Study on Hepatic Artery Chemoembolization Using Temperature-Sensitive Liposome or Lipiodol Emulsion

AtsuO Ono, Isamu Horikoshi, and Masaharu Ueno

Department of Hospital Pharmacy* and Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan. Received July 13, 1994; accepted October 17, 1994

As a novel method for the medical application of liposomes, we have tried hepatic artery chemoembolization using temperature-sensitive liposomes with hyperthermia for the treatment of hepatic tumors. In this study, the effect of temperature-sensitive liposomes was compared with that of Lipiodol emulsion, which has been used clinically. The temperature-sensitive liposomes, consisting of dipalmitoylphosphatidylcholine or Lipiodol emulsions entrapping doxorubicin, were administered into the hepatic artery of hepatic tumor-bearing rats via a cannula. Doxorubicin administered in a liposomal form showed a high accumulative property toward tumors, with heating, while that in the emulsion form showed a slow release property toward tumors. Not only was tumor growth inhibited, but also, an actual diminishing of the tumor was observed in each form. Side effects were also examined: an abnormal rise in GPT, or necrosis of the normal tissues in liver, which was often observed in hepatic artery chemoembolization using Lipiodol emulsion, was remarkably reduced in the liposomal chemoembolization.

Key words temperature-sensitive liposome; Lipiodol emulsion; therapeutic effect; side effect; reverse phase evaporated vesicle; doxorubicin

Liposomes have been widely studied in recent years. The bio-distribution of a drug entrapped in liposomes is different from that of the free drug. This fact brings about advantages such as the extension of drug residence time in the blood, and protection of the drug from inhibitor or metabolizing enzymes. In addition, the liposome is made up of low toxicity lipids. Chemotherapy, as well as surgery and radiotherapy, is important for the treatment of cancer. However, anti-cancer agents have many side effects and many wrong factors. Therefore, patients have not been cured with these drugs in many cases. Liposomes, high-molecular compounds and so on, as drug carriers, have been studied in order to increase the anti-tumor potency and decrease the side effects of cancer chemotherapy agents. Yatkin et al. and Weinstein et al. showed a new method for the medical application of liposomes which allows for the control of drug release from liposomes according to the nature of the phospholipid membrane; the barrier efficiency of the membrane abruptly decreases near the phase transition temperature of the gel-to-liquid crystal of the phospholipid membrane.

Chemotherapy, intra-arterial infusion, arterial embolization and transcatheter arterial chemoembolization during medical treatment have been tested for nonresectable hepatoma, and their efficiencies are recognized.

We have studied chemoembolization therapy of the hepatic artery on hepatic tumor-bearing rats using temperature-sensitive liposomes containing doxorubicin, with local hyperthermia. To date, the formation of an embolus with the liposomes in the hepatic artery, temperature-sensitive drug release from the liposomes with local heating on the liver, high drug accumulative property to the tumor with heating and significant therapeutic effect, not only the inhibition of tumor growth but also a diminishing of the tumor, have been found.

In the present study, the therapeutic effect and side effects of temperature-sensitive liposomes were compared with that of Lipiodol w/o emulsion, which is used in our hospital, on hepatic tumor-bearing rats.

MATERIALS AND METHODS

Materials 1,2-Dipalmitoylphosphatidylcholine (DPPC) was purchased from Nihon Yushi, Tokyo, (no less than 98% purity). Lipiodol Ultra-fluid (Lipiodol) was obtained from Kodama, Tokyo. Adriamycin injection as doxorubicin hydrochloride (DOX) was purchased from Kyowa Hakko, Tokyo. GPT UV-Test Wako was purchased from Wako, Osaka. All other reagents were of reagent grade from Wako, Osaka or Nakalai Tesque, Kyoto.

Seven- or 5-week-old male Wistar rats (Japan Shizuoka Laboratory Center, Shizuoka) were used. Walker 256 carcinosarcoma cells were obtained from Toyama Chemical, Tokyo.

Experiments. Liposome Preparation Liposomes, in a reverse-phase-evaporation vesicle (REV), was prepared according to the methods of Szoka et al. and DOX was encapsulated in it. DPPC (29.4 mg) was added to a 50 ml round-bottom flask, and 12 ml of organic solvent (chloroform: isopropyl ether = 1:1) was added to the flask. DOX was solved in 2-(N-morpholino)ethanesulfonic acid, monohydrate (MES) buffer (pH 6.00), and 2 ml of this DOX solution (10 mg/ml) was added to the organic solvent. The resulting two-phase system was sonicated briefly (5 min) in a bath-type sonicator (Sonorex Super RK156BH, Bandelin electronic) until the mixture became a clear one-phase dispersion that did not separate for at least 30 min after sonication. The mixture was then placed on a rotary evaporator and the organic solvent was removed under reduced pressure (with water aspirator) at 42 °C, rotating at approximately 200 rpm. As the majority of the solvent was removed, the mixture first formed a viscous gel and subsequently became an aqueous suspension. The aqueous suspension was evaporated for an additional 30 min at 47 °C to remove traces of the organic solvent.

© 1995 Pharmaceutical Society of Japan
DOX, which was not encapsulated, was separated by centrifugation as follows: the REV suspension was centrifuged at 3000 × g at 4 °C for 20 min. The precipitate was resuspended in MES buffer after being washed by a cold buffer 3 times. This suspension was adjusted to make 7 mg/ml. The liposome size was evaluated by quasi elastic light scattering using a laser particle analyzing system (Otsuka Electronics Co., LPA 3000/3100). The mean diameter of the liposomes was approximately 1000 nm.

**Lipiodol w/o Emulsion Preparation** Lipiodol w/o emulsion was prepared according to the method used in this hospital as follows. A test tube containing Lipiodol (1.5 ml) and HCO-60 (60 mg), was heated at 60 °C. Then 0.4 ml at a DOX water solution (25 mg/ml) was added drop by drop (1 drop per 10 s) with vortex mixing.

**Implantation of Tumors Cell in the Liver** The Walker 256 tumor cell line was maintained by successive transplantation into the abdomen of a 5-week-old male rat. A total of 0.1 ml of a suspension containing approximately 10⁶ cells of Walker 256 was injected via a 26.5 gauge needle into the parenchyma of the left anterior lobe of the liver. Seven to 9 d after the implantation of the cells into the liver, the tumor was 0.5 to 2.0 cm in long diameter. The animals then underwent laparotomy, and those rats with tumors of about 1.0 cm in long diameter were used for subsequent experiments.

**Drug Administration** The rats were anesthetized and then a laparotomy performed. The gastroduodenal artery was used for hepatic artery cannulation. A polyethylene cannula was inserted from the duodenum side of the gastroduodenal artery into the entrance of the hepatic artery. The blood flow from the common hepatic artery to the hepatic artery was not occluded by the cannula; positioning and flow could be checked by angiography (Fig. 1). The intra-arterial dose was fixed at 2.35 mg (DOX)/kg of body weight. When the drug was administered, the common hepatic artery was closed by a clip for protection against flowing backward. The clip was removed after administration. Two hours after administration of liposomes containing the drug, the tumor, liver and surrounding tissues were heated from the outside using a microwave generator (Minato Med. Sci. Co., Microtizer MT-30i) at 42 °C for 6 min for temperature-sensitive controlled drug release.

**Bio-distribution** Three or four rats were killed at each time interval (at 5 min, 2, 8, 24, 120 h) after the administration. Various organs were removed and weighed. A volume of Kuthoff buffer equal to 5 times the tissue sample weight was added, the tissues were homogenized, and the DOX was extracted with butanol: toluene = 1:1 solution (5 ml per ml of homogenized tissue). The samples were centrifuged at 3000 × g for 10 min. The supernatants were collected, dried and dissolved in PBS: methanol = 1:1 solution (1 ml per ml of homogenized tissue). DOX and metabolite concentrations in these samples were analyzed by HPLC.

**Tumor Growth Inhibition** Rats were randomly separated into 5 groups of 4 animals each. Seven days after implantation, these rats underwent abdominal surgery and tumor size was measured (long diameter and short diameter). Group one was treated with normal saline as a control, group two with the Lipiodol emulsion (drug-free), group three with the liposome (drug-free), group four with the Lipiodol emulsion containing DOX, and group five with the liposome containing DOX. The administration method followed the preceding description. Five days after the administration, tumor size was measured. The tumor growth rate (%G) was calculated using the following equation:

\[
\%G = \frac{\text{tumor volume after dosing}}{\text{tumor volume before dosing}} \times 100
\]

Tumor volumes were approximated by the next equation

\[
\text{tumor volume} = \text{long diameter} \times \frac{1}{2} (\text{short diameter})
\]

**Side Effect Evaluation** Side effects were evaluated by the GPT measurement in plasma and by necrosis in the liver. Normal rats were randomly separated into 4 groups of 5 animals each. The groups were treated as follows: normal saline, liposome containing DOX without heating, liposome containing DOX with heating, and Lipiodol emulsion containing DOX. Blood samples were taken with heparinized syringes from the heart at 0, 1, 5, 7, 14 and 21 d after the dosing and were centrifuged to separate the plasma. The plasma samples were kept frozen until the analyses according to the UV-Rate method of GPT. Liver necrosis was pictured at 24 h after imaging.

**RESULTS**

**Bio-distribution** The DOX concentration in the various organs 8 h after dosing is shown in Fig. 2. The tumor levels were higher in the two treatment groups than in the others. The highest DOX level was obtained in the liposome treatment group. In the spleen and lung, DOX levels of the liposome administration group were high, which compared with those of the others, except for the tumor and liver levels.
Figure 3 shows the time course of DOX concentration in the tumor. The abscissa was expressed in logarithmic scale. The DOX concentration rose abruptly 8 h after the liposome administration. This was presumably due to the fact that DOX was released from the liposomes by local heating 2 h after treatment. On the other hand, a slow release (slow clearance) property was observed for the lipiodol emulsion administration group. For example, the concentration of DOX was 12 μg/g at 5 min, and it was 6 μg/g at 120 h after dosing. In Fig. 4, the clearance of
DOX from the normal liver in the Lipiodol emulsion administration group was slightly faster than that of the liposome administration group. DOX is known to be decomposed by enzymes in the liver. Its metabolites are doxorubicinol, doxorubicinone and aglycone. Doxorubicinol and doxorubicinone can be detected with a fluorescence detector.\(^{21}\) Figures 5 and 6 show the time behaviors of the concentration of DOX and its metabolites in the tumor and the liver. For the liposomal DOX-treated group, a high concentration of DOX and its metabolites in the tumor were observed 8 h after dosing, about 38 μg/g. But a low concentration of metabolites was observed at other times. In Fig. 6, metabolites were observed each time in the liposomal treated-group, although the quantity of metabolites decreased over time and no metabolites were observed after 24 h in the Lipiodol emulsion administration group.

**Anti-Tumor Effect** Tumor growth for 5d after treatment was examined. The anti-tumor effect of liposomes containing DOX was the highest of all in Fig. 7. In the groups with liposome and Lipiodol emulsion (each drug-free) administration, anti-tumor effects were not recognized.

**Side Effect Evaluation** Figure 8 shows the GPT activity at several periods after dosing. To examine the heating effect on GPT activity, the liposome administration group was divided into two groups, that is, with heating and without. One day after treatment, the activity of all groups rose except in the control group. As for the group receiving the Lipiodol emulsion treatment, an especially abnormal activity rise (about 100 times as high as the control) was observed. GPT activity was raised by local heating in the liposome administration group. The same results were obtained in AST activity observation (data not shown). Wide-ranging necrosis was observed in the liver of the Lipiodol emulsion-treated group, as shown in Fig. 9.

**DISCUSSION**

In cancer chemotherapy, the most serious problem is that therapy is often uncompleted. Trans-arterial chemoembolization is an effective method for treating cancer.\(^{11,12}\) Chemoembolization using Lipiodol emulsion has been carried out in nonresectable hepatoma in our hospital. The therapeutic effect, the drug release process, side effects of using liposomes or Lipiodol emulsion have been discussed.

As bio-distribution results, DOX concentrations in the liver 5 min after dosing were almost the same as in the two other groups (liposome and Lipiodol emulsion treatment), but in the tumor they were different, as shown in Figs. 3 and 4. The DOX concentration of the Lipiodol emulsion-treated group was higher than that of the liposome-treated group in the tumor for 2 h after dosing. By local heating, the DOX contained in liposomes showed a high accumulative property toward tumors. DOX concentration in the Lipiodol emulsion-treated group in the tumor and in the liver was almost the same.
within 2 h after the treatment. Walker 256 carcinosarcoma was a solid tumor during the observation, and had not grown any vessels. From the above results, the following release process of each drug was inferred. The drug administered via the hepatic artery, exists in the arterial capillary of the liver instantly after dosing. Liposome and the DOX in it remained in the hepatic arterial capillary until local heating was complete. Liposomes flowed slightly from the liver and arrived at the spleen and the lung (Fig. 2). By the local heating, DOX was released from the liposomes and arrived at the tumor with this slight blood flow. The DOX in the Lipiodol emulsion arrived at the tissues of the liver and the tumor 5 min after dosing. Then, the DOX in the tumor was cleared slowly compared with that in the liver.

As shown in Figs. 8 and 9, the Lipiodol emulsion was severely damaged in the normal liver tissues. It was shown that the GPT activity rose abnormally 24 h after the treatment, and a fall in metabolite concentration, which indicated a decrease in metabolism ability, was observed at the same time, as shown in Fig. 6.

In this paper, it was shown that trans-hepatic-arterial chemoembolization using temperature-sensitive liposomes containing DOX with local heating resulted in an extremely temperature-sensitive controlled drug release effect with good anti-tumor efficiency, while that using a Lipiodol emulsion had anti-tumor efficiency and a low clearance effect in the tumor, but caused wide spread necrosis of the normal tissues in the liver. Temperature-sensitive liposomes are a new material for efficient chemoembolization for nonresectable hepatomas.

REFERENCES