Effects of a Dosing Solution on the Nasal Absorption of Non-glycosylated Recombinant Human Granulocyte Colony-Stimulating Factor in Rats

Hideaki NOMURA,* Satoshi AKAMISAKA, and Kazutoshi MARUYAMA

Pharmaceutical Development Laboratory, Kirin Brewery Co., Ltd., 3 Miyahara-cho, Takasaki, Gunma 370-12, Japan. Received April 17, 1996; accepted July 30, 1996

The nasal absorption of non-glycosylated recombinant human granulocyte colony-stimulating factor produced by *Escherichia coli* (*E. coli*-rhG-CSF) was studied in rats. We investigated the effects of the basic conditions of the dosing solution and the enhancing effects of various substances to find means to improve the nasal absorption of *E. coli*-rhG-CSF. Relative bioavailability following the nasal administration of *E. coli*-rhG-CSF solution to subcutaneous administration was 3.6%. Osmotic pressure of the dosing solution had virtually no effect on the nasal absorption of *E. coli*-rhG-CSF. Absorption was enhanced by a decrease in pH, an increase in buffer concentration at pH 2 and an increase in drug concentration. Sodium cholate, ascorbic acid, tartaric acid, β -cyclodextrin and dimethyl- β -cyclodextrin remarkably increased the nasal absorption of *E. coli*-rhG-CSF. Condition adjustments to the dosing solution and the use of several enhancers were significantly concluded to increase the intranasal absorption of *E. coli*-rhG-CSF.

Key words granulocyte colony-stimulating factor; nasal absorption; pH; osmotic pressure; enhancer

The granulocyte colony-stimulating factor (G-CSF) is capable of stimulating the proliferation of bone marrow precursor cells and their differentiation into granulocyte colonies. Recombinant human G-CSF (rhG-CSF) is quite useful as a therapeutic agent. Glycosylated rhG-CSF produced by Chinese hamster ovary cells (CHO-rhG-CSF), non-glycosylated rhG-CSF produced by *Escherichia coli* (*E. coli*-rhG-CSF) and a non-glycosylated rhG-CSF derivative produced by *E. coli* are presently finding clinical application. The N-terminal amino acids of the third type differ from those of the second type. This study used *E. coli*-rhG-CSF, a single-chain polypeptide with a molecular weight of about 19000 and consisting of 175 amino acids.

The clinical application of rhG-CSF is limited to either subcutaneous or intravenous administration. There are problems such as the inconvenience of the injection dosage form in the clinical use of protein drugs, including rhG-CSF. Alternative administration routes for rhG-CSF should thus be sought. Although the feasibility of the oral administration of rhG-CSF has been reported,¹⁻³⁾ there are problems associated with enzymatic and acid digestion, together with apparently poor gastrointestinal permeability. Alternatively, rhG-CSF is rapidly and effectively absorbed from the lung.^{4,5)} However, delivering the drug to the lung lobes is difficult because most of the administered drug adheres to the mouth, trachea, esophagus and administrative device. The nasal route appears to be a good alternative due to its ease of administration and rich vascularization.⁶⁾ Recombinant human G-CSF is absorbed from rat nasal mucosa using glycosylated CHO-rhG-CSF,^{7,8)} and absorption is slightly dependent on pH and the osmotic pressure of the dosing solution. Also, *E. coli*-rhG-CSF is absorbed from rabbit nasal mucosa and its absorption has been shown to be enhanced by cyclodextrins.^{9,10)}

To find a means of attaining more efficient nasal absorption of *E. coli*-rhG-CSF, examination was made of the effects of basic conditions such as pH, osmotic pressure, buffer concentration and drug concentration on

absorption. An increase in the nasal absorption of *E. coli*-rhG-CSF using various enhancers was also studied.

MATERIALS AND METHODS

Materials *E. coli*-rhG-CSF, a non-glycosylated recombinant human protein, was produced by Kirin Brewery Co., Ltd., as previously reported.¹¹⁾ Propylene glycol, benzyl alcohol, tartaric acid and aprotinin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Polyoxyethylene 20 sorbitan monooleate (Polysorbate 80) and polyoxyethylene hydrogenated castor oil 60 (HCO 60) were kindly supplied by Nikko Chemicals Co. (Tokyo, Japan). Sodium caprylate and lecithin were from Kanto Chemical Co. (Tokyo, Japan) and Nichiyu Liposome Co. (Tokyo, Japan), respectively. Glycine and lysine were supplied by Ajinomoto Co. (Tokyo, Japan). Sodium cholate, benzalkonium chloride and ascorbic acid were from Nacalai Tesque (Kyoto, Japan). β -Cyclodextrin and dimethyl- β -cyclodextrin were kindly supplied by Nihon Shokuhin Kako Co. (Tokyo, Japan). All other reagents were of analytical grade or better.

Preparation of *E. coli*-rhG-CSF Solution as the Dosing Formulation For a comparison of administration routes, solutions for intravenous and subcutaneous administration were prepared by diluting the original bulk solution, which had a pH of 4; osmotic pressure of 14 mOsm/kg; buffer composition of 10 mM acetate and sodium acetate; and concentration of *E. coli*-rhG-CSF of 0.5 mg/ml. Solutions for intranasal administration were obtained by concentrating the original bulk solution. To examine the effects of pH, osmotic pressure, buffer concentration and drug concentration, the four conditions specified in Table 1 were used. The pH of the dosing solution was adjusted with 1 N HCl or 1 N NaOH. The osmotic pressure in all cases was adjusted by NaCl. To enhance the nasal absorption of *E. coli*-rhG-CSF, absorption enhancers were each used at a final concentration of 1%. This concentration is used often and thus the present results

* To whom correspondence should be addressed.

Table 1. Conditions for Dosing Solution Intranasal Administration

	pH	Osmotic pressure (mOsm/kg)	Concentration of buffer composition (phosphate and citrate, mM)	Drug concentration (mg/ml)	Dose (μ g/kg)
1	2–7	283 (isotonic)	100	1.0	50
2	4	28, 283, 821	a)	1.0	50
3	2 and 4	283	4, 50 and 100	1.0	50
4	4	283	100	1.0, 2.0 and 4.0	100

a) Ten mM acetate buffer solution was used.

could be compared with those in the literature.

Animal Studies Male Sprague-Dawley rats were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan), and used at 10 weeks of age (body weight 350–400 g). Surgery for intranasal absorption, following the method of Hirai *et al.*,¹²⁾ was carried out after animals had been anesthetized with an intraperitoneal combination of sodium phenobarbital (100 mg/kg) and sodium pentobarbital (50 mg/kg). Solutions for intravenous and subcutaneous administration were injected *via* the tail vein and back skin, respectively. Doses for intravenous, subcutaneous and intranasal administration were of 1–50, 1–50 and 50 μ g/kg of *E. coli*-rhG-CSF, respectively. Only to examine the effects of drug concentration on nasal absorption was 100 μ g/kg of *E. coli*-rhG-CSF used (Table 1). Blood samples were collected periodically from the jugular vein for 6 h after each administration. The area under the curve of the plasma *E. coli*-rhG-CSF concentration from 0 to 6 h (AUC_{0-6}) was calculated by the trapezoidal rule, and the bioavailability was assessed based on the ratio of the AUC to the dose.

Enzyme-Linked Immunosorbent Assay for *E. coli*-rhG-CSF Plasma *E. coli*-rhG-CSF concentration was determined by an enzyme-linked immunosorbent assay (ELISA) established at our laboratory.¹³⁾

RESULTS

The mean plasma concentration of *E. coli*-rhG-CSF versus time following intravenous (dose, 10 μ g/kg), subcutaneous (dose, 10 μ g/kg) and intranasal (dose, 50 μ g/kg) administrations are compared in Fig. 1. In intravenous and subcutaneous administrations, a dose-dependent increase in the AUC_{0-6} was observed at 1–50 μ g/kg (data not shown). The plasma concentration of *E. coli*-rhG-CSF following intranasal administration reached a maximum at approximately 2 h and gradually decreased thereafter. The AUC_{0-6} following intravenous, subcutaneous and intranasal administrations was 102.9 ± 7.5 (S.E.), 39.1 ± 4.2 and 7.0 ± 1.5 ng·h/ml, respectively. The bioavailability of *E. coli*-rhG-CSF following intranasal administration, compared to intravenous and subcutaneous administration, was 1.37 ± 0.28 and $3.59 \pm 0.74\%$, respectively.

The effects of the dosing solution pH on the intranasal absorption of *E. coli*-rhG-CSF are shown in Fig. 2. AUC s at a low pH of 2 and 3 were much higher than at other pH values. Table 2 shows the effects of the conditions of other dosing solutions. A somewhat higher AUC at low

Fig. 1. Plasma Concentration of *E. coli*-rhG-CSF versus Time after Intravenous (dose, 10 μ g/kg; ■), Subcutaneous (dose, 10 μ g/kg; ▲) and Intranasal (dose, 50 μ g/kg; ●) Administrations

The pH, osmotic pressure and buffer composition of *E. coli*-rhG-CSF of the dosing solution for each administration were 4.0, 14 mOsm/kg and 10 mM acetate, respectively. *E. coli*-rhG-CSF concentrations of the dosing solution for intravenous, subcutaneous and intranasal administration were 20 μ g/ml, 20 μ g/ml and 1.0 μ g/ml, respectively. Values represent means $\pm$ S.E. of more than four rats.

Fig. 2. Effects of the pH of the Dosing Solution on the Intranasal Absorption of *E. coli*-rhG-CSF

Conditions of the dosing solution are given in Table 1. Values represent means $\pm$ S.E. of more than six rats. * $p < 0.01$ compared with the pH 7 group, ** $p < 0.05$ compared with the pH 7 group, *** $p < 0.01$ compared with the pH 4 group.

Table 2. Effects of Osmotic Pressure, Buffer Concentration and Drug Concentration of the Dosing Solution on Intranasal Absorption

Conditions	AUC_{0-6} (ng·h/ml)
Osmotic pressure (mOsm/kg)	
28	10.8 ± 3.3
283	5.6 ± 4.0
821	5.7 ± 1.4
Buffer concentration (mM)	
pH 2	
4	21.1 ± 10.9
50	59.6 ± 10.0*
100	94.5 ± 10.0**
pH 4	
4	9.1 ± 3.1
50	10.9 ± 3.4
100	14.1 ± 3.4
Drug concentration (mg/ml)	
1.0	21.3 ± 3.3
2.0	39.0 ± 9.4
4.0	63.9 ± 27.6

Values represent means ± S.E. of four rats. * $p < 0.05$ compared with the 4 mM group, ** $p < 0.01$ compared with the 4 mM group. Other conditions for dosing solution administration are given in Table 1.

Table 3. Effects of 1% Enhancers Added to the Dosing Solution on the Intranasal Absorption of *E. coli*-rhG-CSF

	AUC_{0-6} (ng·h/ml)	Enhancing index ^{a)}
Control ^{b)}	7.0 ± 1.5	1
Propylene glycol	2.5 ± 1.2	0.36
Polysorbate 80 ^{c)}	4.7 ± 1.3	0.67
HCO 60 ^{d)}	9.0 ± 2.0	1.28
Sodium caprylate	1.8 ± 0.8	0.26
Sodium cholate	210 ± 72 [†]	30.0
Lecithin	10.8 ± 4.4	1.54
Benzalkonium chloride	1.6 ± 0.3	0.23
Benzyl alcohol	13.7 ± 3.8	1.96
Glycine	12.7 ± 12.0	1.81
Lysine	10.1 ± 5.2	1.44
Ascorbic acid	148 ± 34 [†]	21.1
Tartaric acid	258 ± 42*	36.9
β -Cyclodextrin	283 ± 59**	40.4
Dimethyl- β -cyclodextrin	278 ± 11*	39.7
Aprotinin	34.9 ± 24.3	4.99

a) AUC of each substance/ AUC of the control. b) In the control, the original solution of *E. coli*-rhG-CSF (pH 4) was administered intranasally (dose, 50 μ g/kg), with an osmotic pressure, buffer composition, and concentration of *E. coli*-rhG-CSF of 14 mOsm/kg, 10 mM acetate and sodium acetate, and 1.0 mg/ml, respectively. c) Polyoxyethylene 20 sorbitan monooleate. d) Polyoxyethylene hydrogenated castor oil 60. Values represent means ± S.E. of four rats. * $p < 0.01$ compared with the control, ** $p < 0.05$ compared with the control, [†] $p < 0.10$ compared with the control.

osmotic pressure was observed. When the buffer concentration of the dosing solution was changed at pH 4, the AUC remained virtually unchanged. However, at pH 2 the AUC increased remarkably with the increase in the buffer concentration. The concentration of *E. coli*-rhG-CSF in the dosing solution also affected the intranasal absorption. The AUC tended to increase with the concentration of *E. coli*-rhG-CSF.

The absorption enhancing effects of various substances added to the dosing solution following the intranasal administration of *E. coli*-rhG-CSF are indicated in Table 3. Remarkable enhancement was evident in the presence of sodium cholate, ascorbic acid, tartaric acid and cyclodextrins. In contrast, propylene glycol, polysorbate 80, sodium caprylate and benzalkonium chloride inhibited the intranasal absorption of *E. coli*-rhG-CSF.

DISCUSSION

The intranasal bioavailability of *E. coli*-rhG-CSF was found in this study to be $1.37 \pm 0.28\%$ (S.E.). Machida *et al.* reported this parameter for the nasal absorption of CHO-rhG-CSF to be $0.92 \pm 0.40\%$,⁷⁾ and these values are essentially the same. The intranasal bioavailability of rhG-CSF was somewhat more than that of other polypeptides such as insulin¹⁴⁾ and interferon.¹⁵⁾ *E. coli*-rhG-CSF is highly absorbed from the lung mucosa, with a bioavailability following intratracheal instillation of 45.9%.⁴⁾ *E. coli*-rhG-CSF would thus appear to have mucosal permeability exceeding that of other polypeptides.

The enhancing effects of the dosing solution of pH 2 and 3 on the absorption of *E. coli*-rhG-CSF differed from that reported for CHO-rhG-CSF,⁷⁾ whose intranasal absorption is slightly dependent on the pH of the dosing solution. The presence of a sugar chain bonded to the rhG-CSF molecule may be the reason for this. *E. coli*-rhG-CSF is physicochemically stable at approximately pH 4 or lower compared with neutral regions. The pH of an injectable solution for clinical use has thus been adjusted to 4. In this study, maximum absorption was observed at pH 2 and minimum at pH 6 (Fig. 2). pH 6 is consistent with that of the isoelectric point of *E. coli*-rhG-CSF. One reason the intranasal absorption of *E. coli*-rhG-CSF increases at a low pH may be the histological transformation of the nasal mucosa. However, in this study, no change in mucosal tissue was observed under a microscope following the intranasal administration of the dosing solution of pH 2 or 4. Intranasal absorption after administration of the dosing solution containing CHO-rhG-CSF at a pH of 3.0 did not increase in contrast to that at pH 7.⁷⁾ Histological transformation of the mucosal membrane may have been too small for the absorption of rhG-CSF to have been affected. Possible morphological alterations should be confirmed by electron microscope, and measurement of the exsorption of protein and lipid from the mucosa should be included as future study objectives.

The intranasal absorption of *E. coli*-rhG-CSF and CHO-rhG-CSF is enhanced by cyclodextrins^{9,10)} and non-ionic surfactants,⁸⁾ respectively. In this study, bile salts and sodium salts of fatty acids were used for comparison with cyclodextrins and surfactants. A bile salt such as sodium cholate is a well-known enhancer of nasal absorption,^{15,16)} and the sodium salt of fatty acids such as sodium caprylate enhances rectal absorption.¹⁷⁾ Sodium cholate or cyclodextrins have frequently been shown to enhance intranasal absorption by reducing the barrier function of the nasal mucosa. Moreover, it is known that sodium cholate and cyclodextrins inhibit peptidase activity in the mucosa and ensure drug activation.^{14,16)} For the absorption of *E. coli*-rhG-CSF, sodium cholate and cyclodextrins were effective, but sodium caprylate was not (Table 3). Aprotinin as a protease inhibitor to prevent *E. coli*-rhG-CSF degradation in the nasal mucosa failed to have any significant effect (Table 3). A study on the stability of *E. coli*-rhG-CSF in the homogenate of rat nasal mucosa indicated no

degradation of *E. coli*-rhG-CSF (data not shown). Accordingly, sodium cholate and cyclodextrins may be considered effective only for enhancing the membrane transport of *E. coli*-rhG-CSF. This is at variance with Machida *et al.*, who reported that the effect of sodium cholate is not significant.⁸⁾ Physicochemical differences in the two molecules may be the reason for this.

The enhancing effect of ascorbic acid or tartaric acid on intranasal absorption may possibly have been induced by lowering the pH of the dosing solution (Fig. 2 and Table 3). The pH values of the dosing solutions containing ascorbic acid and tartaric acid were 3.0 and 2.2, respectively.

In conclusion, *E. coli*-rhG-CSF is absorbed following intranasal administration, and its plasma concentration profile is essentially the same as that following subcutaneous administration. The intranasal absorption of *E. coli*-rhG-CSF depends on the pH and drug concentration in the dosing solution. Sodium cholate and cyclodextrins have considerable potential for enhancing intranasal absorption. The effects of pH and sodium cholate on the intranasal absorption of *E. coli*-rhG-CSF differed from those on CHO-rhG-CSF as reported by Machida *et al.*⁸⁾ For the clinical intranasal application of *E. coli*-rhG-CSF to be considered, the solution should be administered at a high dose and with a low pH. For greater absorption, an effective and safe enhancer should be added. In any case, the topical safety of the preparation on the nasal mucosa should take priority.

Acknowledgements The ELISA method for *E. coli*-rhG-CSF was kindly conducted by Dr. H. Tanaka, Pharmaceutical Development Laboratory, Kirin Brewery Co., Ltd. The author thanks Professor M. Hayashi, Faculty of Pharmaceutical Science, Science University of Tokyo, for his helpful comments during the preparation of this

manuscript.

REFERENCES

- 1) Takada K., Tohyama Y., Oohashi M., Yoshikawa H., Muranishi S., Shimosaka A., Kaneko T., *Chem. Pharm. Bull.*, **37**, 838—839 (1989).
- 2) Takada K., Nakahigashi Y., Tanaka T., Kishimoto S., Ushirogawa Y., Takaya T., *Int. J. Pharm.*, **101**, 89—96 (1994).
- 3) Ushirogawa Y., Nakahigashi Y., Kiriya A., Takada K., *Int. J. Pharm.*, **81**, 133—141 (1992).
- 4) Niven R. W., Lott F. D., Cribbs J. M., *Pharm. Res.*, **10**, 1604—1610 (1993).
- 5) Niven R. W., Lott F. D., Ip A. Y., Cribbs J. M., *Pharm. Res.*, **11**, 1101—1109 (1994).
- 6) Chien Y. W., Su K. S. E., Chang S. F., "Nasal Systemic Drug Delivery," Dekker, New York, 1989, pp. 1—26.
- 7) Machida M., Sano K., Arakawa M., Hayashi M., Awazu S., *Pharm. Res.*, **10**, 1372—1377 (1993).
- 8) Machida M., Sano K., Arakawa M., Hayashi M., Awazu S., *Biol. Pharm. Bull.*, **17**, 1375—1378 (1994).
- 9) Watanabe Y., Matsumoto Y., Yamaguchi M., Kikuchi R., Takayama K., Nomura H., Maruyama K., Matsumoto M., *Biol. Pharm. Bull.*, **16**, 93—95 (1993).
- 10) Watanabe Y., Matsumoto Y., Kikuchi R., Kiriya M., Ito R., Matsumoto M., Nomura H., Maruyama K., *Int. J. Pharm.*, **110**, 93—97 (1994).
- 11) Souza L. M., Boon T. C., Gabrilove J., Lai P. H., Zsebo K. M., Murdock D. C., Chazin V. R., Bruszewski J., Lu H., Chen K. K., Barendt J., Platzer E., Moore M. A. S., Mertelsmann R., Welte K., *Science*, **232**, 61—65 (1986).
- 12) Hirai S., Yashiki T., Matsuzawa T., Mima H., *Int. J. Pharm.*, **7**, 317—325 (1981).
- 13) Tanaka H., Tokiwa T., *J. Pharmacol. Exp. Ther.*, **255**, 724—729 (1990).
- 14) Irie T., Wakamatsu K., Arima H., Aritomi H., Uekama K., *Int. J. Pharm.*, **84**, 129—139 (1992).
- 15) Maitani Y., Igawa T., Machida Y., Nagai T., *Drug Design and Delivery*, **1**, 65—70 (1986).
- 16) Hirai S., Yashiki T., Mima H., *Int. J. Pharm.*, **9**, 173—184 (1981).
- 17) Nishimura K., Nozaki Y., Yoshimi A., Nakamura S., Kitagawa M., Kakeyama N., Kitao K., *Chem. Pharm. Bull.*, **33**, 282—291 (1985).