Use of Oily Contrast Medium for Selective Drug Targeting to Tumor: Enhanced Therapeutic Effect and X-Ray Image

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ABSTRACT
Highly malignant rabbit tumor (VX-2) was implanted at the periphery of the liver in 63 rabbits. Selective delivery of the anticancer agent copoly(styrene-maleic acid) conjugated to neo-carzinostatin (SMANCS), which was dissolved in an oil contrast medium (Lipiodol), was performed by injection via the proper hepatic artery. The anticancer effect was also evaluated by various parameters. By using low-kVp X-ray examination of the resected rabbit liver, Lipiodol was found to distribute throughout the entire liver arterial lumina and was retained there for about 24 hr, but disappeared from the normal liver arterial lumina gradually. However, Lipiodol was retained in the tumor tissue and vessels for at least 7 days, whereas it was undetectable in any other organs. A radioactive analogue of Lipiodol, a chloroiodinated fatty acid, was prepared by using [14C]linoleic acid. This analogue was used in the study of the distribution by low-kVp X-ray examination, Sudan III staining, and autoradiography. Lipiodol remained in the tumor vessels as well as the tumor cells. The use of the radioisotope yielded a quantitative profile of Lipiodol accumulation in tumor tissues; approximately 1000 times more at 15 min and 100 times more at 3 days after the injection than that of most other organs or plasma. Its major excretion route appeared to be through the bile and then the feces.

The biological activity of SMANCS was also determined and was found to be significant in both tumor and liver even 7 days after injection. No activity was found in any other organ or tissue. The relatively high biological activity of SMANCS in the nonmalignant liver adjacent to the tumor may be the result of continuous drug release from SMANCS-Lipiodol in the tumor tissue. By histological examination, massive tumor necrosis and infiltration of the inflammatory cells were found in the rabbits treated with SMANCS-Lipiodol. In the rabbits treated with Lipiodol alone, necrosis of the tumor was only minimal, and no infiltration of inflammatory cells was observed.

Survival periods of the treated rabbits (n = 14) were significantly longer than those of controls (n = 10): 23.1 ± 5.5 (S.D.) days versus 16.1 ± 2.9 days (p < 0.005), respectively, even though only one injection was used for this highly malignant tumor. Mean tumor size for both groups at laparotomy was 163.3 ± 83.0 sq mm and 160.5 ± 76.5 sq mm, respectively (not significant). At death, the mean tumor size was 517.1 ± 664.7 sq mm in the treated rabbits and 1897.5 ± 665.0 sq mm in the nontreated (mock operation) control rabbits (p < 0.005). Thus, the anticancer effect of SMANCS-Lipiodol was obvious even after a single dose.

INTRODUCTION
The lipid lymphographic agent Lipiodol has been found to remain selectively in tumor tissue of the liver for a long time when injected into the hepatoprostimal lumen of the ligated hepatic artery (9). This phenomenon, which showed no complication, was also found for the nonligated proper hepatic artery (4, 5). Based on this finding, a novel therapeutic approach in humans has been undertaken by using a high-molecular-weight anticancer agent, SMANCS, dispersed and solubilized in Lipiodol (4, 5). SMANCS is a chemical conjugate of a synthetic copolymer of styrene-maleic acid and the proteinaceous anticancer agent NCS, and SMANCS retains the original DNA-damaging properties of NCS in vitro (13). The molecular weight of SMANCS used in the previous study was approximately 15,000 to 25,000 (7). SMANCS can be dissolved in some organic solvents, such as pyridine and acetone, or partially in Lipiodol. The homogeneous suspension in Lipiodol, designated SMANCS-Lipiodol, could be used for administration via the proper hepatic artery of rabbit with VX-2 carcinoma of the liver. Selective accumulation of both SMANCS and Lipiodol and the anticancer effect are described in the present report. Furthermore, the selective deposition of Lipiodol found by the present method indicates that specific enhancement of the tumor X-ray image is possible, and it will thus be an invaluable tool for the accurate diagnosis of solid tumor.

MATERIALS AND METHODS

Drug
SMANCS was prepared as described by Maeda et al. (7). Briefly, the drug is a reaction product of a copolymer of styrene-maleic acid anhydride (mean molecular weight, approximately 2500) and 2 amino groups of NCS (M, 11,000). At least 2 styrene-maleic acid anhydride groups were conjugated through an amide linkage; thus, the drug used in the present study had an approximate molecular weight of about, 15,000 to 18,000 as estimated by polyacrylamide gel electrophoresis with sodium dodecyl sulfate and by elemental analysis. One mg of SMANCS was dissolved in 1 ml of Lipiodol (Laboratoire Guerbet, Paris, France). Lipiodol is an ethyl ester of iodinated poppy seed oil in which most of the unsaturated double bonds in oleic, linoleic, and linolenic acid were iodinated almost completely, and has a specific gravity of 1.28 and an iodine content of 38% by weight.

Preparation of Radioactive 14C-Labeled Analogue of Lipiodol
Linoleic acid uniformly labeled with 14C, with a specific activity of 54.9 mCi/mol (New England Nuclear, Boston, MA), was used, and iodochlo-
rination with monochloroacetic acid (Wako Pure Chemical Co., Osaka, Japan) was carried out in 10 ml of chloroform as follows:

\[
\text{CH}_3\text{CH}_2\text{OH} + \text{HCl} \rightarrow \text{CH}_3\text{CH}_2\text{Cl}
\]

In this reaction, 10 mmol of HCl in 10 ml of acetic acid were added to 1.0 mmol of fatty acid and allowed to react for 10 min. Then, about 30 ml of 1.0% KI solution were used several times as a wash, followed by washing with water and then chloroform removal in a vacuum. The colorless compounds thus obtained had a density of about 1.28 and yielded 24.2% of iodine and 26.3% of chlorine. The derivative had a radioactivity of 2.88 x 10^7 dpm/ml, and was designated as [\(^{14}\text{C}\)]Lipiodol.\(^4\)

### Animals

Sixty-three New Zealand White rabbits about 3 months old, each weighing 1.5 to 2.5 kg, were used. A general anesthesia was applied by injecting pentobarbital sodium (30 mg/kg i.v.) during laparotomy performed through a middle abdominal incision for tumor cell inoculation and for arterial injection of SMANCS-Lipiodol.

### Tumor Cell Inoculation

The VX-2 tumor cell line was maintained by successive transplantation into the hind leg muscles of a rabbit. A total of 0.1 ml of a suspension containing approximately 1.5 to 2.0 x 10^5 VX-2 carcinoma cells was injected via a 25-gauge needle into the subcapsular parenchyma of the left anterior lobe of the liver. Twelve to 15 days after the implantation of the cells into the liver, the tumor was 1 to 3 cm in diameter. The animals then underwent laparotomy, and those rabbits with tumors of about 2.0 cm in diameter were used for subsequent experiments.

### Drug Administration

The hepatoduodenal ligament was stretched by pulling the stomach to the caudal side so that the proper hepatic artery could be seen. Two groups of experiments were carried out. One was designed for assay of biological activity, histological study, and soft (low-kVp) X-ray examination. Another group was to study the distribution of [\(^{14}\text{C}\)]Lipiodol by assaying its radioactivity. SMANCS-Lipiodol was injected into the lumen of the proper hepatic artery without ligation for about 15 sec via a 30-gauge needle connected to a 10-cm polyethylene tube.

### Selective Accumulation of SMANCS-Lipiodol: Bioactivity and Low-kVp X-Ray Analysis

Twenty-eight rabbits were used in this study. Selective accumulation of SMANCS-Lipiodol was evaluated after injection via the proper hepatic artery in 5 rabbits which were killed at 15 min and at 1, 3, and 7 days after the injection. The liver, kidney, and lung were removed at the above indicated times. To evaluate the location of Lipiodol, serial slices of 5-mm-thick liver were radiographed with a Softex instrument using Fuji fine-grain film. The bioactivity of SMANCS, which was parallel to the antitumor activity, was determined by an antibacterial assay using Sar-cina lutea on a soft agar plate as described previously (8). Briefly, 5-mm-thick specimens for assay were obtained by punching out samples from the resected organs or tissues with a cork borer 8 mm in diameter. Measurements of the bioactivity of SMANCS were carried out for the various tissues and organs as described in Table 1.

### Distribution of [\(^{14}\text{C}\)]Lipiodol after Injection via the Proper Hepatic Artery

Six rabbits were killed at each time (at 15 min and at 3 and 7 days) after the injection of 0.2 ml of [\(^{14}\text{C}\)]Lipiodol (about 2 x 10^6 dpm) via the proper hepatic artery. Specimens (about 100 mg) of various organs or tissues were removed, weighed, minced, dissolved in an aliquot of 1 ml NaOH in 20-ml vials on a hot plate, and decolorized by intermittent dropwise additions of 30% hydrogen peroxide solution. A calibration with an internal standard was made afterward when decolorization was incomplete due to the presence of blood cells. To each vial were added 10 ml of liquid scintillator containing toluene and Triton X-100. The \(^{14}\text{C}\) radioactivity of each solution was counted by a Packard Model 3325 scintillation counter (6).

### Autoradiography of Resected Tissues

A 0.2-ml sample of [\(^{14}\text{C}\)]Lipiodol (2 x 10^6 dpm) was injected via the proper hepatic artery into rabbits bearing VX-2 tumor in the liver. They were killed at various times after injection of [\(^{14}\text{C}\)]Lipiodol with a lethal dose of pentobarbital given i.v. Liver containing tumor was obtained by resection and were placed on dry ice. Autoradiography of a thin section on a glass slide prepared by a cryostat was performed by the dipping method to evaluate the location of \(^{14}\text{C}\). Each specimen on the glass slide was coated with Sakura autoradiography Emulsion NR-M2 (Sakura X-ray Co. Ltd., Tokyo) at 40°, and the slides were placed in a sealed box at 4° for about 2 weeks. After development, slides were stained with hematoxylin and eosin and examined under a microscope as described (6). To evaluate the location of Lipiodol further, a microsotex study and Sudan III staining of similar specimens were also performed under lesser magnification.

### Anticancer Effect of SMANCS-Lipiodol

Histological Examination for Pathological Change. Eleven rabbits were divided into the following 3 groups: (a) 5 rabbits were treated with 0.2 ml of SMANCS-Lipiodol (Group 1); (b) 3 rabbits were treated with 0.2 ml of Lipiodol alone (Group 2); and (c) 3 rabbits received 0.2 ml of 0.9% NaCl solution (saline) via the proper hepatic artery (Group 3). In all 3 groups, the injections were done only once. Ten days after the injections, the rabbit were killed, and liver slices including tumor tissue were stained with hematoxylin and eosin. Examinations for pathology were performed with a microscope to evaluate the anticancer effect.

Effect on Survival of Rabbits with VX-2 Tumor. Twenty-four rabbits weighing 1.5 to 2.0 kg were divided into the following 2 groups. In both groups, rabbits with VX-2 tumor of only a limited size range (1.0 to 2.0 cm in diameter) were used. In the first group, 14 rabbits were treated once with 0.2 ml of SMANCS-Lipiodol. In the second group, a mock puncture of the proper hepatic artery was performed in 10 rabbits. The survival periods of the 2 groups after these treatments were compared.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity (μg/ml) of SMANCS at following period from injection to sacrifice</th>
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<tr>
<td></td>
<td>15 min (n = 5)</td>
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<tr>
<td>Tumor</td>
<td>8.3 ± 10.8</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
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<tr>
<td>Nontumorous portion adjacent to tumor</td>
<td>3.1 ± 4.7</td>
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<tr>
<td>Nontumorous portion distant from tumor</td>
<td>4.9 ± 7.7</td>
</tr>
<tr>
<td>Lung</td>
<td>0</td>
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<tr>
<td>Kidney</td>
<td>0</td>
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* Mean ± S.D.  

\(^4\) Microsoftex and Softex are photographs of resected tissue obtained by low-kVp X-ray.
Also, the size of each tumor was measured before treatment and at autopsy, and the 2 groups were compared.

RESULTS

X-Ray Examination: Selective Accumulation of SMANCS-Lipiodol in Tumor. The results of the soft X-ray examination of the slice of resected liver, as shown in Fig. 1A (15 min after injection), Fig. 1B (1 day), Fig. 1C (3 days), and Fig. 1D (7 days), indicate clear deposition of Lipiodol in the tumor tissue; however, deposition in normal tissue was almost undetectable at the later time. The iodine of the Lipiodol, having a high electron density, is visible as white spots and lines, and these were seen mostly in the tumor site and partially in the nontumorous liver capillaries. Such Lipiodol staining of the nontumorous liver tissue disappeared gradually (Fig. 1, C and D).

Biological Activity of SMANCS in Tumor Tissues. The result is shown in Table 1. The bioactivity of SMANCS almost paralleled the deposition of Lipiodol. The bioactivity of SMANCS in the tumor tissue and that in the nontumorous part of the liver adjacent to tumor were found to be very high even 7 days after the injection. On the other hand, the bioactivity of SMANCS in the kidney and lung could not be detected even after 15 min. Thus, selective drug targeting to the cancer appears feasible with the present methodology.

Organ and Tumor Distribution of $^{14}$C after Injection of $[^{14}$C]-Lipiodol via the Proper Hepatic Artery. The radioactivity of the various organs is shown in Table 2. The highest count was obtained for the tumor tissue, followed by tissue adjacent to the tumor at 15 min after injection. At 3 and 7 days after injection, the radioactivity was still highest and distinct only in the tumor compared with other organs or tissues tested. This phenomenon agreed with the result of the Softex® film experiment described above. In other organs or plasma, the radioactivity was very low, about 0.1% of the tumor tissue at 15 min and about 1.0% of the tumor tissue on Day 3. This result is highly suggestive of a selective deposition of Lipiodol in the cancer tissue.

Autoradiography to Locate $[^{14}$C]-Lipiodol in Tumor. Because the tumor accumulated SMANCS to the highest degree, autoradiography of tumor and liver tissues was undertaken. The autoradiogram and Sudan III staining of the same site of the tumor are shown in Figs. 2 and 3, respectively. In Fig. 2, black dots showing the location of $^{14}$C are found in the cytoplasm of tumor cells as well as in the tumor vessels. Staining with Sudan III for lipid particles showed that some capillaries were also occupied by Lipiodol (Fig. 3A). It is emphasized that SMANCS-Lipiodol injected via the proper hepatic artery remained to some degree in the tumor vessels, but its substantial amount also leaked out into the extracapillary space through the neovascularature. It is interesting that there was little recovery through the lymphatics in the tumor, as judged from long-term deposition. In normal tissue, Lipiodol is recovered via the lymphatic system because of its lipid nature; thus, it is used for lymphography. Here again, tumor-specific delivery of Lipiodol is confirmed.

Anticancer Effect. Results of a histological examination of the tumor specimen from rabbit killed 10 days after injection of SMANCS-Lipiodol are shown in Fig. 4, and results of control experiments with either Lipiodol or saline alone are shown in Figs. 5 and 6, respectively. Massive necrosis and inflammatory cell infiltration surrounding the necrotic tissue are seen in Fig. 4B, although the drug was given only once. A very little necrosis without inflammatory cell infiltration was found in the Lipiodol group (Fig. 5B). Neither necrosis nor infiltration could be found in the saline control (Fig. 6B). Thus, the anticancer effect of SMANCS-Lipiodol, injected via the hepatic artery, against VX-2 carcinoma in the liver was obvious. The survival periods of both treated and nontreated rabbits are shown in Chart 1. Either SMANCS-Lipiodol or control material was given only once arterially. Mean survival of the rabbits in the treated group was 23.1 ± 5.5 days, and that of the control group was 16.1 ± 2.9 days. The difference is statistically significant ($p < 0.005$). The major cause of death of the treated rabbits was pulmonary metastasis; i.p. dissemination was rare in this group. On the contrary, both i.p. and pulmonary dissemination were common and were the
major causes of death in the control group.

The growth rate of the tumors of treated and control rabbits is shown in Table 3. The mean size of the tumor at the beginning of treatment was 162.3 ± 83.0 sq mm for the treated group and 160.5 ± 76.5 sq mm (not significant) for the nontreated group. That of the treated group at death was 517.1 ± 644.7 sq mm, and that of the control group was 1897.1 ± 655.0 sq mm (p < 0.005). Thus, the antitumor effect in the treated group was obvious.

**DISCUSSION**

Substantial efforts have been made for targeting cancer drugs to solid tumors including monoclonal antibody conjugation, arterial infusion of ordinary anticancer agents with or without an infusion pump, or, more recently, embolization by sponge gel with or without anticancer agents. None has been shown, however, to be adequate for the treatment of solid tumors. Embolization had at first seemed promising, but extensive studies indicated several disadvantages such as rapid development of collateral vessels within 2 to 3 weeks, and unexpected accidents due to occlusion in the other vital normal organs. In addition, the method can be applied to only small tumors.

As discussed in “Results,” the selective deposition of Lipiodol in the tumor was confirmed by gross Softex film, Microsoftex film (not shown), Sudan III staining, and autoradiography (Figs. 1 to 4). The site of deposition was confirmed by autoradiography to be in the tumor vessels and the extracapillary space of the tumor. Furthermore, the biological activity of SMANCS was correlated very well with the location of Lipiodol during the first 24 hr. Namely, both the biological activity of SMANCS and the location of Lipiodol were detected predominantly in the tumor and its vicinity for a much longer time than anticipated. In VX-2 tumor, Lipiodol appears to persist longer than biological activity by at least 1 week (Table 1). The present results agree quite well with those found with resected livers of patients with hepatocellular carcinoma, in which the drug activity was detected in the tumor tissue and adjacent nontumorous tissue even 22 days after the injection of 4 mg of SMANCS-Lipiodol via the common hepatic artery. Lipiodol was detected for more than 3 months, or sometimes more than 1 year, by computer-assisted tomography scan and abdominal plain X-ray film (5). A similar result was reported for metastatic liver cancer, lung cancer, and other solid tumors (4).

At 15 min after the injection of [14C]Lipiodol via the proper hepatic artery, the radioactivity was detected at very high levels in the tumor, followed by the liver adjacent to the tumor, and, to lesser extent, in the bile and stomach (Table 2). Recoveries from other organs, urine, and blood were very low. The relatively high radioactivity in the bile may indicate an excretion pathway. During the injection procedure via the proper hepatic artery, a backflow to the gastroduodenal artery was found to occur; thus, moderately high radioactivity in the stomach is reasonable. At 3 and 7 days after injection, radioactivity was high only in the tumor, and very low in all other organs (Table 2).

The occurrence of pulmonary embolisms of Lipiodol may be rare, because radioactivity in the lung was low. The particle of Lipiodol flowing through the capillary network of the liver may be small enough to pass through the capillary of the lung. The lipid particles may also be entrapped eventually in lipoprotein and albumin. If pulmonary embolisms do occur, their incidence may be so small that no significant complication may result.

**Table 3**

<table>
<thead>
<tr>
<th>Tumor size of VX-2 carcinoma in rabbits</th>
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<td>Tumor size (sq mm)</td>
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<td>-------------------</td>
</tr>
<tr>
<td>At laparotomy</td>
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<tr>
<td>At autopsy</td>
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<tr>
<td>Growth rate</td>
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* Values were calculated by a multiple of longitudinal and transversal distances (mm).

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vessels did not cause complete embolization, as shown in Fig. 3A. In a recent study of peripheral hepatic artery embolization with polystyrene microspheres by Burgener et al. (2), the survival period was not significantly prolonged and, at best, partial tumor necrosis was found by histological examination.

In conclusion, our present finding of selective Lipiodol deposition in tumor tissue can offer 2 separate benefits: (a) the selective delivery of the anticancer drug to the target tumor, which can be applied to other anticancer agents; and (b) a potential value in the accurate determination of size and location of the tumor by various X-ray systems, and for long-term follow-up study.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Softex film of slices of rabbit liver. A to D are from 15 min, 1 day, 3 days, and 7 days, respectively, after injection of SMANCS-Lipiodol via the proper hepatic artery. A, deposition of Lipiodol in the tumor (T) and normal liver arterial lumina. B, deposition in normal liver arterial lumina was less than that in the tumor. C, Lipiodol deposition in normal liver arterial lumina was much less than that at 1 day, whereas that of the tumor persisted. D, deposition of Lipiodol was confirmed only in the tumor tissue. Lipiodol disappeared completely from the normal liver arterial lumina. Bars, 1 cm. × 1.
Fig. 2. Autoradiogram of the liver specimen at 3 days after injection of [14C]Lipiodol with SMANCS. A, Lipiodol shown as black speck (arrow) in the neovasculature. × 100. B, Lipiodol can be found in tumor cells as tiny black points (arrows). × 400.

Fig. 3. Microscopic findings for the same specimen as in Fig. 2 stained with Sudan III. Fatty acid deposits are shown as black particles (arrows) in the neovasculature (A) and black points (arrows) in the tumor tissue (B). (A, × 100; B, × 200).
Drug Targeting with Lipid Contrast Medium

Fig. 4. Liver specimen of rabbits killed 10 days after administration of SMANCS-Lipiodol. A, Softex film. Lipiodol is found in the tumor tissue as a white area, but not in the normal liver parenchyma. B, microscopic finding (H & E stain). N, massive tumor necrosis; arrows, inflammatory cell infiltration. A and B are of the same slice. Bar, 1 cm. x 1.

Fig. 5. Liver specimen of rabbits killed 10 days after injection of Lipiodol only (no drug was given). A, Softex film. Lipiodol is found in the tumor tissue as white dots. B, microscopic finding (H & E stain). A very slight tumor necrosis is found (N), but infiltration of the inflammatory cell is not observed. Bar, 1 cm. x 1.

Fig. 6. Liver specimen of rabbits killed 10 days after injection of saline. A, Softex film. No high-density object is observed. B, microscopic finding (H & E stain). Neither tumor necrosis nor inflammatory cell infiltration is found. Bar, 1 cm. x 1.
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