The Effects of Absorption Enhancers on the Pulmonary Absorption of Recombinant Human Granulocyte Colony-Stimulating Factor (rhG-CSF) in Rats

Minoru MACHIDA,*a Masahiro HAYASHI,b and Shoji AWAZUc

Formulation Technology Lab., Fuji Gotemba Research Labs., Chugai Pharmaceutical Co., Ltd., 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Science University of Tokyo,b 12 Ichigaya-Funagawara-machi, Shinjuku-ku, Tokyo 162-0826, Japan, and Department of Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Science,c 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan. Received July 23, 1999; accepted September 30, 1999

Pulmonary absorption of recombinant human granulocyte colony-stimulating factor (rhG-CSF) with various surfactants and protease inhibitors were examined in rats. The relative bioavailabilities of rhG-CSF with surfactants, such as polyoxyethylene 9-lauryl ether (Laureth-9) and sodium glycocholate (SGC), after intratracheal (i.t.) administration by intravenous (i.v.) and subcutaneous (s.c.) means were 37% (i.v.), 88% (s.c.), 84% (i.v.) and 197% (s.c.), respectively. These values were evaluated from the ratio of the area under the curve (AUC) of the plasma rhG-CSF concentration versus time for 8 h. In the presence of various kinds of protease inhibitors, such as (p-amidinophenyl)methanesulfonyl fluoride-HCl (p-APMSF), aprotinin and bestatin, an increase in the plasma rhG-CSF concentration was observed, and the effect with p-APMSF was maximal. The relative bioavailabilities of rhG-CSF with p-APMSF after i.t. administration by i.v. and s.c. means were increased about 2-fold. To clarify the absorption mechanism of rhG-CSF, rhG-CSF was intratracheally administered with both Laureth-9 and p-APMSF. The AUC of rhG-CSF increased with both agents, and was approximately equal to that with SGC, which has both an enhancing effect on membrane permeation and an inhibitory effect on enzymatic degradation after i.t. administration. Consequently, it was considered that permeation and enzymatic degradation were rate-determining steps in the pulmonary absorption of rhG-CSF after i.t. administration.

Key words rhG-CSF; pulmonary absorption; absorption enhancer; intratracheal administration; surfactant; protease inhibitor

Human granulocyte colony-stimulating factor (hG-CSF), a hydrophobic glycoprotein of 20,000 Da, is a hematogenic factor which stimulates granulocyte precursor cells in bone marrow and specifically promotes their differentiation and proliferation into granulocytes. Recombinant hG-CSF (rhG-CSF) has recently been made in high purity and in large quantities.2,5 rhG-CSF has been administered intravenously and subcutaneously daily in repeated doses for the treatment of neutropenia during tumor chemotherapy, acute myelogenous leukemia (AML), myelodysplastic syndrome (MDS), aplastic anemia (AA), as well as for bone marrow transplantation therapy. Therefore, the use of more convenient routes of administration would be preferable. We have already discussed some characteristics of the nasal and pulmonary absorption of rhG-CSF in previous reports.3-5

In pulmonary absorption, the relative bioavailability of rhG-CSF after intratracheal (i.t.) administration by intravenous (i.v.) and subcutaneous (s.c.) means was 11.6% and 27.4%, respectively. The pharmacological availability was equal to or greater than the availability after i.v. or s.c. administration. From these results, we confirmed that the pulmonary absorption of rhG-CSF is an effective parenteral route of administration.5

In this study, the enhancing effects of various surfactants on the pulmonary absorption of rhG-CSF were determined by examining the characteristics of the absorption enhancer. Furthermore, the effects of protease inhibitors were examined to clarify the contribution of enzymatic degradation of rhG-CSF during pulmonary absorption.

MATERIALS AND METHODS

Chemicals The drugs, surfactants, protease inhibitors and a bulk solution of rhG-CSF used in this study and their sources are as follows: Tween 20 from Nikko Chemicals Co., Ltd., Tokyo, Japan; sodium pentobarbital (Nembutal® injection, 50 mg/ml) from Sankyo Co., Ltd., Tokyo; and phenobarbital (Phenobar® 0.1 g/ml) from Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. A bulk solution of rhG-CSF (phosphate buffer solution, 440 μg/ml, specific activity 108 U/mg protein) was supplied by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan. Sodium glycocholate (SGC) was from Tokyo Kasei Co., Ltd., Tokyo, Japan; (p-aminophenyl)-methanesulfonyl fluoride-HCl (p-APMSF) was from Wako Pure Chemical Industries, Ltd., Osaka, Japan; polyoxyethylene (POE) (9) lauryl ether (Laureth-9) from Nikko Chemicals Co., Ltd., Tokyo, Japan; aprotinin was from Yoshitomi Pharmaceutical Co., Ltd., Tokyo, Japan; and bestatin was from Sigma Chemicals, St. Louis, MO, U.S.A. Other reagents were of analytical grade or better.

Preparation of rhG-CSF Solution for Intratracheal Administration The pH of the original rhG-CSF bulk solution was adjusted to pH 6.5 with 100 mM of NaH2PO4. Then, osmotic pressure was adjusted to isotonic with NaCl. After adding the surfactants and protease inhibitors, the final rhG-CSF concentration was adjusted to 250 μg/ml with a phosphate buffer (pH 6.5). The final concentration of the surfactants was 1% (w/v), and those of the protease inhibitors were 10 mM for p-APMSF, 500 IU/ml for aprotinin and 1 mM for bestatin.

Animal Experiments for Pharmacokinetic Study Male SD rats (8 weeks old, 250—300 g: Clea Japan, Inc., Tokyo)
were used. The methods of animal surgery, intratracheal administration, and blood collection followed those of our previous report. The dose of rhG-CSF was 100 μg/kg for all administrations. Blood samples were collected periodically for 8 h to determine the plasma rhG-CSF concentration for the pharmacokinetic study.5)

**Assay** The plasma concentration of rhG-CSF was determined by enzyme immunoassay.6)

**Data Analysis** The AUC of the plasma rhG-CSF concentration vs. time for 8 h concerning the bioavailability in the presence of a surfactant and/or protease inhibitor was compared with that following the rhG-CSF i.t. administration in its absence (i.t. control).

### RESULTS

To clarify the absorbability and the mechanism of absorption of rhG-CSF through the pulmonary membrane, changes in rhG-CSF absorption by the addition of absorption enhancers, which were surfactants and protease inhibitors, were studied.

Laureth-9, which enhances the nasal absorption of rhG-CSF, and SGC, a bile salt enhances both insulin 7-10 and human growth hormone 11 permeation and has also shown protease inhibitor activity on nasal administration, were selected as test surfactants, and their effects as a 1% solution on rhG-CSF absorption were evaluated.

In Fig. 1, changes in the plasma concentration of rhG-CSF after i.t. administration of 100 μg/kg rhG-CSF with and without Laureth-9 or SGC and after i.v. and s.c. administration are compared. Table 1 shows the values of AUC until 8 h after the administration (AUC 0-8), and the relative bioavailability. The plasma rhG-CSF concentration and the absorption rate were markedly increased with the addition of Laureth-9 and SGC (Fig. 1). The effect of SGC was greater than that of Laureth-9.

The relative bioavailability after i.t. administration with Laureth-9 was 37% (vs. i.v.) and 88% (vs. s.c.); that after i.t. administration with SGC was 84% (vs. i.v.) and 197% (vs. s.c.).

As for protease inhibitors, the effects of p-APMSF, bestatin, and aprotinin on rhG-CSF absorption were similarly evaluated. AUC 0-8 increased more than 1.5-fold compared with the i.t. control by the addition of all protease inhibitors. Among them, p-APMSF increased the plasma rhG-CSF concentration about 3-fold, from 3.9 to 11.7 ng/ml, at the first sampling point 30 min after i.t. administration. Such early onset of protease inhibitor activity is considered to have resulted in the greater enhancement of rhG-CSF absorption (Fig. 2).

To clarify the absorption mechanism, the surfactant Laureth-9 and/or the protease inhibitor p-APMSF was added to the solution of rhG-CSF for i.t. administration, and the enhancing effects of the two agents on rhG-CSF absorption were evaluated by changes in the plasma rhG-CSF concentration (Fig. 3). While the rhG-CSF concentration 30 min after i.t. administration was 3.9 ± 1.4 ng/ml, it was increased about 123-fold, to 481.5 ± 96.7 ng/ml, by the addition of both Laureth-9 and p-APMSF, and approached the value reached after the addition of SGC (600.5 ± 103.2 ng/ml).

As a result, the relative bioavailability of rhG-CSF administered i.t. with Laureth-9 and p-APMSF (73.6 ± 5.5% vs. i.v.; 172.8 ± 12.9% vs. s.c.) was nearly the same as that with SGC.
Fig. 3. Plasma Concentrations of rhG-CSF Following i.t. Administration without an Absorption Enhancer (Control: ▲), with Laureth-9 (∆), with Laureth-9 + p-APMSF (□) and with SGC (■)

The dose of rhG-CSF was 100 μg/kg. The values represent the mean±S.E. of more than 4 rats.

DISCUSSION

In our previous report, the relative bioavailability of rhG-CSF until 8 h after i.t. administration at 100 μg/kg rhG-CSF was 11.6% vs. i.v. administration, and 27.4% vs. s.c. administration. It was also higher than that by nasal administration, which is another effective non-invasive route of administration. The absorbability of rhG-CSF was markedly increased by the addition of the surfactants Laureth-9 and SGC (Fig. 1). From these results, we considered that absorption through the pulmonary mucosa may provide relative bioavailability and therapeutic effects comparable to those obtained by i.v. and s.c. routes, which have been conventional clinical routes. Since both additives showed marked absorption enhancing effects, a suitable absorption rate is considered to be obtained by regulation of the drug transport through the pulmonary mucosa. In the future, evaluation of the doses of the additives for optimization of the therapeutic effects of rhG-CSF and their histotoxicity to the pulmonary mucosa is considered to be necessary. Also, as the relative bioavailability was increased by the addition of SGC, which showed no absorption enhancing effect in nasal administration, nearly to the level achieved by i.v. administration, the mechanism of transport of rhG-CSF through the mucosa was confirmed to be different between nasal and i.t. routes (Table 1).

All protease inhibitors, such as p-APMSF, bestatin and aprotinin, showed enhancing effects on rhG-CSF absorption, but p-APMSF caused the most characteristic increase in plasma rhG-CSF concentration (Fig. 2). These results suggest that the degradation of rhG-CSF by protease is another rate-determining process in the transport of rhG-CSF through the pulmonary mucosa. Of the protease inhibitors evaluated, p-APMSF has serine protease inhibitor (P.I.) activity, bestatin leucine P.I. activity, and aprotinin trypsin P.I. activity. Therefore, the effect observed in Fig. 2 is not necessarily ascribed to a particular P. I. activity. For this reason, we judged that the estimation of the relative importance of the roles of individual protease inhibitors in an in vitro experiment would be impossible.

The relative bioavailability of rhG-CSF by simultaneous i.t. administration with Laureth-9 and p-APMSF was about twice that by i.t. administration with Laureth-9 alone, and was nearly comparable to that by the administration with SGC, which is considered to have both an enhancing effect of membrane permeation and an inhibitory effect of enzymatic degradation after i.t. administration. The enzymatic degradation mechanism of rhG-CSF in the pulmonary mucosa should be examined in greater detail by in vitro protease inhibitor study and comparative study of local and systemic enzymatic degradation. From these experimental results, however, the absorption rate of rhG-CSF through the pulmonary mucosa is considered to be regulated by both membrane transport and degradation by proteases.

The relationships between the high relative bioavailability values of rhG-CSF by i.t. administration and the absorption enhancing effects of various additives must also be evaluated in relation to the toxicity of the solutions to be administered to the pulmonary mucosal tissue. The results of detailed evaluation concerning this point will be reported in our next paper.

In summary, permeation and enzymatic degradation were rate-determining steps in the pulmonary absorption of rhG-CSF. Co-administration of Laureth-9 and p-APMSF was effective to overcome the above barriers, and a similar plasma rhG-CSF concentration was obtained by i.t. administration.

Acknowledgment The authors wish to thank Ms. Keiko Sano and Mrs. Kiyoe Yoshimori for their excellent technical assistance.

REFERENCES