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Pharmacological effect of recombinant human colony-stimulating factor (rhG-CSF) after administration into rat large intestine

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Abstract

The effects of organic acid and nonionic surfactant on the pharmacological activity of recombinant human granulocyte colony-stimulating factor (rhG-CSF) after administration into the large intestine of rats was investigated in comparison with administration into the small intestine. RhG-CSF was administered to rats (50 and/or 100 μg/kg) as solutions in which citric acid and/or polyoxyethylated castor oil derivative (HCO-60) were formulated as representative of both organic acid and nonionic surfactant, respectively. Test solutions were administered into the rat ascending colon or duodenum. Blood samples were collected for a 72 h period from the rat tail artery and the blood total leukocyte (BTL) counts were measured as a pharmacological index of rhG-CSF. The results are expressed as a relative increase in BTL counts as compared to the pre-dose level. The area under the curves (AUC; % BTL increase $\times$ h) obtained was used as an index for the pharmacological activity of rhG-CSF when comparing the test with the placebo solution. With respect to large intestinal administration, the following observations were made: (1) the effect of citric acid on the pharmacological activity was dependent on the amount of citric acid added to the test solution; (2) HCO-60 exerted a synergistic effect to citric acid on the pharmacological activity of rhG-CSF; (3) dose-dependent pharmacological activity of rhG-CSF was achieved at 50 and 100 μg/kg. As compared to small intestinal administration, stronger pharmacological activity of rhG-CSF was elicited by adminstration into the large intestine. These results are in support of the potential use of colonic delivery of rhG-CSF as an oral dosage form.

Key words: Recombinant human granulocyte colony-stimulatiug factor (rhG-CSF); Organic acid; Nonionic surfactant; Large intestine; Colon delivery; Oral availability; Blood total leukocyte count; Rat

1. Introduction

Recombinant human granulocyte colony-stimulating factor (rhG-CSF) is a protein constituted by 174 amino acids having a molecular mass of 19500 Da. RhG-CSF has considerable biological activity (1.9×10^8 U/mg) which is almost of the same order as that of native G-CSF (Nagata et al., 1986; Crosier and Clark, 1992). Recent developments in biotechnology have made it possible to produce large amounts of rhG-CSF. RhG-CSF is given therapeutically via subcutaneous and intavenous injections. Clinically, rhG-CSF has been administered to patients with

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leukemia (Teshima et al., 1989), Hodgkin's disease, testicular germ cell tumor (Layton et al., 1989) and advanced pulmonary tumor (Eguchi et al., 1989) to accelerate hematologic recovery after high dose chemotherapy or irradiation with or without autologous bone marrow rescue. The duration of neutropenia was shortened in these patients and it was found that rhG-CSF is a useful drug for the treatment of cancer patients with drug- or irradiation-induced myelosuppression. However, these studies were performed with rhG-CSF injections.

To broaden the range of clinical application of this new potent drug, another administration route is required. We have been studying the possibility of oral administration of rhG-CSF (Takada et al., 1989, 1991, 1994; Ushirogawa et al., 1992). Kikuchi et al. (1993) have also been studying the intranasal route of administration with this proteinaceous drug. After both oral and intranasal administration, the pharmacological activities, namely, the increase in total blood leukocyte (BTL) counts of rhG-CSF were determined. The areas under the curve (AUC) of the % BTL count increase-time curve were determined as an index for the total pharmacologicl activity of rhG-CSF. By comparing the AUC values obtained following oral and nasal administration with that after i.v. injection, the availabilities were estimated. However, the availabilities of rhG-CSF after oral and nasal administration were low, at most 5%, as compared to i.v. administration.

Recent progress in biopharmaceutics has made it possible to deliver drugs to the colon where hydrolytic enzyme activity is lower than in the small intestine (Friend, 1992). As colon-specific drug delivery systems, a colon-specific azopolymer (Saffran et al., 1980) and a time-controlled release system such as Pulsincap™ produced by Scherer DDS Ltd have been reported. As we have confirmed that rhG-CSF undergoes extensive hydrolysis in the rat small intestine and is more stable in the large intestine, such a colon-specific drug delivery system would be a valuable dosage form for the oral administration of rhG-CSF (Ushirogawa et al., 1992). In order to evaluate the colonic absorption of rhG-CSF, the pharmacological activity of rhG-CSF solution after administration into the two intestinal sites, duodenum and ascending colon, has been evaluated in in vivo rat experiments.

2. Materials and methods

2.1. Materials

A solution of rhG-CSF (250 μg/ml) was obtained from Kirin Brewery Co., Ltd (Tokyo, Japan), anhydrous citric acid from Wako Pure Chemicals Co. Ltd (Osaka, Japan) and polyoxyethylated castor oil derivative (HCO-60®) from Nikko Chemicals Co., Ltd (Tokyo, Japan). All other reagents were commercial products of reagent grade.

2.2. Preparation of test solutions

The placebo solution was prepared by dissolving 50 mg of citric acid and 12.5 mg of HCO-60 in 2.5 ml of water. Five kinds of rhG-CSF test solutions were also prepared by the addition of known amounts of citric acid and HCO-60, as detailed in Table 1. The concentrations of rhG-CSF in the test solutions were 50 and 100 μg/ml.

2.3. Animal study

Three to four male Wistar rats (SLC, Hamamatsu, Japan), weighing 300–400 g, were used in each experimental group. Before experiments, rats were fed with a standard meal supplied by Oriental Yeast Co., Ltd (Tokyo, Japan) and water was given ad libitum. Under anesthesia induced by an intraperitoneal injection of sodium pentobarbital (45 mg/kg) midline incision was performed. Test solution was administered into rat duodenum using a glass syringe having a 27 gauge needle attached with vinyl tubing (5 mm length) through a pore on the gut near the pylorus. In the case of administration into the ascending colon, test solution was carefully administered into the space between colon epithelia and stools through a pore at the ileo-cecal junction. Administration was performed at 4 p.m. The dose of rhG-CSF

Table 1
Test solutions used in this study

Test solution	Rat group	Administration site	Dose of rhG-CSF (μg/kg)	Amount of citric acid (mg/kg)	Amount of HCO-60 (mg/kg)	Volume of test solution (ml/kg)
A	I	colon	0	20	5	0.5
A	II	duodenum	0	20	5	0.5
B	III	colon	50	20	0	0.5
B	IV	duodenum	50	20	0	0.5
C	V	colon	50	5	0	0.5
D	VI	colon	50	0	5	0.5
E	VII	colon	100	10	2.5	0.25
E	VIII	duodenum	100	10	2.5	0.25

was 50 or 100 μg/kg for all active groups of rats. Group I and II rats were the control experimental groups and received placebo test solution into their duodenum or ascending colon. The other groups of rats received test solutions containing rhG-CSF with pharmaceutical additives such as citric acid and HCO-60. Two groups of rats (III and IV) received the test rhG-CSF solution (50 μg/kg) containing citric acid (20 mg/kg) and the effect of the administration site on the pharmacological activity of rhG-CSF was studied. Group V and VI rats received rhG-CSF at the same dose level (50 μg/kg) into their ascending colon. In group V rats, the amount of formulated citric acid was less (5 mg/kg) as compared to rats of group III. In group VI rats, HCO-60 was formulated (5 mg/kg) instead of citric acid. In rats of groups VII and VIII, the rhG-CSF dose was increased 2-fold (100 μg/kg). The same test solution containing both citric acid (20 mg/kg) and HCO-60 (5 mg/kg) was used in these two groups of rats, although the administration site was different. Single blood samples (100 μl) were taken by rat tail arterial puncture after drug administration. The standard sampling schedule was at 0, 6, 18, 24, 48, and 72 h. The BTL counts were manually determined on gentian violet-stained blood smears. Namely, 0.9 ml of Türk solution was added to 100 μl of the blood sample and leukocytes were stained. The determination of BTL counts was performed using an ultramicroscope. The baseline BTL count was determined on the blood sample prior to dosing. The baseline value was considered as the 100% level and all the following BTL count-time data recorded were expressed as percent of the baseline. These values denote a percent pharmacological response (% BTL count increase). The area under the curve (AUC) of the % BTL count increase-time curve was determined as an index for the total pharmacological activity of rhG-CSF. The AUC values obtained for test solutions were compared to that of the placebo solution.

2.4. Statistics

Comparison of data was performed using Student's *t*-test and *P* values below 0.05 were regarded as statistically significant.

3. Results

The main additives used in this study were organic acid and nonionic surfactant. Therefore, at first, the effect of these additives on the blood total leukocyte (BTL) counts was examined after administration into rat duodenum and ascending colon. Fig. 1 shows the changes in BTL counts following administration of the placebo solution. The BTL counts did not show a significant increase as compared to the pre-dose level. This result supports the feasibiliy of our in vivo evaluation system for the administration of test solutions into rat duodenum and ascending colon.

With other groups of rats (III and IV), test solution containing rhG-CSF (50 μg/kg) and citric acid (20 mg/kg) was injected into the duode-

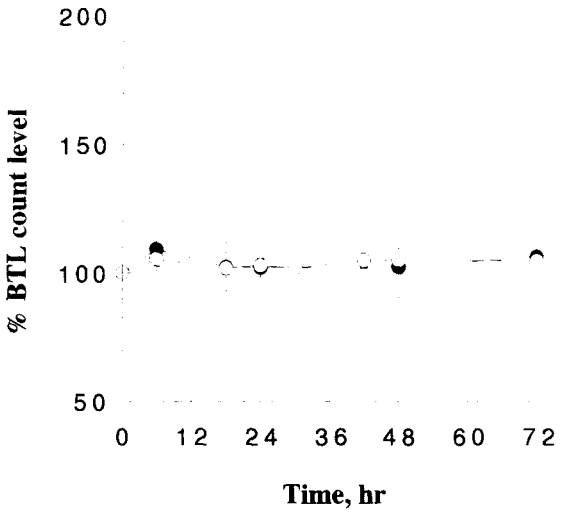

Fig. 1. Time course of blood total leukocyte (BTL) dynamics after administration of placebo solution into rat duodenum (○) or ascending colon (●). Each point represents three or four individual determinations, and is expressed as the mean ± S.E.

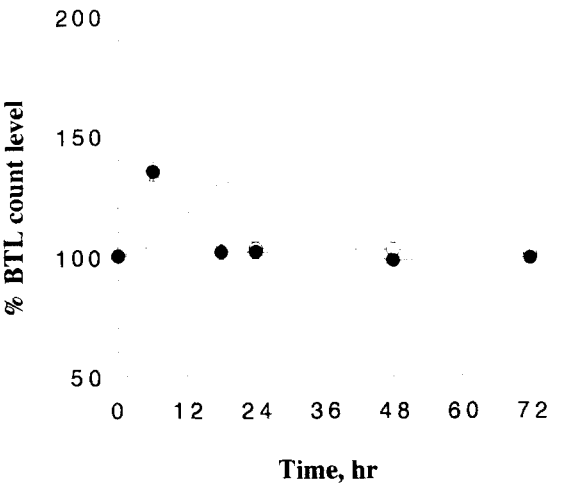

Fig. 2. Effect of citric acid (20 mg/kg) on blood total leukocyte (BTL) dynamics after administration of rhG-CSF solution into rat colon (○) and duodenum (●) (50 μg/kg). Each point represents three or four individual determinations, and is expressed as the mean ± S.E.

num and ascending colon, respectively. Fig. 2 illustrates the results. The time at which the BTL counts reached the maximum level, T_{max}, was found to occur at 18 h after colonic administration of the test solution. However, the T_{max} for intraduodenal administration was observed at 6 h after administration, although the maximum BTL count increase was comparable. To evaluate the total pharmacological activity of the test solution, the areas under the curves (AUC; % BTL increase × h) were calculated and the results are listed in Table 2. Row 1 in Table 2 shows the mean authentic AUC value for each rat group. The value in row 2 was determined by dividing the AUC value of each test group by that of the placebo group. Since two placebo groups (I, intraduodenal and II, ascending colonic administration) were used in this study, the mean AUC value of these two groups was used as the control group value. The AUC observed with group III was almost equal to that with group IV rats. Therefore, the penetration enhancing effect of citric acid is non-specific with respect to the administration site.

In group V rats, the amount of citric acid decreased to 1/4 as compared to those of group III and IV, namely, 5 mg/kg. The administration site was the ascending colon and the results are

Table 2
Comparison of pharmacologial activities with several rhG-CSF solutions

Parameter	Experimental rat group							
	I	II	III	IV	V	VI	VII	VIII
AUC [a]	153 ± 46	132 ± 132	289 ± 105	318 ± 22	286 ± 61	693 ± 191	1095 ± 390	445 ± 36
Relative pharmacological activity (%)	100 [b]		195	215	193	468	740	301

[a] AUC was determined to represent the total pharmacological activities of the test solutions and was calculated by multiplying the % BTL count increase with time (h). Each value represents the mean ± S.E.
[b] As the control value for AUC, the mean AUC value of group I and II rats was used.

shown in Fig. 3. In group V rats, the T_{max} and AUC were 6 h and 286 ± 61%, respectively. In the case of group VI rats, nonionic surfactant (HCO-60, 5 mg/kg) was formulated instead of citric acid. As shown in Fig. 3, the T_{max} appeared at 18 h after administration into the ascending colon and the AUC was 693 ± 191%, respectively. This result suggests that the pharmacological activity of rhG-CSF was considerably amplified with HCO-60. The maximum increase in BTL counts in group VI rats is significantly greater than that in group V rats. Therefore, we may state that the nonionic surfactant HCO-60 elicits the pharmacological activity of rhG-CSF more strongly than organic acid, citric acid, after the administration of rhG-CSF into the rat ascending colon.

Test solution containing 2-fold more rhG-CSF, where the amounts of pharmaceutical additives were half of those in group II rats (HCO-60, 2.5 mg/kg and citric acid, 10 mg/kg), was prepared and the effect of increased dose on the pharmacological activity of rhG-CSF was examined. At first, the test solution was administered into the ascending colon, the results being shown in Fig. 4. The AUC was significantly increased although

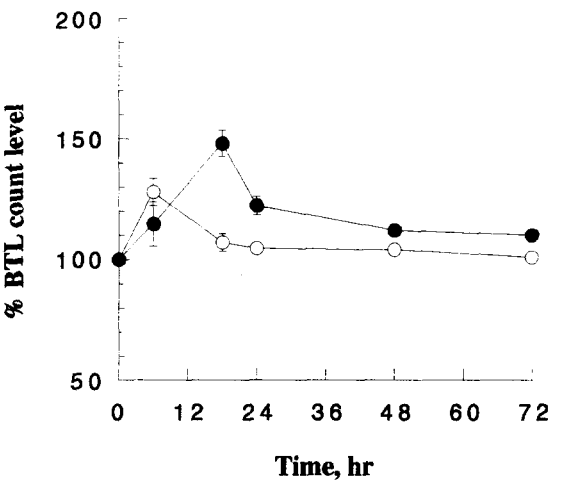

Fig. 3. Comparison of the effect of citric acid (5 mg/kg) (○) and HCO-60 (5 mg/kg) (●) on blood total leukocyte (BTL) dynamics after administration of rhG-CSF solution into rat colon (50 μg/kg). Each point represents three or four individual determinations, and is expressed as the mean ± S.E.

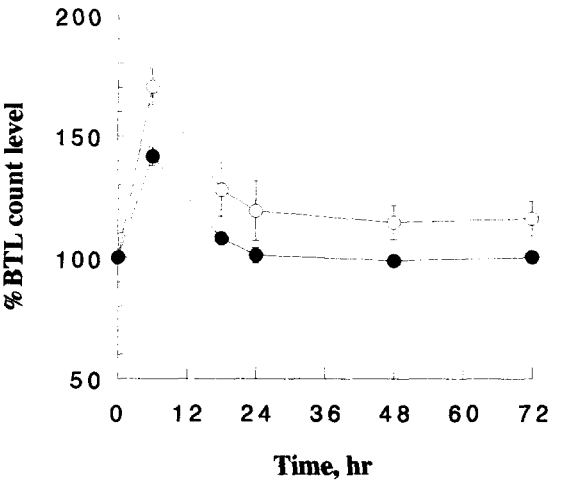

Fig. 4. Comparison of the synergistic effect of HCO-60 (2.5 mg/kg) to citric acid (10 mg/kg) on blood total leukocyte (BTL) dynamics after administration of rhG-CSF solution into rat colon (○) and duodenum (●) (100 μg/kg).

T_{max} remained unchanged. To compare the pharmacological activity of rhG-CSF, the same test solution was administered to the duodenum of group VIII rats. As is evident from Fig. 4 and Table 2, lower pharmacological activity of rhG-CSF was elicited by the administration of rhG-CSF into the rat duodenum than into the ascending colon.

4. Discussion

To improve the compliance of patients, oral dosage forms such as tablets and capsules are generally preferred. Recent developments in biotechnology have produced many clinically important new drugs such as rhG-CSF, erythropoietin and interferons. On the other hand, in the field of biopharmaceutics, formulation studies have been performed to improve the bioavailability of proteinaceous drugs after oral administration of rhG-CSF (Takada et al., 1994) and insulin (Touitou, 1992). However, the availabilities estimated by comparing the pharmacological index between oral and i.v. routes were at most 5%.

For the oral delivery of proteinaceous drugs, two kinds of technologies are needed: namely, (1)

protection of protein from the attack of hydrolytic enzymes in the gastrointestinal tract; and (2) absorption enhancement of the protein. In our previous study using an in vitro experimental system, the rate of hydrolysis of rhG-CSF by digestive enzymes was low under acidic conditions, and the stability of rhG-CSF increased on addition of a protease inhibitor, for example, typsin inhibitor (Takada et al., 1991). However, the hydrolysis of rhG-CSF was not completely inhibited. Therefore, to render rhG-CSF more stable in the gastrointestinal tract, rhG-CSF must be delivered to sites where protease activity is low. Recently, a colon-specific delivery method has attracted the interest of several pharmaceutical researchers (Saffran et al., 1980; Friend, 1992). However, even if the colon-specific delivery of proteinaceous drugs does become possible, the second problem remains to be solved. According to our previous reports, the combination of organic acid and nonionic surfactant, especially citric acid and HCO-60, elicited improved pharmacological activity after intraduodenal administration of rhG-CSF (Takada et al., 1991). In this study, we examined whether this method was also applicable after the administration of rhG-CSF to the large intestine. According to our experiments, HCO-60 exerted a synergistic effect to citric acid on the pharmacological activity of rhG-CSF after administration into the ascending colon. Therefore, in order to produce the synergistic effect of HCO-60 and citric acid, both the drug and pharmaceutical additives must be delivered into the colon after oral administration.

Saffran et al. (1986) reported on the use of new azo-polymer as a colon-specific delivery system. Although unique, the azo-polymer has not previously been used as a pharmaceutical additive. Considerable experimentation is needed to develop an oral protein delivery system using such a new pharmaceutical adjuvant. For instance, many safety studies must be performed. Besides the report of Saffran et al., many investigations on oral peptide- and protein-delivery systems have been published (Lee and Yamamoto, 1990; Lee et al., 1991; Pitt, 1990; Nellans, 1991; Ritschel, 1991; Smith et al., 1992; Swenson et al., 1992). Furthermore, microparticles, nanoparticles and liposomes have been widely studied as an oral delivery system for vaccines (Gilligan and Li Wan Po, 1991; Morishita et al., 1992; O'Hagan, 1992). However, these technologies cannot deliver proteinaceous drugs to the large intestine.

As a practically applicable and easily clinically introducible colon-specific delivery system, novel capsules offer great possibilities. Ritschel and co-workers reported on the usefulness of a gelatin capsule of which the surface was modified with formaldehyde vapor for insulin delivery (Kraeling and Ritschel, 1992; Rao and Ritschel, 1992). Another candidate is the pulsed-release dosage form designated as Pulsincap™. Our previous study was performed to evaluate several oral rhG-CSF tablets composed of citric acid and HCO-60 in in vivo rat experiments (Takada et al., 1994). Although this study was performed with rhG-CSF solutions, it should be possible to prepare our formulation contained in the colon-specific delivery system, once citric acid, HCO-60 and rhG-CSF have been formulated into solid dosage form. By combining the colonic capsule and formulation described in this report, it should be feasible to develop a novel oral rhG-CSF delivery system.

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