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Development of a Colon Delivery Capsule and the Pharmacological Activity of Recombinant Human Granulocyte Colony-stimulating Factor (rhG-CSF) in Beagle Dogs

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Abstract

A peroral dosage form was examined to deliver recombinant human granulocyte colony-stimulating factor (rhG-CSF) to the colon in beagle dogs. A new gelatin capsule with its inside surface coated with ethylcellulose was prepared for this purpose.

RhG-CSF was dissolved with propylene glycol and was filled in the capsule. Several kinds of ethylcellulose-gelatin capsules with an ethylcellulose layer of thickness 46 to 221 mm were used. The capsule was filled with propylene glycol solution containing fluorescein as an absorption marker, castor oil derivative and citric acid. The hardness of the capsule was tested after the gelatin layer was dissolved using a hardness tester and was dependent on the thickness of the ethylcellulose layer of the capsule.

The time, T_{max}, at which plasma fluorescein level reaches its maximum following oral administration of ethylcellulose capsules was used as a parameter for the in-vivo disintegration time of the ethylcellulose capsule into the colon. Capsules of thickness 84 mm with a T_{max} of 4–6 h were filled with rhG-CSF solution containing fluorescein and were administered to dogs. After administration, blood samples were collected for 96 h and the blood total leucocyte (BTL) counts were measured as a pharmacological index of rhG-CSF.

The maximum BTL count appeared at 10 h then gradually decreased and returned to its normal level at 48 h. These results suggest the usefulness of ethylcellulose capsules for the delivery of rhG-CSF to the colon and the possibility of a new oral rhG-CSF dosage form has been elucidated.

With recent advances in biotechnology, clinically important proteins such as erythropoietin, granulocyte colony-stimulating factor (G-CSF) and granulocyte monocyte colony-stimulating factor (GM-CSF) have been produced on a large scale (Szkrybalo 1987). These compounds are highly potent therapeutic agents compared with traditional drugs which are simple organic compounds (Bienz-Tradmor 1993). However, their clinical uses are limited to parenteral administration (Chen 1992). For chronic therapy, parenteral administration is inconvenient and uncomfortable. Therefore, we have studied the peroral delivery of recombinant human G-CSF (rhG-CSF) as a model drug for these protein drugs (Takada et al 1989a, b; Takada & Ushirogawa 1991; Ushirogawa et al 1992; Takaya et al 1994;). As rhG-CSF is a proteinaceous drug, it may undergo extensive hydrolysis in the gastrointestinal tract (Takada & Ushirogawa 1991). Therefore, a colon-specific oral drug delivery system has attracted much interest. Although colon-specific delivery systems using an azo-polymer (Saffran et al 1986), Pulsincap and a gelatin capsule treated with formaldehyde vapour (Kraeling & Ritschel 1992) have been reported, safety problems have not been overcome and we have tried to prepare a new safe colon-delivery system.

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Materials and Methods

Gelatin capsules (#00) were obtained from Yoshida Co., Ltd (Himeji, Japan). A solution of rhG-CSF (250 $\mu g\,mL^{-1}$) was obtained from Kirin Brewery Co., Ltd (Tokyo, Japan). Propylene glycol, anhydrous citric acid and fluorescein were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Sulphasalazine was obtained from Sigma Chemical Co. (St Louis, MO). Sulphapyridine was obtained from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). Ethylcellulose (EC, 7G and 22G) was a gift from Shin-etsu Chemical Industrial Co., Ltd, (Tokyo, Japan). Polyoxyethylated castor oil derivative (HCO-60) was obtained from Nikko Chemicals Co., Ltd. (Tokyo, Japan). Beagle dogs used in the study were obtained from Oriental Yeast Co., Ltd (Tokyo, Japan). All other materials were of reagent grade and were used as received.

Preparation of fluorescein solution

Fifty milligrams of HCO-60 and 350 mg citric acid were dissolved in 0·8 mL propylene glycol and 30 mg fluorescein was dissolved in this propylene glycol solution. The fluorescein concentration was 37·5 $mg\,mL^{-1}$ and the dose of fluorescein was 3 $mg\,kg^{-1}$.

Preparation of rhG-CSF solution

Two hundred and fifty milligrams of lyophilized rhG-CSF was dissolved with 0·8 mL of the propylene glycol solution

Table 1. Physical properties of ethylcellulose capsules.

Ethylcellulose gelatin capsule	Concn of coating fluid	Thickness (μm)					Hardness (N)
		Cap		Body			
		side	bottom	side*	side**	bottom	
#1	7G 10%	213 ± 5	321 ± 10	208 ± 4	221 ± 5	325 ± 21	17·4 ± 1·9
#2	7G 5%	117 ± 6	231 ± 3	108 ± 3	125 ± 4	222 ± 5	ND
#3	7G 3·5%	80 ± 1	206 ± 3	80 ± 2	85 ± 2	179 ± 7	ND
#4	7G 2·5%	80 ± 1	171 ± 3	70 ± 2	81 ± 2	170 ± 5	ND
#5	22G 5%	116 ± 25	243 ± 22		76 ± 7	309 ± 21	24·4 ± 4·5
#6	22G 3·75%	82 ± 14	153 ± 11		72 ± 5	178 ± 13	17·2 ± 2·6
#7	22G 3·5%	66 ± 6	163 ± 24		58 ± 2	178 ± 10	16·6 ± 3·4
#8	22G 3·25%	66 ± 6	144 ± 8		60 ± 7	170 ± 17	15·0
#9	22G 3%	82 ± 2	144 ± 4		47 ± 3	167 ± 20	ND

* 10 mm from the top. ** 15 mm from the top. ND, not determined.

prepared above. The rhG-CSF concentration was 312·5 mg mL^{-1} and the dose of rhG-CSF was 25 $\mu g\,kg^{-1}$.

Preparation of ethylcellulose-coated gelatin capsules

As shown in Table 1, several kinds of ethylcellulose-coating solutions were prepared. A mixture of methylene chloride and methanol (4 : 1) was used as the solvent. To the body and the cap of a #00 gelatin capsule, 1 and 0·55 mL of the solution were applied, respectively. The solvent was evaporated overnight at 6°C in a refrigerator. The cap was attached to the body, using a glue of concentrated ethylcellulose solution, and the coated capsule was filled with either fluorescein solution or rhG-CSF solution through a 1-mm hole at the top of the cap, made by a syringe. The capsule was sealed with concentrated ethylcellulose solution.

Physical properties of the ethylcellulose capsule

The thickness of the ethylcellulose layer was measured after the gelatin layer was dissolved in water at 37°C, at several sites on each capsule. The bottom and wall portions of both cap and body were measured using a micrometer and the mean thickness of each portion was determined. The hardness of the capsule containing approximately 1·0 mL fluorescein solution was measured using a hardness tester (Erweka apparateban GmbH Heusenstamm, Germany).

Beagle experiments

Absorption of fluorescein. Three adult male beagle dogs were fasted overnight for at least 12 h. A standard solid meal of commercial food was given at 6 h after drug administration. All experiments were carried out at the same time of day in the morning. Water was not given during this experiment. Thirty minutes before drug administration, 0·5 mL blood was removed from the jugular vein. After the oral administration of test capsules containing fluorescein, 0·5 mL samples of blood were collected. As a control experiment, gelatin capsules containing fluorescein and lactose (80 mg kg^{-1}) were orally administered to the same dogs, according to the same method described above. The standard sampling schedule was at 0·5, 1, 1·5, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 h. Plasma was immediately prepared by centrifugation and frozen at −20°C until analysed.

Measurement of colon arrival time in beagle dogs. According to the method of Kennedy et al (1979), colon arrival time in our beagle dogs was measured using sulphasalazine, a prodrug of 5-aminosalicylic acid. The cleavage of the azo-bond in the prodrug occurs in the colon releasing sulphapyridine and 5-aminosalicylic acid. The colon arrival time in the beagle dogs used in our study was estimated by determining the time of the appearance of sulphapyridine in the systemic circulation.

Pharmacodynamic study of rhG-CSF. The same dogs were used in this study under the same conditions described above. The test capsule filled with propylene glycol solution containing 25 $\mu g\,kg^{-1}$ rhG-CSF was administered orally. Thirty minutes before drug administration, 0·6 mL blood was removed from the jugular vein; these dogs also received placebo capsules. After administration, 0·1 mL blood samples were collected at 10, 24, 36, 48, 60, 72 and 96 h. Fifty microlitres of the blood sample was used for the blood total leucocyte (BTL) count, using a microcell counter CC-180A (Sysmex, Toa Medical Electronics, Kobe, Japan). The BTL count was expressed as a relative value, obtained by dividing the BTL count at each time after drug administration by the appropriate control value (the pre-dose BTL). From the remaining blood sample, 0·2 mL plasma was obtained by centrifugation and was used for the assay of plasma fluorescein.

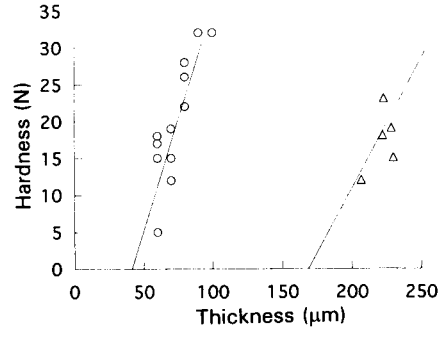

FIG. 1. Relationship between the thickness of the ethylcellulose layer and hardness of the capsules. O 22G ethylcellulose capsules (the correlation coefficient = 0·854), △ 7G ethylcellulose capsules (correlation coefficient = 0·808).

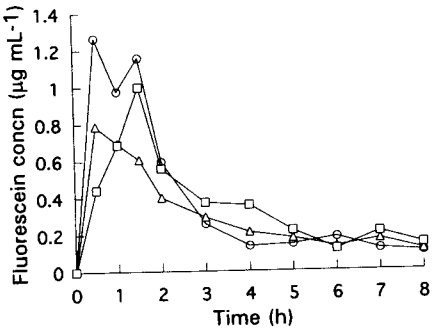

FIG. 2. Plasma fluorescein concentration–time curves after oral administration of fluorescein in gelatin capsules to beagle dogs (n = 3).

Plasma fluorescein assay

To 0·2 mL plasma, 0·5 mL methanol was added and the resulting mixture was shaken and centrifuged at 12 000 rev min^{-1} for 5 min to remove protein components. The supernatant fluid was diluted with 1 mL 0·1 M NaOH and 2 mL distilled water. The plasma fluorescein concentration was estimated by measuring the fluorescein intensity of the extracted sample in a Shimadzu RF-500LC spectrofluorometer.

Plasma sulphapyridine assay

To a 15 mL extraction tube, 100 μL plasma, 1 mL 1/15 M phosphate buffer (pH 7·4) and 2 mL ethyl acetate were added. The tube was shaken for 10 min and centrifuged at 3000 rev min^{-1} for 10 min. The organic phase was transferred to a clean glass tube, and evaporated to dryness. The residue was dissolved in the mobile phase, and an aliquot was injected onto the HPLC system.

The HPLC system for sulphapyridine analysis consisted of a Shimadzu LC-6A pump (Kyoto, Japan) and a Shimadzu SPD-10A, UV detector, connected to a Shimadzu C-R4A Chromatopac. Samples were injected using a Shimadzu SIL-6A auto-injector. The analytical column was a Chemcosarb 5-ODS-H (4·6 mm i.d. × 250 mm) (Chemco Scientific Co. Ltd, Osaka, Japan) and was maintained at 60°C. The mobile phase was a mixture of water, acetonitrile and acetic acid (89 : 10 : 1). The flow rate was 1·0 mL min^{-1}.

Data treatment

The baseline BTL count was determined as the mean of the duplicate blood samples before dosing. The mean baseline value was considered as the 100% level and all the following BTL counts were expressed as % of the baseline. These values denote a % pharmacological response (% BTL increase). All values at or above baseline were considered 100%. Significance between treatments was tested by Student's *t*-test.

Results

Physical properties of ethylcellulose capsules

Nine kinds of capsules were prepared as shown in Table 1. As shown in Fig. 1, there is a good correlation between the thickness and the hardness of the capsules, where the correlation coefficient is 0·854 for 22G capsules and 0·808 for 7G capsules.

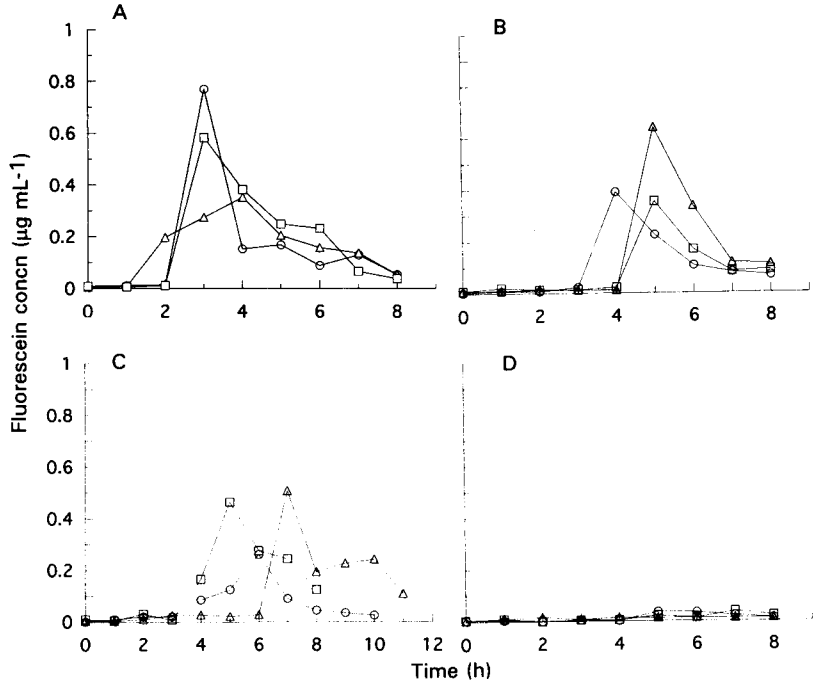

FIG. 3. Plasma fluorescein ethylcellulose concentration–time curves after oral administration to beagle dogs. A. Control capsule, B. Capsule #3, C. Capsule #7, D. Capsule #2.

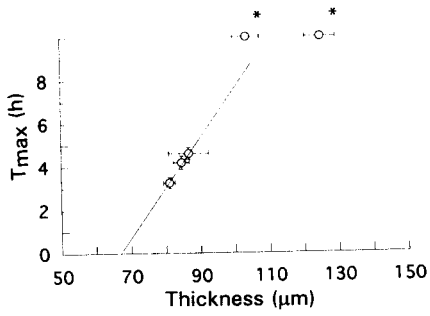

FIG. 4. Relationship between the thickness of the ethylcellulose layer and plasma fluorescein T_{max} after oral administration to beagle dogs. * These capsules did not disintegrate in the gastrointestinal tract. The correlation coefficient is 0·997.

Absorption of fluorescein in beagle dogs

Fig. 2 shows the plasma fluorescein concentration–time profiles when fluorescein was administered in a gelatin capsule to three beagle dogs. Fig. 3 shows the plasma fluorescein profile when ethylcellulose capsules were administered. From Fig. 3, it is suggested that as the thickness of the ethylcellulose capsule increases the absorption of fluorescein is delayed. Fluorescein was used as an absorption marker to estimate the time when the test capsule disintegrates in the gastrointestinal tract, and the observed T_{max} becomes an important parameter for the estimation of the disintegration time of the capsule in the gastrointestinal tract. Fig. 4 shows the relationship between the T_{max} and hardness of the capsules. From this figure, it is suggested that T_{max} is well correlated with the hardness, and therefore the thickness of the capsules.

Measurement of colon arrival time in beagle dogs

Fig. 5 shows the plasma sulphapyridine–time curves in our three dogs. Sulphapyridine appeared in the blood circulation at 4 h after oral administration of sulphasalazine. Accordingly, colon arrival time has been estimated to be 4 h in our beagle dogs.

Pharmacodynamic study of rhG-CSF in beagle dogs

The effect of rhG-CSF after oral administration as an ethylcellulose capsule was studied using capsules having a thickness of 84 mm based on the results mentioned above. The ethylcellulose capsule used in this study contained both rhG-CSF and fluorescein as an absorption marker. The

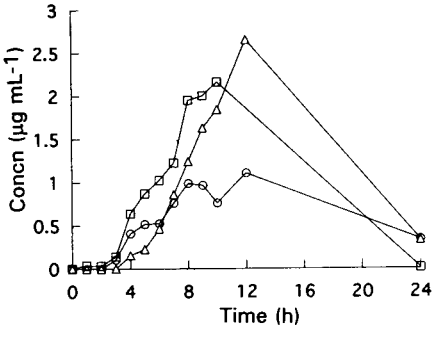

FIG. 5. Plasma sulphapyridine concentration–time curves after oral administration of sulphasalazine (25 mg kg^{-1}) to beagle dogs.

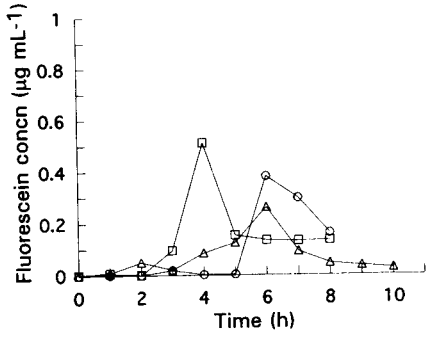

FIG. 6. Time courses of plasma fluorescein concentration after oral administration of ethylcellulose capsules containing fluorescein (3 mg kg^{-1}) and rhG-CSF (25 μg kg^{-1}) to beagle dogs.

results are shown in Figs 6 and 7. T_{max} of the absorption marker was 4–6 h, which is almost the same value as expected from the previous result shown in Fig. 3. The T_{max} observed in these beagle dogs was almost the same as that obtained in the sulphapyridine for the estimation of colon arrival time. Therefore, it may be concluded that this capsule did not disintegrate in the small intestine and was delivered to the colon. The pharmacological activity of rhG-CSF was also elucidated in this study. The BTL count started to increase at 10 h after administration and reached 1·3 times the pre-dose level. Thereafter, the BTL count gradually decreased and returned to the normal level at the end of the experiment at 96 h.

Discussion

For the oral administration of protein drugs, two important pharmaceutical technologies are required. One is to protect the hydrolysis of protein drugs in the gastrointestinal tract, and the other is to enhance the absorption of the drug from the gastrointestinal tract into the enterocytes. With respect to the second problem, the combination of citric acid and non-ionic surfactant, HCO-60, elucidated a stronger pharmacological activity of rhG-CSF after colonic administration to rats than after intraduodenal administration (Takaya et al 1994). Our previous report suggested the possibility of a nonparenteral route of rhG-CSF. However, intracolonic administration of drugs is not clinically applicable with ease, although a pharmaceutical study has been performed

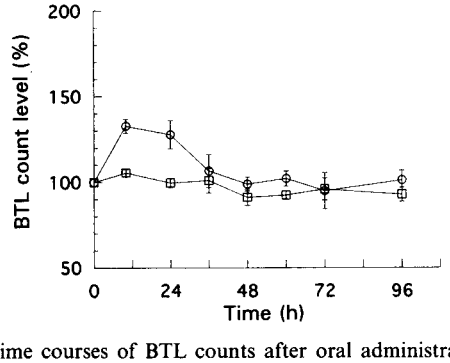

FIG. 7. Time courses of BTL counts after oral administration of ethylcellulose capsules containing fluorescein and rhG-CSF. □ Control (placebo capsules), O capsules containing fluorescein and rhG-CSF. Each point represents three individual determinations expressed as the mean ± s.e.

with recombinant human calcitonin (Hastewell et al 1994). Therefore, a colonic delivery system is required for the oral administration of rhG-CSF. Saffran et al (1986) demonstrated the usefulness of an azo-polymer for the oral administration of insulin in rabbits. Several new polymers have been synthesized (Kopecek 1977; Ashford et al 1993), and many investigators have studied oral delivery systems for peptide or protein drugs (Pusztai 1989; Pitt 1990; Lee & Yamamoto 1990; Lee et al 1991; Nellans 1991; Ritschel 1991; Smith et al 1992; Swenson & Curatolo 1992; Brøndsted & Kopecek 1992; Cummings et al 1993). However, except for the colon-specific oral delivery system, most of the administered drugs are degraded in the gastrointestinal tract by hydrolytic enzymes. For colon-specific oral drug delivery systems, Pulsincap and gelatin capsules denatured with formaldehyde vapour (Ritschel 1991) have been reported. In both types of capsule, the drugs are incorporated in the capsule in the solid state. In general, the drug molecule must be dissolved in the gastrointestinal tract before it can be absorbed. However, less water is present in the colon than in the small intestine. In preliminary experiments not presented in this report, ethylcellulose capsules containing fluorescein suspension were also administered to the dogs in our laboratory. However, poor absorption of fluorescein was observed, and we concluded that the dissolution of the drug molecule in the colon is a serious problem for the colon-specific oral delivery system. As the Pulsincap and formaldehyde-denatured gelatin capsules contain drug molecules in the solid state, the dissolution process of the drug molecule in the colon must be improved by alternative pharmaceutical technology. Our ethylcellulose capsules contain drug in the liquid state. Our previous report using cyclosporin A, a decapeptide of molecular weight 1200 Da, suggested that propylene glycol is a good solvent for the absorption of drugs from the gastrointestinal tract (Takada et al 1989b). Based on these results, we formulated rhG-CSF in propylene glycol, HCO-60 and citric acid mixture. In this formulation, rhG-CSF is well dissolved and the dissolution process in the colon will not be a problem. Thus, our system fulfills both functions; protection of the degradation of proteins in the gastrointestinal tract; and the efficient absorption of drug. In addition, our system consisted of pharmaceutical additives which have already been widely used in pharmaceutical technology, especially in oral dosage forms. Therefore, safety would not appear to be a problem for developing an oral delivery system for rhG-CSF.

Acknowledgements

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