

Liposomes as Carriers of Cisplatin into the Central Nervous System

—Experiments with 9L Gliomas in Rats—

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Abstract

The anticancer agent cis-diamminedichloroplatinum (cisplatin) has several disadvantages, including extreme nephrotoxicity, rapid binding to plasma proteins, and poor penetration of the central nervous system. In this study liposomes, which can cross the blood-brain barrier, were investigated for their potential in delivering therapeutic agents to brain tumors. Liposomes prepared from egg phosphatidylcholine and cholesterol in a 3 : 1 molar ratio were divided into 1-ml aliquots and either labeled with ^{14}C or treated with horseradish peroxidase (HRP). The preparations were administered *via* the carotid artery to rats bearing 9L glioma. Radioactive uptake by brain tumor and normal tissues was measured with a liquid scintillation counter. The presence of HRP-containing liposomes in capillary endothelium and brain tumor cells was demonstrated by light and electron microscopic histochemical techniques. Thirty minutes after injection of ^{14}C -labeled liposomes, radioactive uptake was higher in the spleen than in normal brain, brain tumor, liver, and kidney. Also uptake was greater in brain tumor and lower in kidney than that of cisplatin given alone. Light microscopy showed HRP-containing liposomes in brain tumor tissue 30 minutes after injection. On electron microscopy, liposomes were found to be regularly distributed in surface invaginations and vesicles of capillary endothelial cells. They were also observed within tumor cells. These results indicate that liposomes can penetrate the blood-brain barrier and hold promise as drug carriers in the treatment of brain tumors with cisplatin.

Key words: liposome, cisplatin, 9L rat brain glioma, drug delivery system, blood-brain barrier

Introduction

Cis-diamminedichloroplatinum (cisplatin) is known to be effective in the treatment of malignant glioma. Unfortunately, however, it does not easily cross the blood-brain barrier. In the hope of overcoming this problem, as well as that of nephrotoxicity, we have been investigating a cisplatin delivery system that involves liposomes as carriers.^{12,13)}

In the study reported here, we injected ^{14}C -labeled liposomes into the internal carotid arteries of rats with implanted 9L gliomas and measured their uptake by brain tumor and certain organs. In addition, we injected liposomes containing horseradish peroxidase (HRP) and studied their distribution in capillary endothelium and tumor cells by light and

electron microscopy.

Materials and Methods

I. Rat 9L glioma model

N-ethyl-N-nitrosourea was administered to Fisher 344 rats and the resultant brain tumors were cultivated in complete medium for many generations, until independent gliosarcoma cells were obtained. Using 15 male Fisher 344 rats, 15-day-old, we drilled trephined holes 1.5 mm anterior to the bregma and 3 mm lateral to the sagittal sinus. The 9L glioma cells were suspended in fluid (15 cells/10 μl) and implanted stereotactically into the left caudate nucleus at a depth of 5 mm.

II. Preparation of liposomes

^{14}C -labeled liposomes: Egg phosphatidylcholine

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(50 μmol) and cholesterol (15 μmol) were dissolved in 2 ml of a chloroform solution, to which 1 μCi of ^{14}C -dipalmitoyl phosphatidylcholine was added. The fluid was then evaporated under negative pressure, leaving a thin lipid film. A 1-ml saline solution was added to this film and it was centrifuged repeatedly, thus forming a multilamellar vesicle (MLV).

HRP-containing liposomes: A 1-ml saline solution containing 100 mg of HRP was added to the above-described lipid film to moisten it. Repeated centrifugation was applied to remove HRP deposits. A saline solution was added to bring the volume to 1 ml.

III. Injection of liposomes and preparation of tissues

Under halothane anesthesia, the left common, internal, and external carotid arteries were exposed. The occipital, superior thyroidal and pterygopalatine arteries were coagulated and divided, and a catheter was inserted into the left external carotid artery toward the bifurcation of the carotid artery.

In Experiment 1, 1 ml of ^{14}C -labeled liposomes was injected into five rats through the catheter. Thirty minutes later, the brain was irrigated through the ascending aorta with a saline solution containing heparin and the brain, the tumor, liver, spleen, and kidney were removed. The organs were sliced and each 100 mg of the specimens was solubilized. Radioactivity was measured with a fluid scintillation counter (LCS 900, Aloka Co. Ltd., Tokyo).

In Experiment 2, 1 ml of liposomes containing 20 mg of HRP was administered to 10 rats through a catheter. Thirty minutes later, the brain was irrigated with a mixture of 1.25% glutaraldehyde and 4% paraformaldehyde inserted through a cannula into the left ventricle of the heart. Brain slices were fixed, washed, and prepared as 40- μm frozen sections. These were incubated in a diaminobenzidine- H_2O_2 solution for 10 minutes to allow the reaction product to develop. The sections were then rinsed and stained with methylgreen for light microscopic examination. Some specimens were refixed, dehydrated, embedded in Epon, and cut into ultrathin sections for electron microscopic study. For control purposes, similar specimens were prepared from three rats injected with 20 mg of HRP alone.

Results

I. Distribution of ^{14}C -labeled liposomes

Using an uptake index of 1 ml of ^{14}C -labeled liposomes per 100 mg of organ, the uptake rates 30 minutes after intracarotid injection were: spleen,

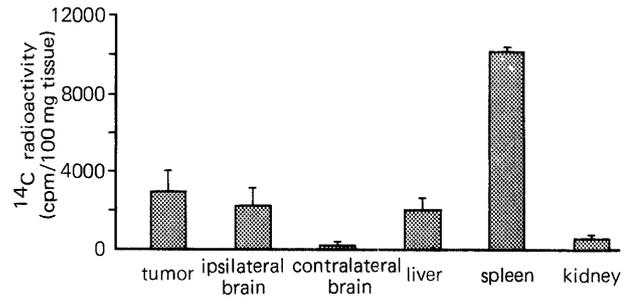


Fig. 1 Radioactive uptake by various tissues 30 minutes after injection of ^{14}C -labeled liposomes in five rats.

0.74%; brain tumor, 0.22%; liver, 0.15%; kidney, 0.05%; and contralateral brain, 0.02%. Thus, uptake of liposomes was greatest in the spleen, relatively low in the kidney, and slightly higher in brain tumor than in the liver (Fig. 1).

II. Distribution of liposome-encapsulated HRP

Figure 2A is a light photomicrograph of a control brain specimen, removed 30 minutes after intracarotid injection of HRP 20 mg alone, showing that HRP was minimally distributed in the brain tumor tissue. As Fig. 2B shows, however, when HRP was carried by liposomes, it was diffusely distributed throughout the brain tumor. Mild staining was observed in the brain tissue surrounding the tumor.

Electron microscopy demonstrated the liposomes as MLVs 0.1–0.4 μm in diameter, positive for HRP. There were many liposomes in the lumen, lodged in the surface infoldings, and in the pinocytotic vesicles of the capillary endothelium of the tumor (Fig. 3A). Liposomes were also seen under the basal lamina. In one specimen, two liposomes were observed inside a tumor cells (Fig. 3B). Figure 3C shows macrophages ingesting liposomes.

Discussion

Cisplatin is the only available antitumor drug that contains a heavy metal complex. Although cytotoxic *in vitro*,^{6,7,11} this agent is blocked by the blood-brain barrier *in vivo* because it is soluble in water. Therefore, it has not well been effective against central nervous system (CNS) tumors.^{1,2,5,15,16} Moreover, cisplatin is nephrotoxic, and numerous potential antidotes to this side effect (selenious acid, bismuth subnitrate, sodium thiosulfate, and fosfomycin) have proved unsuccessful. Addition of water is apparently the only solution to the problem of the nephrotoxicity.

We have been directing our efforts to the develop-

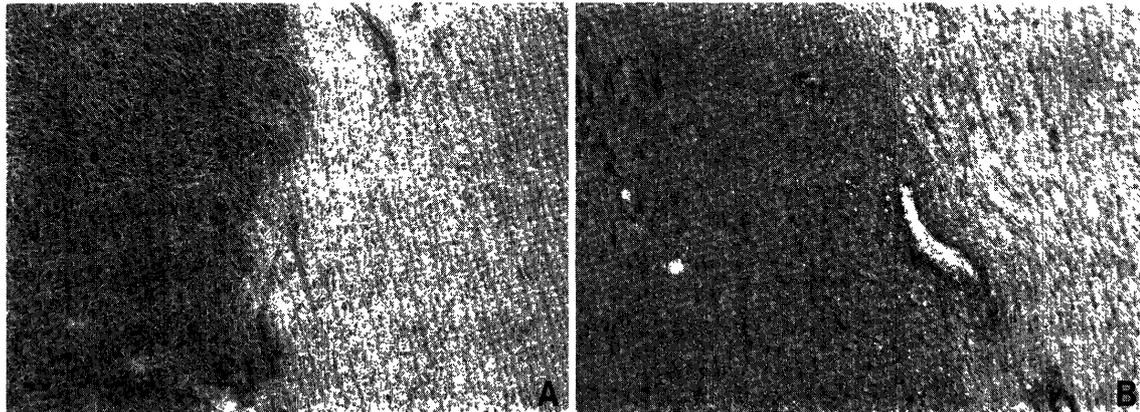


Fig. 2 A: Thirty minutes after injection of HRP alone, no uptake is seen in the tumor. Methylgreen stain, $\times 82.5$. B: Thirty minutes after their injection, HRP-containing liposomes are distributed within the tumor. Methylgreen stain, $\times 165$.

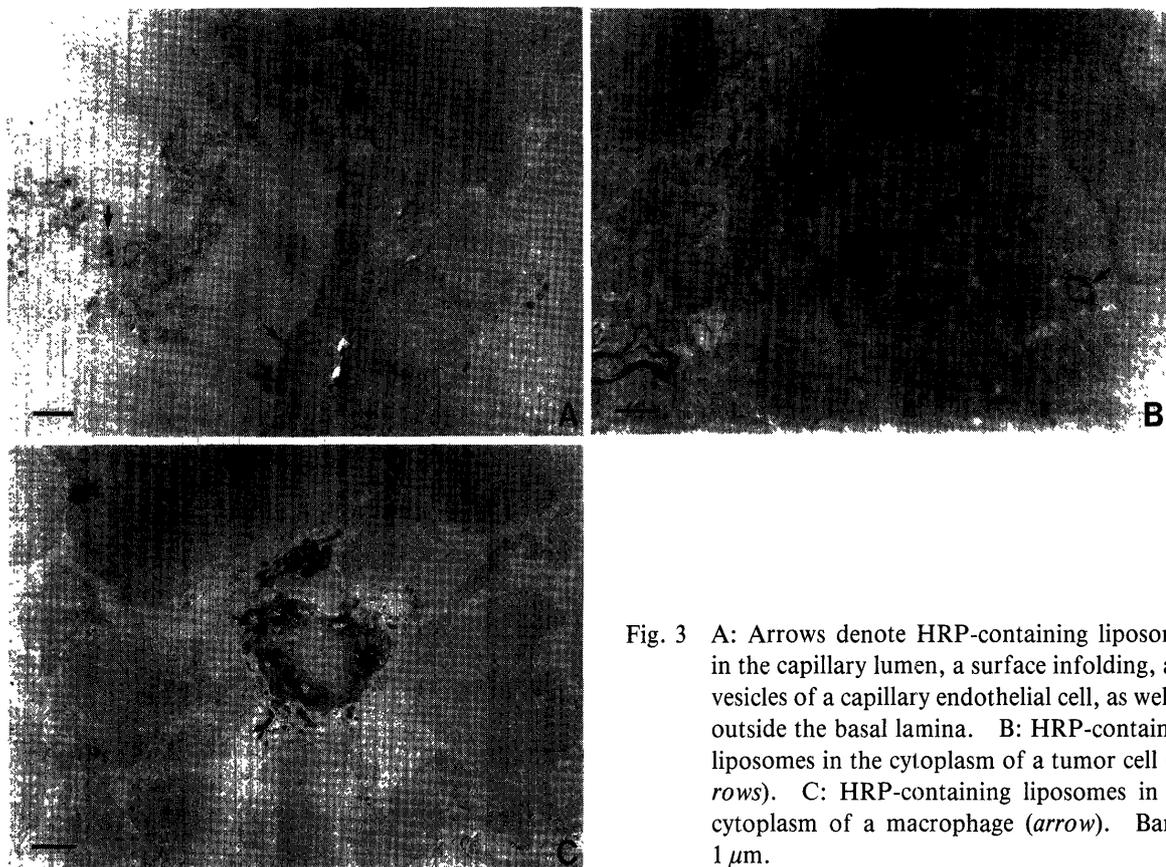


Fig. 3 A: Arrows denote HRP-containing liposomes in the capillary lumen, a surface infolding, and vesicles of a capillary endothelial cell, as well as outside the basal lamina. B: HRP-containing liposomes in the cytoplasm of a tumor cell (arrows). C: HRP-containing liposomes in the cytoplasm of a macrophage (arrow). Bar = $1 \mu\text{m}$.

ment of a system whereby liposomes deliver cisplatin past the blood-brain barrier. As oil-soluble carrier capsules, liposomes would reduce nephrotoxicity and increase the therapeutic effect within the CNS.^{3,4,9,10,20,21)}

In the study presented here, we evaluated the transport of ^{14}C -labeled and HRP-containing liposomes into brain tumors and found it to be slightly greater than that into liver. In samples of brain

tumor, liposomes were observed, under electron microscopy, in the vesicles of endothelial cells, outside of the basal lamina, and inside tumor cells. As we reported previously,^{12,13)} we obtained similar findings when we injected ferritin-containing liposomes into the carotid arteries of rats with ethylnitrosourea-induced gliomas. We believe that these studies of vascular transport of liposomes provide a strong basis for consideration of liposome-encapsulated cis-

platin in the treatment of brain tumors.

In this study, liposome accumulation was considerably greater in the spleen and liver than in the kidney. Thus, while the use of liposomes may significantly reduce the nephrotoxicity of cisplatin, the possibility of damage to the liver and spleen must be considered. Cisplatin is thought not to affect the hepatic enzymes that metabolize drugs, but a means must be sought to inhibit its uptake into the reticuloendothelial system. It is also important to reduce phagocytosis of liposomes by macrophages that migrate into the brain tumor.^{8,14)}

The liposomes used in this experiment were electrically neutral. In a subsequent report we will describe the results of a study involving positively and negatively charged as well as neutral liposomes, in which we found the neutral liposomes to have the highest uptake by rat 9L glioma. Also, at the suggestion of our colleague Dr. J. Sunamoto,¹⁷⁻¹⁹⁾ we have produced liposomes coated with polysaccharides and intend to study their potential as drug carriers.

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