Pharmacodynamics and Pharmacokinetics of Recombinant Human Granulocyte Colony-Stimulating Factor (rhG-CSF) after Administration of a Rectal Dosage Vehicle¹⁾

Yoshiteru WATANABE,^{*,a} Miyuki KIRIYAMA,^a Ritsuko ITO,^a Rie KIKUCHI,^a Yuko MIZUFUNE,^a Hideaki NOMURA,^b Masanao MIYAZAKI,^b and Mitsuo MATSUMOTO^a

Department of Pharmaceutics, Showa College of Pharmaceutical Sciences,^a Higashi-Tamagawagakuen, Machida, Tokyo 194, Japan and Pharmaceutical Development Laboratory, Kirin Brewery Co., Ltd.,^b Miyahara, Takasaki, Gumma 370-12, Japan. Received February 1, 1996; accepted May 8, 1996

The pharmacodynamic activities (leukopoietic effect) and pharmacokinetics of recombinant human granulocyte colony-stimulating factor (rhG-CSF) administered rectally in various doses as hollow-type suppositories were investigated in rabbits. Three kinds of rhG-CSF hollow-type suppositories were employed: suppository I containing rhG-CSF (100–1000 µg/kg) in 10 mM acetate buffer solution (ABS) at pH 4.0, suppository II containing rhG-CSF in ABS with α -cyclodextrin (α -CyD) as an absorption-enhancing agent, and suppository III containing lyophilized rhG-CSF powder. We found that the total count of leukocytes in peripheral blood (total blood leukocyte count) and serum granulocyte colony-stimulating factor (G-CSF) concentration showed a dose-dependent increase. Consequently, the area under the increased total blood leukocyte count–time curve (*AUL*), an index of pharmacodynamic activity, and the area under the serum G-CSF concentration–time curve (*AUC*), a pharmacokinetic parameter, increased with an increase in the rhG-CSF dose administered rectally. Good correlation was found between *AUL* and *AUC*; thus, it is concluded that an increase in *AUC* leads to an increase in the effect of rhG-CSF in inducing leukopoiesis in rabbits following rectal administration. We report for the first time the rectal absorption of rhG-CSF using a new rectal dosage vehicle (rhG-CSF hollow-type suppository) and its leukopoietic effect. The rhG-CSF hollow-type suppository is found to be a promising drug delivery system.

Key words granulocyte colony-stimulating factor; leukopoietic effect; serum G-CSF concentration; hollow-type suppository; α -cyclodextrin; rabbit

The recombinant human granulocyte colony-stimulating factor (rhG-CSF), which stimulates granulocyte precursor cells in the bone marrow and promotes their differentiation and proliferation into granulocytes, has been produced with high purity and in large quantities by means of recombinant DNA technology.^{2,3)} We recently found that following the intranasal administration of a formulation comprising an aqueous solution of rhG-CSF (non-glycosylated protein type) and cyclodextrins (natural type and its derivatives such as dimethyl- β -cyclodextrin), significant increases in the serum granulocyte colony-stimulating factor (G-CSF) concentration and the total count of leukocytes in peripheral blood (total blood leukocyte count) in rabbits were observed.^{4,5)} Machida and coworkers⁶⁾ reported the effect of surfactants (polyoxyethylene 9-lauryl ether and sodium glycocholate) on the nasal absorption of rhG-CSF (glycoprotein type) in rats. It was suggested that a promising rhG-CSF delivery system has been developed in the form of intranasal administration of rhG-CSF,^{1,7)} since it might improve the mucosal membrane permeability of rhG-CSF and induce leukopoiesis. These findings led us to investigate whether rhG-CSF absorption *via* another delivery route might be possible. To our knowledge, no experiment on the rectal absorption of rhG-CSF and the observation of subsequently induced leukopoiesis has been reported.

To evaluate the effectiveness of rhG-CSF *via* a new administration route, we investigated the rectal absorption and blood leukocyte dynamics of rhG-CSF using the hollow-type suppository developed by Watanabe *et al.*^{8,9)} When a suppository containing protein or a peptide drug is prepared, it is necessary to eliminate the effect of the

heating process of the conventional method of suppository preparation on these drugs, because this method includes a heating procedure to melt the suppository base and allow it to mix with the peptide drugs. The advantages of the use of hollow-type suppository are that it eliminates the need for heating during preparation with protein drugs, so that differences in the release rate of drugs are minimized.¹⁰⁾ The cavity can contain aqueous preparations as well as powdered forms. We successfully applied these suppositories as a dosage vehicle for the rectal delivery of polypeptide drugs such as insulin.^{11,12)}

To investigate the leukopoietic effect and pharmacokinetics of rhG-CSF following rectal administration in rabbits, we chose a new rhG-CSF formulation using a hollow-type suppository which can contain various amounts of rhG-CSF in the aqueous solution form. The rectal absorption of rhG-CSF and the effect of formulation factors such as the concentration and use of absorption-enhancing agents were evaluated.

MATERIALS AND METHODS

Materials A lyophilized powder of rhG-CSF (filgrastim, Kirin Brewery Co., Tokyo, Japan) which is a non-glycosylated recombinant human protein consisting of 175 amino acid residues (molecular weight: 18600 daltons),³⁾ and a concentrated bulk solution of rhG-CSF were used. α -Cyclodextrin (α -CyD), which is a new absorption-enhancing agent,¹³⁾ was supplied by Nihon Shokuhin Kako Co., Tokyo, Japan. A suppository base, Witepsol H-15 (H-15), was obtained from Hüls Troisdorf, Troisdorf, Germany. All other reagents used were of

* To whom correspondence should be addressed.

Table 1. Amounts of rhG-CSF and Additives in Hollow-Type Suppositories

Preparation	Dose	
	rhG-CSF ($\mu\text{g/kg}$)	α -CyD (mg/kg)
Suppository I (Aqueous solution) ^{a)}	100	—
	300	—
	600	—
	1000	—
Suppository II (Aqueous solution) ^{a)}	100	10
Suppository III (Lyophilized powder)	1000	—

a) rhG-CSF and α -CyD were dissolved in 10 mM acetate buffer solution (pH 4.0).

analytical grade.

Preparation of Suppositories Formulations of four kinds of hollow-type suppositories (approximately 2 g), containing rhG-CSF aqueous solution alone (suppository I), rhG-CSF aqueous solution with α -CyD (suppository II) and lyophilized rhG-CSF powder (suppository III), were prepared using Witepsol H-15 by the fusion method reported by Watanabe *et al.*⁹⁾ For the control experiments, a suppository containing α -CyD without rhG-CSF was prepared using Witepsol H-15. The amounts of rhG-CSF and additives are summarized in Table 1. A freshly prepared aqueous solution of rhG-CSF, with or without additives, was obtained by diluting the concentrated bulk solution of rhG-CSF (20 mg/ml) to an appropriate concentration with 10 mM acetate buffer solution⁷⁾ at pH 4.0. The prepared solution at a dosage volume of 50 μl per kg body weight of rabbit was filled into the cavity (approximately 0.4 cm^3) of the suppository, and then the opening at the hind part of the suppository was sealed with the suppository base by melting. All suppositories were refrigerated overnight after preparation before being used in the experiments.

Animal Experiments Male albino rabbits weighing 2.8 to 3.0 kg were used. They freely received food and water and were housed individually in cages in a forced-air facility that was maintained at $23 \pm 1^\circ\text{C}$ and 55% relative humidity with a 12-h light/dark cycle. Animals with free access to water were fasted for one night prior to each experiment. Each suppository was administered into the rectum according to the method described in our previous reports.^{10–12)}

After rectal administration, 2 ml blood samples were taken from the auricular vein by a syringe with or without edetate disodium (EDTA-2Na) at predetermined time intervals. These samples were centrifuged at 3000 rpm for 15 min to separate the serum. Each serum sample was stored at -30°C until the assays for rhG-CSF were performed.

Determination of Serum G-CSF Concentration and Total Blood Leukocyte Count Serum G-CSF concentration was determined by the enzyme immunoassay (EIA) method reported by Tanaka and Tokiwa.¹⁴⁾ For the determination of the total blood 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®, Nikkaki, Tokyo,

Japan). The determination of the total blood leukocyte count was performed using a Coulter Counter (Model ZM, Nikkaki, Tokyo, Japan) as described in our previous papers.^{1,7)}

Pharmacokinetic and Pharmacodynamic Data Analysis

As pharmacokinetic parameters, the peak serum G-CSF level (C_{max}) and the time to reach peak serum G-CSF concentration (t_{max}) were obtained from individual serum G-CSF concentration–time curves. The area under the individual serum G-CSF concentration–time curves from 0 to 12 h after rectal administration (AUC_{0-12}) was calculated using the trapezoidal rule.¹⁵⁾ The increased total blood leukocyte count was calculated using Eq. 1.

increased total blood leukocyte count

= (leukocyte count at each time point following administration)

– (leukocyte count before administration) (1)

Then, the area under the increased total blood leukocyte count–time curve (AUL),⁷⁾ an index of pharmacodynamic activity, was determined from 0 to 96 h following rhG-CSF administration using the trapezoidal rule.

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

RESULTS AND DISCUSSION

Serum G-CSF Concentration and Total Blood Leukocyte Count in Rabbits Following Rectal Administration of rhG-CSF in Aqueous Solution To investigate the time courses of serum G-CSF concentration and the total blood leukocyte count after rectal administration, suppository I containing rhG-CSF at various doses in buffer solution was inserted into the rectum of rabbits. The primary advantage of the hollow-type suppository is that it can deliver rhG-CSF in a solution dosage form to the rectal lumens. The releasing process of rhG-CSF in aqueous solution is not the critical limiting step because drugs can release rapidly from this suppository.^{9,11)} The mean semi-log serum G-CSF concentration–time curves and the change in total blood leukocyte count profiles (increased total blood leukocyte count) are shown in Figs. 1 and 2, respectively. Mean values of the pharmacokinetic parameters of G-CSF are summarized in Table 2. Serum G-CSF concentrations were rapidly increased, and t_{max} was reached within 1 h after the administration of suppository I containing rhG-CSF (100–1000 $\mu\text{g/kg}$). Therefore, rhG-CSF was rapidly absorbed from the rectal mucosa following the administration of hollow-type suppositories. At a dose of 100 $\mu\text{g/kg}$, the increased total blood leukocyte count reached a level of approximately 5000/ μl between 12 and 96 h, and serum G-CSF concentrations were observed in a level between 1 and 10 ng/ml. It is reported¹⁶⁾ that a serum G-CSF level of greater than 1 ng/ml was obtained at 10 $\mu\text{g/kg}$ of the subcutaneous dose, and this concentration supports a near-maximum neutrophil proliferation *in vitro*. We previously estimated that the minimum serum G-CSF level required to exert a leukopoietic effect is approximately

Fig. 1. Serum G-CSF Concentration–Time Curves Following Rectal Administration of Hollow-Type Suppositories Containing rhG-CSF in Various Doses in Acetate Buffer Solution (pH 4.0) in Rabbits

RhG-CSF dose ($\mu\text{g/kg}$): $\circ$, 100; $\square$, 300; $\triangle$, 600; $\diamond$, 1000. Each point represents the mean $\pm$ S.E. (vertical bar) of at least three rabbits.

Fig. 2. Increased Total Blood Leukocyte Count Following Rectal Administration of Hollow-Type Suppositories Containing rhG-CSF in Various Doses in Acetate Buffer Solution (pH 4.0) in Rabbits

RhG-CSF dose ($\mu\text{g/kg}$): $\circ$, 100; $\square$, 300; $\triangle$, 600; $\diamond$, 1000. Each point represents the mean $\pm$ S.E. (vertical bar) of at least three rabbits. Dotted line indicates values obtained after administration of suppository without rhG-CSF (control experiment). Statistically significant differences: a) $p < 0.05$ in 600 or 1000 $\mu\text{g/kg}$ vs. control value (without rhG-CSF); b) $p < 0.01$ in 300, 600 or 1000 $\mu\text{g/kg}$ vs. control value (without rhG-CSF).

1 ng/ml following intranasal administration. A serum G-CSF concentration of greater than 1 ng/ml was sustained for 4 h after rectal administration at a dose of 100 $\mu\text{g/kg}$ (the lowest dose in this study), and this result is in agreement with those obtained after intranasal administration. When the dose of rhG-CSF was increased to 300 $\mu\text{g/kg}$, serum G-CSF concentration increased significantly and exceeded 1 ng/ml for approximately 8 h (Fig. 1). The increased total blood leukocyte count, reaching a maximum of 14000/ μl , was obtained at 12 h after the administration of rhG-CSF and this increased count was maintained for 96 h (Fig. 2). Furthermore, the increased total blood leukocyte count was significantly larger than that observed between 6 and 24 h when higher rhG-CSF doses of 600 and 1000 $\mu\text{g/kg}$ were administered, and the maximum value reached approximately 16000/ μl .

Table 2. Pharmacokinetic Parameters of rhG-CSF Following Rectal Administration of Hollow-Type Suppositories Containing rhG-CSF in Rabbits

rhG-CSF dose ($\mu\text{g/kg}$)	AUC_{0-12} (h $\cdot$ ng/ml)	C_{max} (ng/ml)	t_{max} (h)
Rectal			
100	12.5 $\pm$ 11.8	4.7 $\pm$ 4.5	0.8 $\pm$ 0.1
300	168.9 $\pm$ 73.1	90.5 $\pm$ 37.1	0.8 $\pm$ 0.2
600	357.9 $\pm$ 128.9	176.5 $\pm$ 46.5	0.7 $\pm$ 0.2
1000	549.5 $\pm$ 268.1	163.2 $\pm$ 65.6	1.0 $\pm$ 0.3
Intravenous			
1	68.5 $\pm$ 12.5	—	—
10	919.3 $\pm$ 97.4	—	—

Each value represents the mean $\pm$ S.E. of at least three experiments.

Fig. 3. Serum G-CSF Concentration–Time Curves Following Rectal Administration of Hollow-Type Suppositories Containing rhG-CSF Solution with or without α -CyD in Rabbits

rhG-CSF dose: 100 $\mu\text{g/kg}$. Key: $\circ$, without α -CyD; $\bullet$, with α -CyD (10 mg/kg). Each point represents the mean $\pm$ S.E. (vertical bar) of at least three rabbits.

As shown in Table 2, the values of AUC_{0-12} and C_{max} increased with an increasing dose of rhG-CSF. However, no significant difference was found between these parameters obtained at 600 and 1000 $\mu\text{g/kg}$. The release of rhG-CSF from a hollow-type suppository is not the critical limiting step, as stated above. Thus, it may be concluded that rhG-CSF was absorbed by a non-linear mechanism.

To enhance the rectal absorption of rhG-CSF when a low dose of rhG-CSF was administered, we investigated the coadministration of rhG-CSF with α -CyD as an absorption-enhancing agent.¹³⁾ We found previously that natural CyDs (α - or β -type) and their derivatives, such as dimethyl- β -CyD, enhanced the nasal absorption of rhG-CSF.^{4,5)} Although several studies concerning the effect of CyDs on the absorption of polypeptide drugs from nasal mucosa have been reported,¹⁷⁻²⁰⁾ little is known about the effect of CyDs on the rectal absorption of a polypeptide drug from suppositories.¹²⁾ We chose α -CyD, which enhanced rhG-CSF absorption following intranasal administration in rabbits.^{1,7)} Figure 3 illustrates the serum G-CSF concentration–time curves following the rectal administration of suppository II containing rhG-CSF (100 $\mu\text{g/kg}$) with or without α -CyD (10 mg/kg). The rectal absorption of rhG-CSF was

Fig. 4. Increased Total Blood Leukocyte Count Following Rectal Administration of Hollow-Type Suppositories Containing rhG-CSF Solution with or without α -CyD in Rabbits

rhG-CSF dose: 100 μ g/kg. Key: $\circ$, without α -CyD; $\bullet$, with α -CyD (10 mg/kg). Each point represents the mean $\pm$ S.E. (vertical bar) of at least three rabbits. Statistically significant differences: a) $p < 0.05$ in rhG-CSF with α -CyD vs. without α -CyD.

markedly enhanced by coadministration with α -CyD compared with the administration of rhG-CSF alone. The serum G-CSF level significantly increased ($C_{\max}$: 20.8 ± 8.1 ng/ml, an approximately four-fold increase; AUC_{0-12} : 66.6 ± 27.8 h $\cdot$ ng/ml, an approximately five-fold increase). Consequently, the increased total blood leukocyte count upon coadministration of rhG-CSF and α -CyD increased significantly in comparison with that following the rectal administration of rhG-CSF without α -CyD (Fig. 4). Of course, α -CyD administered rectally without rhG-CSF did not lead to a leukopoietic effect in the control experiments. The enhancement of rhG-CSF absorption by α -CyD from the rectal mucosa (AUC_{0-12} , 66.6 ± 27.8 h $\cdot$ ng/ml) was less than that (AUC_{0-12} , 199.8 ± 42.5 h $\cdot$ ng/ml) from the nasal mucosa.⁷⁾ However, rectal coadministration of rhG-CSF and α -CyD exerted efficient leukopoietic effects. Thus, this formulation is quite effective.

It was assumed that a high concentration of drug on the luminal surface of the rectum would increase the rate and extent of absorption. For instance, we found that the extent of rectal absorption of gentamicin, a poorly absorbed antibiotic, in the powdered form was significantly higher than that of gentamicin in aqueous solution when it was coadministered with salicylic acid, an absorption-enhancing agent.²¹⁾ Generally, the lyophilized powder of a protein or peptide drug is water-soluble. The rectal absorption of rhG-CSF administered in a powdered form may be increased because rhG-CSF exists in a high concentration at the absorption sites. A hollow-type suppository can contain a powdered form of rhG-CSF as well as an aqueous solution. To elucidate the effect of a dosage form on the rectal absorption of rhG-CSF, we examined rhG-CSF absorption after the administration of suppository III which contained the lyophilized powder of rhG-CSF (1000 μ g/kg). Unfortunately, an increase in the total blood leukocyte count was significantly decreased 6 to 24 h after administration, as compared with the case

Fig. 5. Increased Total Blood Leukocyte Count Following Rectal Administration of Hollow-Type Suppositories Containing rhG-CSF in Aqueous Solution or Lyophilized Powder Forms in Rabbits

rhG-CSF dose: 1000 μ g/kg. Key: $\diamond$, aqueous solution (pH 4.0); $\blacklozenge$, lyophilized powder. Each point represents the mean $\pm$ S.E. (vertical bar) of at least three rabbits. Statistically significant differences: a) $p < 0.01$ in aqueous solution vs. lyophilized powder.

Fig. 6. Relationship between AUC of G-CSF and AUL Values Obtained after Rectal or Intranasal Administration of rhG-CSF

Each point represents the mean $\pm$ S.E. (vertical bar) of at least three rabbits. Key: ∇ , rectal administration; $\blacktriangledown$, intranasal administration.

of rhG-CSF in aqueous solution (Fig. 5). Also, serum G-CSF concentration was decreased by the rectal administration of rhG-CSF powder. This result is probably related to the low dissolution rate in rectal fluid, since lyophilized rhG-CSF powder is dissolved in a pH 4.0 buffer solution, but is poorly dissolved at neutral pH.

Relationship between Pharmacodynamic and Pharmacokinetic Parameters This study demonstrated that rhG-CSF can be absorbed from the rectal mucosa in rabbits when rhG-CSF aqueous preparations are administered. We found that the serum rhG-CSF concentration showed a dose-dependent increase; consequently, the AUC_{0-12} and $C_{\max}$, pharmacokinetic parameters, increased with increases in the rhG-CSF dose administered rectally (Table 2). Figures 6 or 7 show the relationships between AUL_{0-96} , a pharmacodynamic parameter (an index of leukopoietic effect), and the AUC_{0-12} or $C_{\max}$ of G-CSF obtained following rectal administration (open symbols). Additionally, the same relationship observed after intranasal

Fig. 7. Relationship between $C_{\max}$ of G-CSF and AUL Values Obtained after Rectal or Intranasal Administration of rhG-CSF

Each point represents the mean $\pm$ S.E. (vertical bar) of at least three rabbits. Key: ∇ , rectal administration; $\blacktriangledown$, intranasal administration.

administration (filled symbols) in a previous investigation⁷⁾ is shown in Figs. 6 and 7. A good correlation was found between AUL and each pharmacokinetic parameter; thus, it can be concluded that an increase in AUC_{0-12} or $C_{\max}$ leads to an increase in the effect of rhG-CSF in inducing leukopoiesis in rabbits following rectal administration. Interestingly, the AUL_{0-96} value reached a maximum level (approximately 0.8×10^6 h·count/ μ l) when AUC_{0-12} and $C_{\max}$ increased to values of 200 h·ng/ml and 100 ng/ml, respectively. The estimated dose of rhG-CSF required to attain these two increased values was approximately 300 μ g/kg. We report for the first time the rectal absorption of rhG-CSF using a new rectal dosage vehicle (rhG-CSF hollow-type suppository) and its leukopoietic effect. Thus, the rhG-CSF hollow-type suppository is found to be a promising drug delivery system.

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