Size of Lipid Microdroplets Effects Results of Hepatic Arterial Chemotherapy with an Anticancer Agent in Water-in-Oil-in-Water Emulsion to Hepatocellular Carcinoma

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ABSTRACT
We have initially prepared a new drug delivery system for hepatocellular carcinoma (HCC). Using sonication and a detergent, iodinated poppy seed oil (IPSO) can be mixed with an aqueous solution of epirubicin to make a water-in-oil emulsion. The water-in-oil emulsion is further passed through a micro-porous glass membrane and split into saline to make a long-term inseparable water-in-oil-in-water emulsion (W/O/W) that consists of IPSO microdroplets. To investigate the effect of the size of IPSO microdroplets on the efficacy of injection chemotherapy with W/O/W in patients with HCC, 32 HCC patients were randomly assigned and treated with W/O/W of small IPSO microdroplets (30 µm in diameter) containing 60 mg of epirubicin (n = 16, group A) or W/O/W of large IPSO microdroplets (70 µm) containing the same amounts of epirubicin (n = 16, group B). Effects were assessed by measuring the percentage of decline of the α-fetoprotein (AFP) level in a week from the AFP level immediately before the treatment. The decline was significantly larger in group B (50.5 ± 19.8, mean ± S.D.) compared with group A (18.9 ± 33.1; p < .005). The size of IPSO microdroplets injected into the hepatic artery determines the decrease of serum AFP levels of the patients with HCC.

Hepatocellular carcinoma (HCC) is a malignant disease, strongly linked to chronic infection of the hepatitis B and/or C virus, that has been prevalent particularly in Asia, Africa, and southern Europe. In the past, the treatment for HCC was restricted to hepatic resection and i.v. or hepatic arterial administration of the anthracyclines. Both treatments have some disadvantages; the former can rarely be applied on patients who had impaired functional reserve of the liver, and the latter complicates severe myelosuppression without exception and lacks in sufficient clinical effect.

Recently, in Japan and Europe, ethyl esters of iodinated fatty acids obtained by hydrolysis of poppy-seed oil (IPSO) have been used for hepatic arterial injection chemotherapy (Nakakuma et al., 1983; Konno et al., 1983; Palma, 1998). In this conventional hepatic arterial injection chemotherapy using IPSO, oil-in-water emulsion has been prepared by mixing (by hand) IPSO with an aqueous solution of an anticancer drug in a set of two syringes. Particle size distribution of IPSO droplets in this emulsion widely ranges from a few to 1,000 µm. Moreover, the aqueous solution is easily separated from the lipid (Kanematsu et al., 1984) and the anticancer drug is washed away from the liver, leaving IPSO alone unless embolic substances such as gelatin sponge particles are also injected. Because the embolic substance inevitably delivers unexpected tissue damage of the liver (Belli et al., 1997), the therapy has been rarely applied on patients with multiple HCC.

We previously reported a new drug delivery system for HCC using a long-term inseparable water-in-oil-in-water emulsion (W/O/W; Higashi et al., 1993, 1995). The W/O/W is prepared by a new technique, membrane emulsification, using a glass membrane with labyrinthine pores almost equal in diameter. Through this membrane, IPSO enclosing minute vesicles of an aqueous solution of epirubicin [water-in-oil emulsion (W/O)] is split and dripped into a physiological saline solution containing 1% hydrogenated castor oil treated with ethylene oxide (polyoxyethylene 60 stearate, 1:60 molar ratio) to make a W/O/W. IPSO microdroplets are almost equal in size and show remarkable stability; separation to lipid and water layers or change in microdroplet size does not

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ABBREVIATIONS: IPSO, ethyl esters of iodinated fatty acids obtained by hydrolysis of poppy-seed oil; HCC, hepatocellular carcinoma; W/O, water-in-oil emulsion; W/O/W, water-in-oil-in-water emulsion; polyoxyethylene 60 stearate, hydrogenated castor oil treated with ethylene oxide (1:60, molar ratio); AFP, α-fetoprotein; sGOT, glutamic oxaloacetic transaminase; sGPT, glutamic pyruvic transaminase; LDH, lactate dehydrogenase.
occur for at least 40 days when stored at room temperature (Higashi et al., 1996). Because of its stability, the therapy does not require any embolic substance. When this W/O/W is injected into the hepatic artery or its branch, IPSO is distributed to the entire liver tissue covered by (a branch of) the hepatic artery immediately after injection into (the branch of) the hepatic artery and disappears in 1 to 2 weeks from nontumorous tissue, leaving a thick accumulation in the tumor tissue.

Technology for preparing particles with a definite size had not been available until the membrane emulsification technique was developed. When producing W/O/W, IPSO microdroplets can be controlled to change size in a range of 1 to 70 μm by using a different glass membrane of an appropriate pore size. In our clinical trials, the size of IPSO microdroplets was first designed to be 30 μm in diameter, based on the knowledge that albumin or starch microspheres with a diameter of 30 to 50 μm had been used in arterial embolization for metastatic liver cancer (Miller et al., 1987). During the trials, we wondered if the 30 μm diameter of IPSO microdroplets was appropriate because the treatment sometimes failed to attain an adequate effect.

To confirm the appropriate size for the treatment, IPSO microdroplets with a diameter of 10 or 70 μm were injected into the proper hepatic artery of rabbits. We obtained a preliminary result that the larger microdroplets accumulated more intensively in the liver than the smaller microdroplets (S. Higashi et al., unpublished data). In this clinical study, we report that the size of IPSO is important for the treatment of HCC patients.

Experimental Procedures

Materials. Epirubicin hydrochloride was donated by Kyowa Hakko Co., Ltd. (Tokyo, Japan). IPSO (Lipiodol Ultrafluid) was purchased from Kodama Co., Ltd. (Tokyo, Japan). Polyglycerol esters of polycondensed fatty acids of castor oil were purchased from Saka-moto Yakuhin Kogyo Co., Ltd. (Osaka, Japan). Polyoxethylene 60 stearate was purchased from Nikko Chemicals Co., Ltd. (Tokyo, Japan). Controlled pore glass was prepared as a thin membrane with 10.6 or 20.0 μm pore size.

Preparation of W/O/W Containing an Aqueous Solution of Epirubicin. A volume containing 60 mg of epirubicin was dissolved in 5 ml of a 5.8% (w/v) glucose solution and mixed with 5 ml of IPSO and 500 mg of polyglycerol esters of polycondensed fatty acids of castor oil. The mixture was sonicated for 10 min to make a W/O. The W/O was injected through a controlled pore glass membrane at a rate of 10 ml/h into 5 ml of physiological saline containing 1% (w/v) polyoxethylene 60 stearate to prepare a W/O/W. Final volume of W/O/W containing 60 mg of epirubicin and 5 ml of IPSO was 15 ml.

The W/O/W consisted of numerous spherical oil IPSO microdroplets containing vesicles of the aqueous solution of epirubicin. The oil microdroplets were suspended in physiological saline containing polyoxethylene 60 stearate. The size of IPSO microdroplets was determined with a laser diffraction particle-size analyzer (SALD-2000, Shimadzu Corp., Kyoto, Japan).

Hepatic Arterial Injection Chemotherapy with W/O/W. We studied 32 patients of HCC, in whom the serum α-fetoprotein (AFP) level exceeded 10 ng/ml, with or without cirrhosis. Patients were randomly assigned to receive W/O/W of small IPSO microdroplets (30 μm in diameter) containing 60 mg of epirubicin (group A, n = 16) or W/O/W of large IPSO microdroplets (70 μm) containing the same amount epirubicin (group B, n = 16). In both groups, the dose of epirubicin was determined as 40 mg/m² of body surface. There were no significant differences between groups A and B in age, number of tumors, the maximum size of tumors, 15-min value of indocyanine green clearance test, or serum AFP levels before treatment (Table 1). W/O/W was infused through either the proper hepatic artery or its branch at a rate of approximately 5 ml/min. The injection site of the artery was determined based on the number and location of the tumors. No embolic substances such as gelatin sponge particles were injected.

Serum AFP level was measured immediately before and essentially 7 days after treatment. To assess the post-treatment change in serum AFP level precisely, a decline of serum AFP level in a week after treatment from the level immediately before treatment was measured. When the level was not measured on the 7th day, the 7th day level was calculated proportionally as a linear decline from the level measured immediately before the treatment, although the decline was not actually linear. Because initial levels were distributed in a wide range, the decline was expressed as the percentage of the initial level.

Serum levels of glutamic oxaloacetic transaminase (sGOT), glutamic pyruvic transaminase (sGPT), lactate dehydrogenase (LDH), and total bilirubin were measured 24 h after injection of W/O/W. Computed tomograms were obtained 7 days after treatment to confirm the accumulation of IPSO in tumors. Based on the findings of computed tomograms, deposition of IPSO in the tumors was graded as: 1) good—a complete deposition of IPSO, 2) fair—an incomplete deposition of IPSO, and 3) poor—a faint deposition of IPSO.

Results

Characteristics of W/O/W. Mean diameters of IPSO microdroplets prepared with controlled pore glass membranes with pore sizes of 10.6 and 20.0 μm were 30.1 ± 5.1 and 70.0 ± 6.7 μm, respectively (Fig. 1). Changes in the morphology and size of IPSO microdroplets and separation to oil and water were not observed for at least 40 days in a vial stored at room temperature.

Effects of Hepatic Arterial Injection Therapy with W/O/W. Thirty-two patients were graded by intratumor accumulation of IPSO in computed tomograms as good in 16 patients (50.0%), fair in 12 (37.5%), and poor in 4 (12.5%; Table 2). There was no significant difference in grading of tumors across the groups (Table 2).

TABLE 1

| Number of patients | 16 | 16 |
| Male/Female | 15/1 | 13/3 |
| Age (years) | 61.6 ± 8.7 | 61.9 ± 9.5 |
| Pretreatment examinations | 21.2 ± 7.8 | 20.1 ± 11.9 |
| Indocyanine green 15' | 2,610 ± 5,493 | 2,506 ± 6,955 |
| Serum AFP level | 6.7 ± 6.7 | 70.0 ± 6.7 |
| Number of tumors | 3 | 3 |
| Solitary | 6 |
| 2–4 | 6 |
| 5 or more | 6 |
| Maximum tumor size (cm) | 3.2 | 2.8 |
| W/O/W emulsion used | 30.1 ± 5.1 | 70.0 ± 6.7 |
| IPSO microdroplet diameter (μm) | 40 | 40 |

Group A: Patients treated with W/O/W of small IPSO microdroplets (30 μm in diameter) containing 60 mg of epirubicin. Group B: Patients treated with W/O/W of large IPSO microdroplets (70 μm) containing the same amounts of epirubicin.

% Mean ± S.D.

Maximal diameter of the maximum tumor in computed X-ray tomograms.
IPSO accumulation between groups A and B, although all patients graded as poor belonged to group A.

Serum AFP levels decreased in 26 (81.3%) of 32 patients, and the remaining six patients whose serum AFP level increased or was unchanged belonged exclusively to group A. There was a significant difference in the percent decline of serum AFP levels in 1 week from the level of AFP immediately before the treatment between the A and B groups: 18.9 ± 33.1% (mean ± S.D.) in group A and 50.5 ± 19.8% in group B, p < .005 (Table 2 and Fig. 2).

In all patients, serum levels of sGOT, sGPT, LDH, and total bilirubin, which were almost within normal range before treatment, increased within 24 h after treatment and returned to the levels before treatment within 7 days (Table 2). There were no significant differences between two groups in the values 24 h after the treatment.

**TABLE 2**

<table>
<thead>
<tr>
<th>Degree of IPSO deposition</th>
<th>Number of cases</th>
<th>Group A</th>
<th>Group B</th>
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<tbody>
<tr>
<td>Good</td>
<td>7 (44%)</td>
<td>9 (56%)</td>
<td></td>
</tr>
<tr>
<td>Fair</td>
<td>5 (31%)</td>
<td>7 (44%)</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>4 (25%)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

**Liver function tests with serum AFP levels in 1 week from the AFP levels immediately before the treatment.**

The decline of serum AFP level in the first week from the initial level immediately before the treatment. The value is expressed as a percentage of the initial.

- Group A, patients treated with small IPSO microdroplets (30 μm in diameter);
- Group B, patients treated with large IPSO microdroplets (70 μm).

**Fig. 1.** Distribution of the size of IPSO microdroplets of W/O/W used in groups A and B. Relative frequency of diameter of the microdroplets is expressed as a percentage of the maximal value in each.

**Fig. 2.** The decline of serum AFP level in the first week from the initial level immediately before the treatment. The value is expressed as a percentage of the initial.

**Discussion**

The IPSO has an important role in transcatheter hepatic arterial injection chemotherapy for HCC because it has been proven to accumulate in the tumor when injected to the liver via the hepatic artery (Nakakuma et al., 1983; Konno et al., 1983). An idea of lipiodolization or chemoembolization using IPSO was initially advocated intending a drug delivery system using IPSO as a carrier substance (Kanematsu et al., 1984). Our W/O/W, which is prepared by passing W/O through a controlled pore glass membrane, consists of lipid microdroplets containing vesicles of aqueous solution of drugs. When the drugs are not lipophilic, it seems adequate in the treatment of HCC to enclose an aqueous solution of anticancer drugs in the lipid microdroplets of IPSO.

In arterial injection therapy with microdroplets enclosing an aqueous solution dissolving anticancer drug, a grade of intratumor accumulation of IPSO must influence the antitumor effect. Precise mechanisms of the accumulation are not yet clear. Phagocytosis by sinusoidal cells including Kupffer cells and tight impact in vasculature may influence the effects of microdroplets (Miller et al., 1987).

In the liver, the hepatic artery branches into capillaries and then enters the terminal part of the portal vein just proximal to the confluence of the vein with the sinusoid (Cho and Lunderquist, 1983). HCC tissue, except for relatively uncommon forms such as highly differentiated HCC, is fed only by the hepatic artery, which terminates in a sinusoid-like component (tumor sinusoid). IPSO microdroplets injected into the hepatic artery may be trapped in any part of the liver according to their diameter. If the IPSO microdroplets are too small for the inner diameter of the tumor vasculature, they may pass through the tumor tissue as well as normal tissue. If they are too large, the IPSO microdroplets will be trapped proximal to the tumor vasculature, resulting in complete necrosis of the tumor confirmed by histological examination of the surgically excised specimen.
in a meaningless accumulation. From that point, the size of IPSO microdroplets becomes a key factor; if W/O/W is prepared without controlling the particle size of IPSO, accumulation of IPSO in HCC will occur in smaller amounts.

In this study, the sizes of IPSO were strictly designed to be 30 or 70 μm in diameter. A method that can prepare lipid microdroplets of a definite diameter has not been previously reported, therefore, these results are noteworthy in showing clinical effects that are brought out using two kinds of IPSO microdroplets with completely different diameters. To assess the clinical effects of the therapy, we presented a decline of AFP in a week from the levels of AFP immediately before the treatment. A marked difference in the decline was observed between the two groups. In three-fourths of the patients treated with W/O/W of large IPSO microdroplets, values of the percent decline were distributed near the level of 50%, which corroborates one case in our past experience in which complete necrosis was attained after the therapy and its percent decline of AFP in a week was 52% (Fig. 2). In our experience, a decrease of serum AFP levels to an extent approximately 50% in an initial week means almost total necrosis of tumor cells and is a minimal requirement for attaining long survival after the therapy.

From the results obtained, the size of IPSO microdroplets injected into the hepatic artery determines the decrease of serum AFP levels of the patients with HCC. This brings forward the importance of regulating the size of IPSO microdroplets in hepatic arterial injection chemotherapy for HCC.

References


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