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Report

Enhanced Intestinal Absorption of a Hydrophobic Polymer-Conjugated Protein Drug, Smancs, in an Oily Formulation

Kiichiro Oka,[1,2] Yoichi Miyamoto,[1] Yasuhiro Matsumura,[1] Shohei Tanaka,[1,3] Tatsuya Oda,[1] Fujio Suzuki,[1] and Hiroshi Maeda[1,4]

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Intestinal absorption of neocarzinostatin (NCS) and smancs (copolystyrene maleic acid-conjugated NCS), in aqueous and oily formulations, was investigated after oral administration in mice. Blood concentrations of NCS and smancs were determined with a cytotoxicity assay employing the highly sensitive Epstein–Barr (EB) virus-transformed B-lymphoblastoid cell line, TK/B. Smancs was more efficiently absorbed from a medium-chain triglyceride solution (oily smancs) than from an aqueous solution in phosphate-buffered saline (PBS). The maximum blood concentration and the area under the concentration curve versus time course (AUC) of oily smancs were 9 and 11 times greater than those of the aqueous form of smancs, respectively. At 5 hr after administration of oily smancs, 0.044% of the total smancs dose was found in blood, whereas the parent compound NCS was not detectable at any time. When oily smancs was administered orally to sarcoma 180 tumor-bearing mice, a selective accumulation of smancs in tumor tissue was observed. These results indicated that a biologically active protein, which cannot be used orally, may be rendered orally active drug by conjugation with a hydrophobic polymer in combination with an oily formulation.

KEY WORDS: smancs; oral administration; protein conjugation; oily formulation; polymer drugs; intestinal absorption.

INTRODUCTION

Genetic engineering technology presents the large-scale production of recombinant proteins with potent biological functions. Protein drugs, however, may suffer from antigenicity, low solubility, and short half-lives *in vivo*. These drawbacks can be minimized by conjugation with biocompatible polymers (1–3). Further, protein drugs are often ineffective orally because of their lability toward digestive enzymes, denaturant bile acids, and the acidic environment in the digestive tract. We report here on an approach to enhancing the present gastrointestinal absorption of protein drugs, using smancs as a model. Smancs is a conjugate of the proteinaceous anticancer agent, neocarzinostatin, with a hydrophobic synthetic polymer (styrene-comaleic acid; SMA)[5] (1,2,4). An oily formulation of smancs is used for arterial injection into the tumor artery (5–7). The present report evaluates smancs and oily smancs for possible peroral administration.

MATERIALS AND METHODS

Materials

NCS (MW 11,000) was obtained from Kayaku Laboratories Co., Ltd., Tokyo. Copolystyrene-maleic acid (SMA) and smancs (MW 15,500) were prepared by Kuraray Co. Ltd., Osaka, Japan, as described by Maeda *et al.* (2,4,5). Water-soluble carbodiimide was from Dojin Chemical Laboratories, Kumamoto, Japan. ^{14}C-Glycine (113.0 mCi/mmol) was obtained from New England Nuclear, Inc., Boston, MA. Phosphatidylcholine, medium-chain triglyceride (MCT), and polyglycerine(6)dioleate were kind gifts from Nippon Oil and Fats Co., Ltd., Tokyo. MCT is a synthetic compound containing about 75% caprylic acid and 25% capric acid. All other chemicals were reagent-grade products obtained from commercial sources.

Animals and Tumor

Approximately 12-week-old ddY mice weighing about 40 g were used for the pharmacological studies. Mice were fasted overnight prior to the experiment but water was given

[1] Department of Microbiology, Kumamoto University Medical School, Kumamoto 860, Japan.
[2] Present address: Central Research Laboratories, Kuraray Co. Ltd., Kurashiki 710, Japan.
[3] Present address: Yamanouchi Pharmaceutical Co., Ltd., Azusawa 1-1-8, Itabashi, Tokyo 174, Japan.
[4] To whom correspondence should be addressed.
[5] Abbreviations used: NCS, neocarzinostatin; SMA, a synthetic copolymer of styrene-maleic acid anhydride; smancs, copolystyrene maleic acid-conjugated NCS; PBS, 0.01 *M* phosphate-buffered 0.15 *M* saline, pH 7.4; AUC, area under the concentration curve in time course; MCT, medium-chain triglyceride.

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ad lib. Sarcoma 180 cells grown for 8 days in ascitic fluid of ddY mice were injected into an intracutaneous site on the femoral skin at a dose of 5×10^6 cells/site. After 1 week, when the tumor diameter had reached 8–10 mm, the mice were used for the tissue distribution study.

Preparation of Smancs and NCS in Oily Formulation

Smancs (100 mg) and phosphatidylcholine (200 mg) were dissolved in 70 ml of 0.01% $(NH_4)_2CO_3$ solution and lyophilized. The powder obtained was added to MCT containing 5% polyglycerine(6)dioleate and suspended using a vortex mixer. The above procedure facilitated dissolution of the drug in oil. The final concentration of oily smancs was 4 mg/ml. NCS, which has highly hydrophilic properties, was subjected to the same procedure for oily formulation, and it resulted in a suspension.

Cell Line

TK/B cells (Epstein–Barr virus-transformed B-lymphoblastoid cell line), obtained from Dr. Katsuki of this department, were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 10% heat-inactivated fetal bovine serum under 5% CO_2 in humidified air at 37°C. HeLa S_3 cells were cultured similarly except Eagle's minimal essential medium (MEM) with 10% newborn calf serum was used.

Blood Concentration of NCS and Smancs

Blood concentrations of NCS and smancs resulting from oral administration to healthy mice were determined with a bioassay by measuring the *in vitro* growth inhibition of TK/B cells, which are highly sensitive to these drugs (8). Doses of 33 and 100 mg/kg of oily smancs, smancs in 10 m*M* phosphate-buffered 0.15 *M* saline (PBS), pH 7.4, 100 mg/kg of oily NCS, and NCS in PBS were orally administrated to mice using a narrow metallic tube with a round-tip end. Blood samples were then taken from a single mouse by cutting the tail vein with a razor at 0, 1, 3, 5, 7, 9, and 12 hr after administration. At each time point a 20-μl blood sample was taken with a heparinized Eppendorf pipette, and it was placed into a well of a 96-well plate with a V bottom (Costar No. 3896), each containing TK/B cells at a density of 1×10^5/ml in 130 μl of RPMI 1640 medium with 10% fetal bovine serum (GIBCO). After incubation for 48 hr at 37°C under 5% CO_2 in humidified air, the cells were centrifuged at 1500 rpm for 10 min and the medium was aspirated. Pellets were resuspended with 200 μl of distilled water, which lysed the red blood cells while leaving the lymphoblastoid cells intact. After 1–2 min, a 100-μl aliquot of the cell suspension was taken and mixed with 100 μl of a twofold-concentrated RPMI 1640 medium with 10% fetal bovine serum in the wells of another plate. Surviving TK/B cells were counted using the trypan blue dye exclusion method, and the concentrations of drugs at different time points were determined from a standard dose vs growth inhibition curve for each drug. The minimum detectable drug concentration was 1 n*M* for both NCS and smancs using 20 μl of whole blood.

Labeling of Smancs with ^{14}C-Glycine

Smancs was labeled with ^{14}C-glycine, which causes minimum gross structural modification as described previously (9). Briefly, 20 mg of smancs was added to 1 ml of deionized water, and the pH of the solution was adjusted to 6.0 to solubilize it completely by dropwise addition of 0.8 *M* $NaHCO_3$. Then 20 mg water-soluble carbodiimide was added to the solution. After 5 min, 100 μl of a ^{14}C-glycine solution (3 mCi/ml, pH 6.0) was added. Coupling was carried out at room temperature for 1.5 hr under constant stirring in the dark. ^{14}C-Glycine-labeled smancs was separated from free glycine on a column of Sephadex G-25 (2.3 × 10.5 cm) which had been equilibrated with water, and the fraction containing ^{14}C-smancs was lyophilized. ^{14}C-Glycine-labeled smancs had a specific radioactivity of 6.0 μCi/mg, and it showed the same cytotoxicity in HeLa S3 cells as native smancs (data not shown).

Accumulation of Orally Administered Oily Smancs in Tumor and Other Organs

To examine the accumulation of smancs in the solid tumor, 0.1 ml of ^{14}C-glycine-labeled oily smancs with 2.2×10^6 dpm (1 μCi) was orally administered to each mouse. The mice were sacrificed at 1, 3, 7, 24, and 48 hr for removal of tumors in the femur and of the femoral muscles without tumor used as control. All specimens were weighed (range, 20–100 mg). One milliliter of 2 *M* NaOH was added to the specimens to dissolve the muscle or tumor tissue. Following a 30-min incubation at 70°C, the solution was neutralized with 1.5 ml of 10% ascorbic acid. The solution was decolorized with 1.5 ml of a 30% H_2O_2 solution for 30 min at 55°C, then the mixture was added to 10 ml of scintillation liquid (Triton X-100, PPO, dimethyl POPOP system) and analyzed by liquid scintillation counting (Packard Model 3385). Blood and other organ tissue distribution of orally administered ^{14}C-smancs was also measured as described above.

RESULTS

Blood Concentration of Smancs After Oral Administration in Mice

In order to examine the intestinal absorption of smancs, its blood concentrations were determined by bioassay after oral administration of 33 and 100 mg/kg in mice (Fig. 1). At a dose of 33 mg/kg, only the oily formulation of smancs gave detectable blood levels (Fig. 1A). After oral administration of 100 mg/kg of smancs in PBS, the smancs blood level reached a maximum of 0.1 μg/ml at 1 hr, then gradually decreased, and was undetectable after 5 hr. In contrast, oily smancs (100 mg/kg) gave a maximum of 0.87 μg/ml 5 hr after administration, and bioactive material remained detectable in blood after 9 hr. The area under the curve of oily smancs was 11 times greater than that of smancs in PBS. In contrast to smancs, NCS was not detectable at any time after oral administration of NCS suspension in oil or solution in PBS.

Accumulation of Orally Administered Oily Smancs in Tumor and Normal Tissues

Oily ^{14}C-smancs was administered orally to tumor-

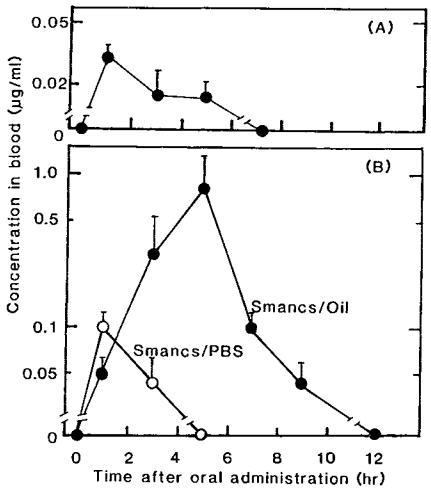

Fig. 1. Plasma concentrations of smancs and NCS after oral administration to mice at the dose of 33 mg/kg (A) or 100 mg/kg (B). (●——●) Oily smancs in medium-chain triglyceride; (○——○) smancs in PBS. NCS in PBS or in oily formulation did not show any detectable plasma concentration. Data are means ± SE; $n = 5$.

bearing mice, and the radioactivity in tumors, blood plasma, and normal muscle tissue was determined at various time intervals. Analysis of blood plasma with Sephadex G-50 and Sephacryl S-200 columns showed that free ^{14}C-glycine was not released significantly from smancs, and most of the radioactivity found at the fractions was similar in size to globulin and albumin. In contrast, free glycine given perorally was present in plasma and urine in significant amounts (not shown). Radioactivity in the tumor was higher than in the femoral muscle in which tumor resided (Fig. 2). The ratio of tumor/muscle was 3.4, 2.5, and 6.5, at 3, 7, and 24 hr, respectively, indicating that smancs selectively accumulated in the tumor. Similar results were observed after i.v. injection of smancs in PBS (5,10,11).

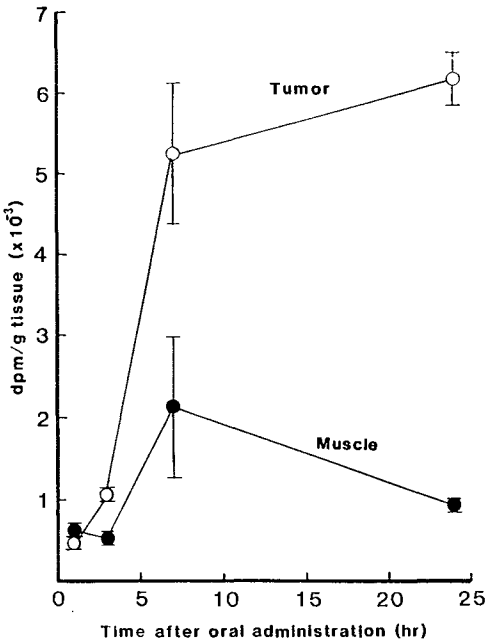

Fig. 2. Accumulation of ^{14}C-smancs in tumor tissue after oral administration of 33.3 mg (200 μCi) of oily ^{14}C-smancs. Radioactivity of tumor (○) and femoral muscle (●). Each point represents the average of results from two mice.

The distribution of ^{14}C-smancs to normal organs and blood after administration of oily smancs is shown in Table I. Approximately 4.4% at 3 hr and 3.0% at 7 hr of the total smancs dose were recovered from major organs (Table I). The low recovery value at 7 hr may be attributed to the lowered radioactivity present in the stomach, which is one of the most dominant organ of accumulation after the intestine. The residual radioactivity in the intestinal tract was 49% of the dose after 5 hr (not shown). The stomach also had a very high radioactive count (Table I). Autoradiography showed radioactive grains detectable in the *mucosa* and *submucosa* (not shown). Except for the stomach, the level of ^{14}C-smancs in all other tissues and blood increased after 7 hr as compared to those at 3 hr (Table I). These results indicate that oily smancs is gradually absorbed from the gastrointestinal tract and retained in tissues and blood.

DISCUSSION

Our main objective was to determine whether bioactive high molecular weight protein drugs can be absorbed from the intestine and traverse into the blood circulation in intact form without loss of their biological activity. In rats, 2% of orally administered bovine albumin was intestinally absorbed in the native form (12). In addition, insulin (about MW 35,000 as a hexamer) (13), horseradish peroxidase (MW 40,000) (14), IgG (MW 150,000) (15), and polysaccharide KS-2 (approx MW 80,000) (16) were intestinally absorbed at about 1 to 3% of the dose. Hence, small amounts of protein can be absorbed intact after oral administration, but the bioavailability of biologically active conjugated proteins, such as smancs (MW 15,500), is unknown.

We describe here that smancs was absorbed from the intestine and circulated in plasma of mice after oral administration of the aqueous solutions (Fig. 1). Further, smancs was absorbed much more efficiently in the lipid formulation of MCT solution (oily smancs) after oral administration. The maximum blood concentration obtained with oily smancs is 9 times greater than that of smancs in PBS, and the AUC is 11 times greater. The recovery of smancs in blood was 0.044% assuming a total blood volume of 2 ml, while the recovery in plasma was 0.039% at the 3-hr point and 0.09% at the 7-hr point (Table I), and these values are similar to those found by bioassay (0.044%, Fig. 1). Considering the very short plasma half-life of smancs when administered intravenously (19 min) (1), a much larger amount must have been absorbed from the intestine.

The following reasons for the enhanced absorption rate of smancs dissolved in MCT were considered. (a) MCT or its fatty acid degradation products enhanced the absorption rate of smancs as a mixture of glyceryl mono-, di-, and tricaprylate was previously shown to enhance the absorption of certain drugs (17). (b) Smancs was protected from the attack of proteinases and other hydrolytic enzymes and from the acidic milieu in MCT. (c) The adhesive character of MCT to the intestinal wall facilitated the absorption of MCT and smancs.

Proteins that are conjugated with lipophilic polymers,

Table I. Tissue Distribution of Orally Administered Oily ^{14}C-Smancs[a]

Tissue (mean weight; g)	After 3 hr		After 7 hr	
	% dose/g tissue	% dose/whole tissue	% dose/g tissue	% dose/whole tissue
Plasma (1.0)	0.039	0.039 ± 0.014	0.090	0.090 ± 0.017
Liver (0.93)	0.342	0.317 ± 0.057	0.633	0.587 ± 0.063
Kidney (0.26)	0.224	0.059 ± 0.032	0.391	0.103 ± 0.005
Spleen (0.084)	0.103	0.09 ± 0.0063	0.156	0.013 ± 0.003
Lung (0.16)	0.104	0.017 ± 0.0053	0.195	0.032 ± 0.00
Muscle (14.2)	0.018	0.266 ± 0.027	0.0337	0.506 ± 0.0039
Stomach (0.36)[b]	9.608	3.46 ± 2.28	3.295	1.19 ± 0.49
Duodenum (0.25)[b]	0.521	0.130 ± 0.043	1.088	0.272 ± 0.056
Sum	—	4.378	—	2.973

[a] Oily ^{14}C-smancs (0.5 mg; 3 μCi) was orally administrated to each mouse.
[b] The contents in these tissues were removed by washing with PBS before analysis.

such as SMA, may become readily absorbable after oral administration when solubilized in MCT. MCT is preferentially absorbed into the portal vein rather than into the lymphatic duct (18). It is not clear, however, which route smancs in MCT traversed. The results in Fig. 1 demonstrate that biologically active smancs molecules were indeed absorbed via the intestine and appeared in the blood after oral administration of oily smancs.

We have previously reported that macromolecules such as plasma albumin, IgG, or smancs accumulated more in tumor tissue than in normal tissues after i.v. injection (10), a result which was confirmed here with ^{14}C-glycine-labeled smancs after oral administration (Fig. 2). The preferential tumor uptake of macromolecules is attributed to a number of factors such as enhanced vascular permeability and a retention effect (EPR effect) because of little lymphatic recovery (1,10,11,19). The enhanced permeability is caused by permeability enhancing factors (19) and defective vascular architecture and hypervasculature in solid tumors. The potentially augmented therapeutic efficacy of macromolecular and lipid drugs is well recognized in cancer chemotherapy (19).

In conclusion, biologically active proteins and peptides can be administered in an orally active form by conjugation with a hydrophobic polymer such as SMA in combination with an oily formulation.

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