

Antitumor Effect of Liposome-entrapped Adriamycin Administered via the Portal Vein

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We examined the distribution in tissues and antitumor effect of freeze-dried liposome-entrapped adriamycin (Lipo-ADM) administered via the portal vein to rabbits bearing VX2 tumors. Liposomes composed of egg phosphatidylcholine (cholesterol 50 mol%) were used as drug carriers. The liver concentration of ADM increased after delivery and cardiac uptake decreased compared with free drug treatment. The *in vivo* antitumor effect of Lipo-ADM was determined in rabbits inoculated with VX2 tumor. Repeated injections of free ADM via the portal vein prolonged the life span of tumor-bearing rabbits. The life span was further prolonged by Lipo-ADM treatment compared with the control group and the free ADM group. Histological examination revealed that the damage to the liver caused by Lipo-ADM administered via the portal vein did not differ from that observed in animals treated with free ADM. These results indicate that portal vein administration of Lipo-ADM may be more effective in dealing with liver metastases than treatment with free ADM and may be therapeutically useful without toxic side effects.

Key words: Liposome-entrapped adriamycin — Liver metastasis — VX2 tumor — Portal vein administration

Liposomes have been extensively examined as a vehicle for improving the delivery of various therapeutic agents.¹⁻⁴ Their potential usefulness to enhance the therapeutic efficiency and to reduce certain types of toxicity of drugs has been reported.⁵⁻¹⁰ Recent reports have demonstrated the effectiveness of liposomal delivery of antitumor agents against experimental liver metastases.^{11, 12} Intravenously injected liposome-entrapped forms are taken up by the liver more efficiently than free forms, which results in an increase in antitumor activity against liver metastases. Furthermore, to increase the uptake and the therapeutic index of drugs it is important to determine the optimum route of administration. Target-oriented topical administration is expected to improve the target specificity and uptake of chemotherapeutic agents. We report here that portal vein administration of liposome-entrapped adriamycin was more effective than free ADM in the treatment and prophylaxis of experimental liver metastases in rabbits.

MATERIALS AND METHODS

Animals Male Japanese white rabbits weighing 2.5 to 3.0 kg were used for this study. They were given free access to water and normal diet.

Chemicals Adriamycin-HCl (ADM, 10 mg of potency per vial) was a gift from Kyowa Hakko Kogyo (Tokyo). Egg lecithin (COATSOME NC-10, Nichiyu Liposome

Co. Ltd., Tokyo) specified as a pharmaceutical adjuvant for parenteral use was used. Cholesterol (special grade, Wako Pure Chem. Co. Ltd., Osaka) was recrystallized from ethanol under aseptic conditions. Mannitol (20% w/v) was purchased from Nikken Kagaku Co., Ltd. (Tokyo).

Preparation of freeze-dried liposome-entrapped ADM (Lipo-ADM) ADM supplied as a freeze-dried form containing 10 mg of ADM and 100 mg of lactose per vial was reformulated to a freeze-dried form of egg lecithin-ADM mixture under aseptic conditions. A required amount of egg lecithin and cholesterol mixture (molar ratio, 2:1; hexane-ethanol (95/5) stock solution) was taken into a sterilized round-bottomed flask and the solvent was removed using a rotary evaporator. The resulting lipid thin film was dissolved in ether, to which an appropriate amount of water for injection containing mannitol was added. The mixture was vortexed for about 5 min to give a W/O emulsion and the solvent was removed according to the reverse-phase evaporation method.¹³ The resulting liposome suspension was distributed into ADM vials, and vortexed until the original freeze-dried cake was completely dissolved. Each vial contains 50 mg of lecithin, 12 mg of cholesterol and 10 mg of ADM in 3 ml. The content was freeze-dried and stored at 4°C until use. Liposomes were reconstituted for use by adding water for injection to the dried cake through a rubber cap, and sonicated using a ultrasonic

apparatus (UD-200, Tomy Seiko Co. Ltd., Tokyo) equipped with a cup horn-type irradiation unit (CH-063, Tomy Seiko). The unit was employed to reduce the particle size of the suspension in the vial without contamination, during which process the temperature of the unit was controlled by water circulation. The reconstituted liposomes were further extruded through 0.45 μm Nucleopore filters prior to experiments. The mean particle size was measured by dynamic light scattering (DLS-700, Otsuka Electronics Co. Ltd., Osaka).

Tumor cell culture VX2 carcinoma cells were obtained fresh from a donor rabbit by mincing the tumor in Hanks' balanced salt solution (HBSS), filtering it through 150 mesh stainless steel gauze and disaggregating the cells in 0.25% trypsin. The cell suspension was washed and resuspended in HBSS to a concentration of 5.0×10^5 cells per ml. The tumor strain was maintained by injection of tumor cells into the thighs of new rabbits each week.

Tissue distribution study VX2 tumor cells (5×10^5) were injected into the mesenteric vein. Two weeks after tumor inoculation, rabbits were injected with free ADM or Lipo-ADM (3 mg/kg, respectively) and killed after various periods of time. Tissues including the tumor, liver, spleen, heart, lung and kidney were removed, frozen rapidly on dry ice, and stored at -30°C for later analysis. The diameters of tumors removed from the liver were 5 to 7 mm. Blood samples were obtained from the femoral vein 5, 10, 15, 30, 60 and 120 min after injection of the drug via the portal vein. ADM was determined by high-performance liquid chromatography with a fluorescence detector (Ex. 470 nm and Em. 585 nm).

Cannulation into the portal vein Rabbits were anesthetized by an iv injection of pentobarbital sodium (25 mg/kg) and laparotomy was carried out in each animal. A catheter (diameter, 1.0 mm) was inserted from the appendiceal vein into the portal vein. A reservoir was placed in the subcutaneous layer of the abdomen.

Antitumor effect Tumor cells (5×10^5) were injected into the appendiceal vein of 18 rabbits followed by cannulation. The animals were divided into three groups with six rabbits in each group. The ADM group and Lipo-ADM group were injected with free ADM or Lipo-ADM (3 mg/kg, each), respectively, on days 1, 4, 7, 14, 21, and 28 thereafter using the reservoir. The control group was established with 1 ml of water per body on the same days. Rabbits were inspected daily and survival curves were recorded.

Biochemical study Blood samples were obtained from the ear vein. GOT, GPT and alkaline phosphatase were determined by using a Raba Super System (Chugai, Pharm. Co., Tokyo).

Histological examination Light microscopical examinations were performed upon organs from normal and

tumor-inoculated rabbits treated with free ADM and Lipo-ADM using routine paraffin fixation and hematoxylin and eosin stains.

Statistical analysis The statistical significance of differences was determined by using Student's *t* test or the generalized Wilcoxon test.

RESULTS

Characterization of freeze-dried Lipo-ADM The size distribution of liposome preparations after reconstitution with water and sonication in the cup horn was investigated. The liposomes had a mean diameter of 256.2 nm before filtration and 219.8 nm after. The ADM content of liposomes was $32 \pm 6\%$ and the prepared

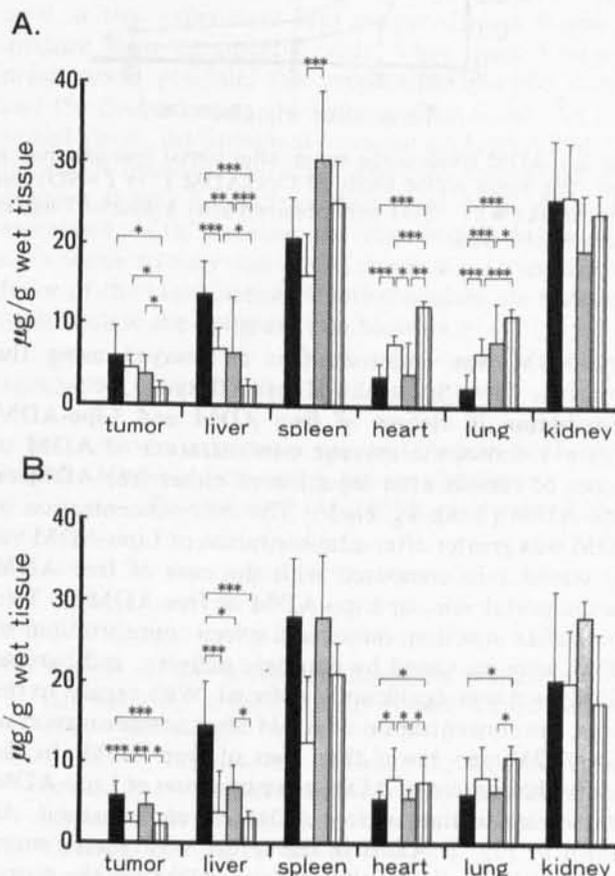


Fig. 1. Tissue distribution of ADM 1 h (A) and 2 h (B) after portal vein administration and iv injection in tumor-inoculated rabbits. The mean levels of ADM were obtained after a dose of 3 mg/kg. ■, portal vein administration of Lipo-ADM; □, portal vein administration of free ADM; ▨, iv injection of Lipo-ADM; ▩, iv injection of free ADM. Each value represents the mean \pm SD of 7 rabbits. Significant differences: *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$.

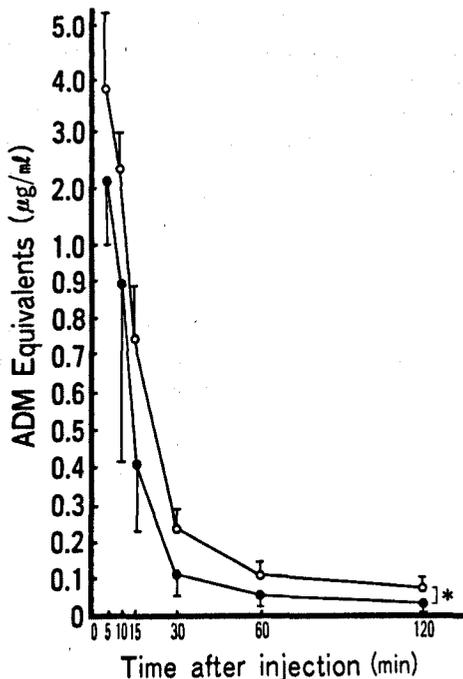


Fig. 2. ADM levels in the serum after portal vein administration. The mean ADM levels of Lipo-ADM (○) (+SD) and free ADM (●) (-SD) were obtained after a dose of 3 mg/kg each. *, $P < 0.05$.

Lipo-ADM was endotoxin-free as assayed using the Toxicolor Test (Seikagaku Kogyo, Tokyo).

Distribution in tissues of free ADM and Lipo-ADM Figure 1 shows the average concentration of ADM in tissues of rabbits after injection of either free ADM or Lipo-ADM (3 mg/kg, each). The liver concentration of ADM was greater after administration of Lipo-ADM via the portal vein compared with the case of free ADM via the portal vein, or Lipo-ADM or free ADM iv. Two hours after injection, tumor and spleen concentrations of ADM were increased by liposome delivery, and cardiac ADM level was significantly reduced. With regard to the lungs, the concentration of ADM after administration of Lipo-ADM was lower than that of free ADM. In the kidney the content of ADM after injection of Lipo-ADM was similar to that of free ADM for each method. As shown in Fig. 2, ADM in the serum was cleared more slowly after administration of Lipo-ADM via the portal vein as compared with administration of free ADM.

Therapeutic effects Figure 3 shows the results of the experiment on the effect of treatment with free ADM and liposome-entrapped ADM administered via the portal vein on survival of rabbits inoculated with VX2 tumor. The mean survival time of the control group was 32 days after tumor inoculation; that of the free ADM group was

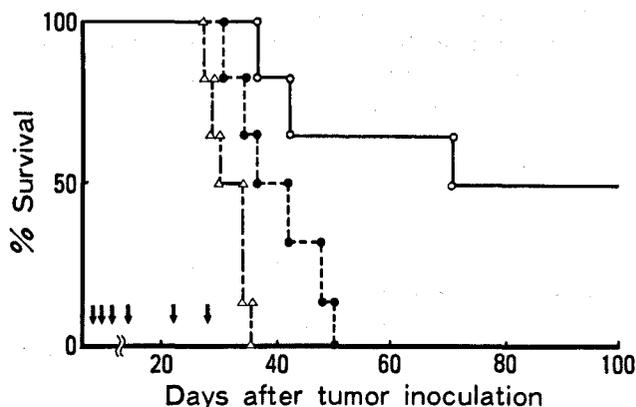


Fig. 3. Survival of rabbits bearing VX2 tumor after portal vein treatment. Eighteen rabbits were injected into the appendiceal vein with 5×10^5 VX2 tumor cells and then cannulated into the portal vein. There were 6 rabbits per treatment group. The ADM (●) and Lipo-ADM (○) groups were injected with free ADM or Lipo-ADM (3 mg/kg, each), respectively, on days 1, 4, 7, 14, 21 and 28, and control groups (△) were treated with water (1 ml/body) using the reservoir. The schedule of treatment is indicated by arrows. Significant differences: controls < ADM group, $P < 0.05$; controls < Lipo-ADM, $P < 0.01$; ADM group < Lipo-ADM group, $P < 0.05$. The statistical significance of differences was determined by use of the generalized Wilcoxon test.

40 days, and compared with that of controls, free ADM significantly prolonged the life span of rabbits ($P < 0.05$). The mean survival time of the Lipo-ADM group was 75 days and Lipo-ADM further prolonged the life span of rabbits in this experiment compared with that of the controls ($P < 0.01$) and that of the free ADM group ($P < 0.05$). In addition, all dead animals were autopsied and all exhibited visible liver metastases.

Biochemical study and histopathological study The results of the biochemical study revealed that GOT, GPT and alkaline phosphatase of the rabbits treated with Lipo-ADM (3 mg/kg) via the portal vein were similar to those of the animals before being treated. Histological study revealed that in the livers of rabbits treated with either form of ADM there was no significant toxic effect. However, in the spleen, atrophy of both red and white pulps was observed with these histological changes being similar to those previously reported.^{14,15} No significant damage was noted in the heart, lungs or kidneys.

DISCUSSION

In this experiment, it was demonstrated that administration of Lipo-ADM through the portal route led to greater accumulation of ADM in the liver than did

administration of free ADM, resulting in significant inhibition of hepatic metastasis. It is believed that most hepatic metastases from gastrointestinal cancer are dispersed through the portal system, and that the blood flow differs according to the stage of tumor growth.^{16,17)} Lipo-ADM administered at an early stage via the portal vein is effectively taken into the liver, inhibiting metastasis, and the remainder circulates systemically from the central vein. The decrease in the clearance in the serum is assumed to reflect the amount of this systemically circulated Lipo-ADM. There was no increase in the uptake of the heart and lungs, in agreement with previous reports on the intravenous injection of Lipo-ADM.^{18,19)} Factors affecting the amount of Lipo-ADM uptake by the liver when it is administered locally include the electric charge of liposomes and the diameter of the particles. Although the intake into the liver can be increased by introducing liposomal components with surface charges,³⁾ this is not a desirable approach for a drug carrier because the alteration causes the liposomes to become toxic.²⁰⁾ Concerning the diameter of the particles, since in the portal vein area the small pores of the endothelial openings of the hepatic sinusoids are 0.1 μm in diameter, and those of the large fenestrations average 1–3 μm ,^{21,22)} intake of small liposomes into the liver is expected to be better than that of large ones.²³⁾ In this experiment using Lipo-ADM averaging 219.8 nm in diameter, the ADM concentration in the liver after 2 h became approximately 3 times that obtained by using free ADM, and the uptake by the tumor increased as well as the hepatic intake, following administration via the portal vein. Repeated administration via the portal vein resulted in a significant increase in the survival rate of rabbits with transplanted tumors. Moreover, no side effects due to the liposomes were recognized. However,

further studies are necessary to clarify the optimal liposome size for administration via the portal vein. The observed prolongation of life span seems to be due not only to the increase in hepatic intake, but also to the fact that Lipo-ADM administered via the portal vein somehow affects the hepatic monocyte-macrophage system. Since ADM²⁴⁻²⁶⁾ and Lipo-ADM²⁷⁾ are known to be activators of macrophages, and since Lipo-ADM in the sinusoids is considered to be taken into Kupffer cells, Lipo-ADM is speculated to augment Kupffer cell activity.²⁸⁾ This matter is presently under investigation.

Many studies on liposomes have been conducted, proving that they enhance the selective delivery of drugs, alter the drug kinetics in the body, and can be effective as drug carriers for anti-cancer agents. However, liposomes cannot easily be used clinically because of their physicochemical properties and complicated method of preparation. Taking those factors into consideration, Lipo-ADM used in this experiment was prepared as a freeze-dry mixture using commercial vials. They made long-term preservation possible, the preparation method simple, and the dose accurate, facilitating clinical use. As mentioned above, the liposomal formulation Lipo-ADM contains 32% liposome-entrapped and 68% free ADM. This mixture should be distinguished from 100% liposome-entrapped ADM because the distribution behavior of each seems to vary and could produce a series effect in favor of the combination. Further studies are necessary to formulate the optimum combination.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid from the Japanese Ministry of Education, Science and Culture.

(Received April 23, 1990/Accepted July 14, 1990)

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