mg/kg did not decrease the total leukocyte count. Consequently, leukopenia was not induced by multiple dosing of CPA (10 mg/kg/d). On the other hand, a higher dose (60 mg/kg) of CPA caused severe leukopenia in which the total blood leukocyte count decreased below levels of 3000/μl in many CPA-treated rabbits. However, some rabbits which received this single dosing of CPA (60 mg/kg) died within a few days. It was found that the toxic dosing range of CPA causing leukopenia without death was narrow in rabbits. When CPA was administered at a dose of 30 mg/kg/d, the mean values of the total blood leukocyte count significantly decreased to levels below 5000/μl approximately 4 d after the initiation of CPA multiple dosing (Fig. 1). The decreased level (<5000/μl) of the total blood leukocyte count was maintained throughout the period of multiple dosing of CPA. From these results, we concluded that leukopenia in rabbits was induced by the i.n. administration of CPA at 30 mg/kg/d, because the minimum level of
Fig. 1. Total Blood Leukocyte Count Following Multiple i.n. Administration of rhG-CSF (1000 µg/kg) with α-CyD in Rabbits which Received CPA for Two Weeks

Each rabbit received CPA at a dose of 30 mg/kg/d during the experimental period of 15 d. Each point represents the mean ± S.E. (vertical bar) of at least three experiments. Key: -- - - -, rhG-CSF with α-CyD (10 mg/kg); --- x ---, α-CyD (10 mg/kg, control experiment). Statistically significant differences: * p < 0.05 in rhG-CSF with α-CyD vs. α-CyD.

Fig. 2. Total Blood Leukocyte Count Following the Single i.n. Administration of rhG-CSF (1000 µg/kg) in Normal and Leukopenic Rabbits

Leukopenic rabbits received CPA at a dose of 30 mg/kg/d during the experimental period of 7 d before rhG-CSF administration. Each point represents the mean ± S.E. (vertical bar) of at least three experiments. Key: ----, normal rabbit receiving rhG-CSF; --- - - -, leukopenic rabbit receiving rhG-CSF with α-CyD (10 mg/kg).

The normal physiological leukocyte count in blood is approximately 5000/μl in rabbits.\(^{10}\)

In our previous studies,\(^{1,9}\) a significant increase in the total blood leukocyte count in normal rabbits was observed following a single i.n. administration of rhG-CSF (1000–10000 µg/kg) with CyDs as an absorption-enhancing agent.\(^{11,14}\) A peak level of total leukocyte count was attained (denoted by filled squares in Fig. 2) within one day after the i.n. administration of rhG-CSF (1000 µg/kg) in normal rabbits. However, the total blood leukocyte count in rabbits with leukaemia induced by CPA did not improve from the decreased level (indicated by filled circles in Fig. 2) following the single i.n. administration of rhG-CSF even at the highest dose of 1000 µg/kg with α-CyD. Therefore, we attempted multiple dosing of the rhG-CSF preparation. When an aqueous solution containing rhG-CSF (1000 µg/kg) and α-CyD (10 mg/kg) was given once a day for 3 d in CPA-treated rabbits via i.n. administration 1 d after the induction of leukopenia (i.e., the 6th day from the initiation of CPA administration), the total blood leukocyte count (indicated by a solid line in Fig. 1) significantly increased to the physiological level and reached a peak level (approximately 15000/μl) at 3 d after the start of the rhG-CSF multiple dosing regimen. The total blood leukocyte count returned to the normal physiological level after the withdrawal of rhG-CSF administration, and tended to decrease to levels below 5000/μl during the period of CPA administration. In rabbits with leukopenia, it seems that multiple dosing of rhG-CSF at a relatively high dose is required to return leukocyte counts to the normal physiological level.

Figure 3 shows the time-course of serum G-CSF concentration following multiple dosing of rhG-CSF in leukopenic rabbits. The mean values of C\(_{\text{max}}\) and t\(_{\text{max}}\) of G-CSF obtained after i.n. administration of rhG-CSF (1000 µg/kg) with α-CyD (10 mg/kg) are approximately 460 ng/ml and 1 h, respectively (serum levels are indicated by filled circles in Fig. 3). Serum G-CSF concentrations were significantly increased by the co-administration of rhG-CSF and α-CyD and exceeded the minimum serum G-CSF level (approximately 10 ng/ml)\(^{11}\) required to exert a leukopoietic effect following each i.n. administration. This co-administered CyD may be a suitable adjuvant for enhancing the nasal absorption of rhG-CSF in rabbits with leukaemia. Even after the serum G-CSF concentration reached an effective level, the beginning of an increase in the total blood leukocyte count was delayed in leukopenic rabbits compared with those of normal rabbits. It is presumed that the time lag (2 d) between the start of rhG-CSF administration and the time at which an increase in the total blood leukocyte count is noted may be accounted for by the time required by hematopoietic progenitor cells to proliferate and differentiate.

In the case of placebo administration (aqueous solution containing α-CyD without rhG-CSF), indicated by a dotted line in Fig. 1, the decreased total blood leukocyte...
leukopoietic efficacy obtained by rhG-CSF at a dose of 300 μg/kg reached a maximal level. The fact that a low dose of rhG-CSF was able to exert a similar effect as that of a high dose of rhG-CSF presents many advantages for therapeutic formulations.

The findings stated above led us to investigate whether i.n. administration of rhG-CSF might be effective in CPA-treated rabbits who had leukopenia for a longer duration. Figure 6 illustrates the change in mean values of the total blood leukocyte count following i.n. administration of rhG-CSF preparation in the case of long-term CPA treatment. Six days after the initiation of CPA multiple dosing, the total blood leukocyte count decreased to minimum physiological levels (5000/μl) and was maintained during the period of CPA multiple dosing. Eight days after the beginning of leukopenia (the 14th day after the initiation of CPA multiple dosing), the rhG-CSF of 1000 μg/kg with α-CyD of 10 mg/kg was given once a day for 3 d using the same procedure stated above. Interestingly, a marked leukopoietic effect of rhG-CSF was observed even in long-term CPA-treated rabbits (indicated by the solid line in Fig. 6). These results suggest that the i.n. administration of rhG-CSF may be effective even in leukopenia induced by CPA multiple treatment for two weeks.

In conclusion, this study demonstrated that rhG-CSF was efficiently absorbed by co-administered α-CyD through the nasal cavity of CPA-treated rabbits, and it induced a significant increase in the total blood leukocyte count decreased by CPA administration. A marked effect of rhG-CSF in CPA-treated rabbits was found when rhG-CSF i.n. administration was initiated during leukopenia induced by multiple dosing of CPA. RhG-CSF reduced the period of leukopenia induced by CPA. It is suggested that i.n. administration of rhG-CSF is promising for reducing the risk of cytotoxic chemotherapy (CPA)-induced leukopenia as an adverse side effect.
REFERENCES AND NOTES

1) This paper is Part II of “Studies of Drug Delivery Systems for Granulocyte Colony-Stimulating Factor.” Part I: Watanabe Y., Matsumoto Y., Kikuchi R., Kiriyama M., Nakagawa K., Nomura H., Maruyama K., Matsumoto M., J. Drug Targeting, in press. Part of this study was presented at the 12th International Congress of Pharmacology, Montreal, July, 1994.


