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Drug-carrier Property of Albumin Microspheres in Chemotherapy. V.[1] Antitumor Effect of Microsphere-entrapped Adriamycin on Liver Metastasis of AH 7974 Cells in Rats

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Bovine serum albumin microspheres containing adriamycin were prepared by heat solidification of albumin in albumin-adriamycin aqueous solution in cottonseed oil emulsions. The efficiency of the microspheres as a drug carrier of adriamycin was evaluated in liver metastasis caused by the injection of AH 7974 tumor cells into the portal vein of rats as a model of release of such cells into the vein during the course of surgical removal of gastric cancer. Adriamycin entrapped in the microspheres exhibited sustained *in vitro* release which followed first-order kinetics. After intraportal injection in rats, the microspheres and entrapped agent distributed mainly in the liver, and the disappearance rate from the tissue was very slow in comparison with that of free drug. The survival times of rats bearing AH 7974 liver metastasis were prolonged by intraportal administration of microspheres containing adriamycin. In contrast, free adriamycin or microspheres without entrapped drug did not significantly increase the life span over the control. These results suggest that albumin microspheres containing adriamycin may be applicable as a drug carrier in the adjuvant chemotherapy of liver metastasis.

Keywords——albumin microsphere; drug carrier; adriamycin; sustained release; preferential distribution; AH 7974 liver metastasis; antitumor effect; adjuvant chemotherapy

One of the goals in cancer chemotherapy is directing drugs selectively into tumor tissues to minimize the side effects on normal tissues. In the past, considerable efforts have been directed toward the development of drug carriers, *i.e.* DNA complexes,[2] liposomes,[3] and emulsions,[4] able to deliver antitumor agents selectively into tumor tissues. We have been studying the utility of albumin microspheres containing 5-fluorouracil as a delivery system, in the hope of enhancing the drug accumulation in the liver of mice after intravenous injection[5,6] and obtaining prolonged action against Ehrlich ascites and solid tumors.[1,7] This series of studies was undertaken to investigate the ability of antitumor agents entrapped in albumin microspheres to prevent liver angiosarcoma or liver metastasis caused by the release of tumor cells through the portal vein during the course of surgical removal of gastric cancer.

The present investigation demonstrates the antitumor effect of adriamycin-containing microspheres on the liver metastasis in rats which had been injected with AH 7974 cells into the portal vein.

Experimental

Materials——Non-labeled and $^{3}H(G)$-labeled adriamycin were from Kyowa Hakko Co., Ltd. Bovine serum albumin (BSA) (Fr. V. Powder) was purchased from Seikagaku Kogyo Co., Ltd. and ^{125}I-human serum albumin from Japan Radioisotope Association. Cottonseed oil was selected as a vegetable oil. Nonionic surfactants, Span 85 and polysorbate 80 were obtained from Wako Pure Chemicals. All other chemicals were commercial reagent-grade products.

Animals——Male Donryu rats, weighing about 150 g, were used in all animal experiments.

Preparation of BSA Microspheres——The basic procedure of Scheffel *et al.*[8] was modified for the preparation of albumin microspheres containing adriamycin. A mixed aqueous solution containing 100 mg of adriamycin and 250 mg of BSA in a volume of 4 ml was emulsified into 100 ml of cottonseed oil containing 10% (v/v) Span 85 by the method described in a previous paper.[6] The solidification temperature of albumin

in oil emulsions was 170—180°. The average diameter and drug content of the final product were about 1.44 μm and 0.15 mg/mg.

***In Vitro* Drug Release**——Adriamycin release from microspheres was determined by means of a dynamic dialysis system employing cellulose tubing. The procedure was described elsewhere.[7] Adriamycin was determined by fluorescence intensity measurement at 470 nm (excitation) and 560 nm (emission)[9] in a Hitachi 650-60s fluorescence spectrophotometer.

Procedure for Preparing Liver Metastasis——AH 7974 cells were maintained by weekly transplantation in ascites form in rats. For preparing liver metastasis, a rat was anesthetized by intraperitoneal injection of sodium pentobarbital and the portal vein was exposed by a midline abdominal incision. AH 7974 ascites cells (10^6 cells/0.1 ml) were implanted into the portal vein, and the bleeding after administration was stopped by finger pressure. The abdominal cut was sewn up, and chloramphenicol ointment (Sankyo Co., Ltd.) was applied to prevent infections.

Microsphere and Adriamycin Distributions in Rats——^{125}I-labeled microspheres containing non-labeled adriamycin or non-labeled microspheres containing ^{3}H(G)-labeled adriamycin (2 mg of microspheres containing 300 μg of adriamycin) suspended in 0.9% NaCl solution containing 0.2% (v/v) polysorbate 80, or 300 μg of free ^{3}H(G)-adriamycin in 0.9% NaCl solution was injected into the portial vein or tail vein in rats. At 10 min, 1 hr, and 1 day after administration, 1 ml of blood was taken by heart puncture and each rat was sacrificed by decapitation. Several tissues (heart, lung, spleen, kidney, liver, and intestine) were removed and weighed wet. ^{125}I-labeled microspheres in blood and isolated tissues were determined with a Packard 5110 Auto-gamma scintillation spectrometer. Tritium levels in blood and isolated tissues were measured with an Aloka LSC-651 liquid scintillation spectrometer after combustion in an Aloka ASC-111 sample oxidizer.

The amount of tritium released from the microspheres in the liver (*i.e.*, total tritium in the liver minus microsphere-entrapped tritium) was determined as follows. The liver was homogenized with 3 volumes of 0.9% NaCl solution. Two ml of homogenate was used for each determination. To each sample, 1 ml of 16.5% $AgNO_3$ solution was added as a deproteinization reagent. The tubes were shaken vigorously for 20 min then centrifuged at 1500 rpm for 10 min, and the supernatant was subjected to liquid scintillation counting after combustion in a sample oxidizer.

Quenching correction was done by the external standard ratio method for tritium radioactivity. The tissue distribution of radioactivity is represented as % of administered dose in whole tissue (blood) or per gram of tissue (ml of blood).

Effect of Drug-containing Microspheres on Liver Metastasis——The antitumor effect of free or entrapped adriamycin against AH 7974 liver metastasis was studied in terms of animal survival (10 mice per group). AH 7974 cells alone were administered into the portal vein in rats of the control group. Three hundred μg of adriamycin or 2 mg of microspheres containing 300 μg of adriamycin was co-administered with AH 7974 cells in the treated groups, and 2 mg of microspheres without entrapped drug was administered to 5 rats to check the effect of the microspheres themselves on the tumor.

The animals were housed in standard rat cages and observed for 60 days. The median survival time of treated rats against that of control rats (T/C%) was used as a measure of antitumor effect.

Results

Figure 1 shows the *in vitro* release of adriamycin from albumin microspheres. It is clear that, in contrast to the rapid release of free adriamycin from a Visking dialysis sac, the release of adriamycin from microspheres and through the Visking sac was small. The half-release time of the free drug through the Visking sac, $T_{50\%}$, was about 0.5 hr. The release of adriamycin from microspheres followed first-order kinetics for about four days after the start of the experiment. The half-release time of adriamycin from microspheres was about 7.3 days and the cumulative amount released during 7 days was 42.5%. Entrapment in microspheres resulted in a remarkable retardation of the release of adriamycin.

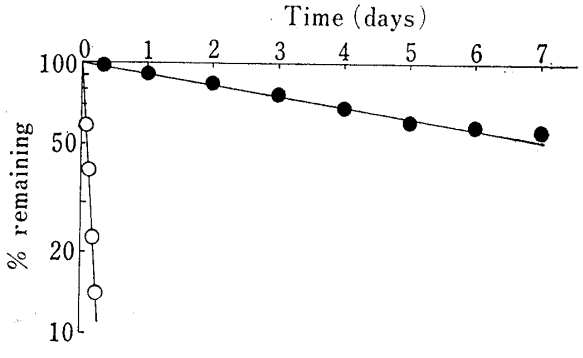

Fig. 1. Release of Adriamycin from a Dialysis Sac

○; free adriamycin,
●; microspheres containing adriamycin.

Figure 2 shows the distribution of ^{3}H(G)-adriamycin to various organs at 10 min, 1 hr,

and 1 day after intravenous or intraportal injection. When the route of administration was changed from the tail vein to the portal vein, the distribution of adriamycin in the liver at 10 min after injection increased from 3.9%/g liver to 6.3%/g. Although the data are not shown, the blood concentration after intraportal injection was lower than that in all tissues measured in this experiments. After intraportal injection, the drug levels in the heart and kidney, which are adversely affected by adriamycin, were lower than those after intravenous injection.

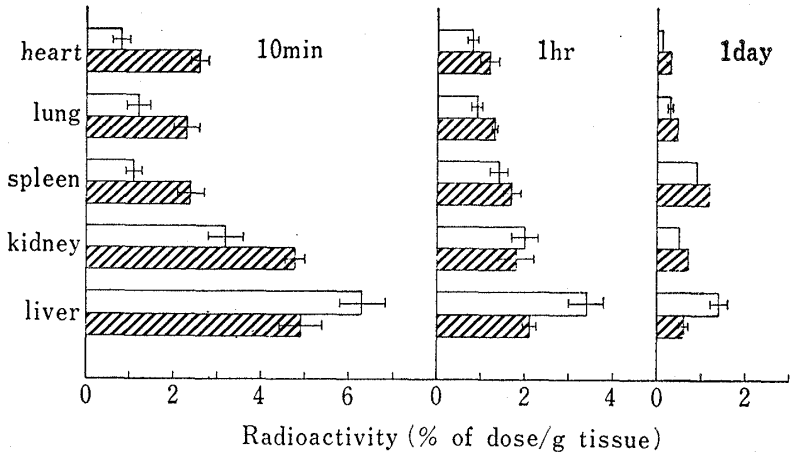

Fig. 2. Tissue Distributions at 10 min, 1 hr, and 1 day after Injection of Free ^{3}H(G)-Adriamycin

▨; intravenous injection,
□; intraportal injection.
Each column represents the mean value of 4—7 rats. Vertical bars indicate S.E.M.

Figure 3 shows the distribution of ^{125}I- and ^{3}H-radioactivities after intraportal injection of ^{125}I-labeled microspheres containing non-labeled adriamycin or non-labeled microspheres containing ^{3}H(G)-adriamycin. As ^{125}I-radioactivity is rigidly bound to the microspheres,[10] the distribution of ^{125}I-radioactivity corresponds to the microsphere distribution in rats. Since adriamycin is little metabolized,[11] the distribution of adriamycin entrapped in microspheres and released from microspheres. These were named microsphere fraction (M) and free fraction (F), respectively. At 10 min, 1 hr, and 1 day after intraportal injection of ^{125}I-microspheres, the percentages of dose per gram of liver were 13.9, 13.9, and 13.4, respectively. ^{125}I-radioactivity in other tissues and blood amounted to only a trace. After intraportal injection of microspheres containing ^{3}H(G)-adriamycin, tritium radioactivity was mainly distributed in the liver. However, the percentage of dose in the liver after administration of microspheres containing ^{3}H(G)-adriamycin was smaller than that after injection of ^{125}I-microspheres. The difference might result from release of adriamycin from microspheres in the blood and liver and rigid binding of ^{125}I-radioactivity. The adriamycin released might distribute to other tissues. This view was supported by the finding that tritium levels (%) in tissues other than the liver were higher than ^{125}I-levels (%), as shown in Fig. 3. At 10 min, 1 hr, and 1 day after injection of microspheres containing ^{3}H(G)-adriamycin, the tritium levels of the free fraction of adriamycin were 1.21, 0.90, and 0.37% per gram of liver, and the free fractions were 1/10, 1/13, and 1/32, respectively.

Figure 4 shows the total amount of radioactivity in the liver (% of dose) at 10 min, 1 hr, and 1 day after intraportal injection of free of microsphere-entrapped ^{3}H(G)-adriamycin, or ^{125}I-microspheres, as well as the total amount of radioactivity in the liver after intravenous injection of free ^{3}H(G)-adriamycin or ^{125}I-microspheres. Adriamycin in the liver was almost eliminated at 1 day after intravenous or intraportal injection of the free drug. In contrast,

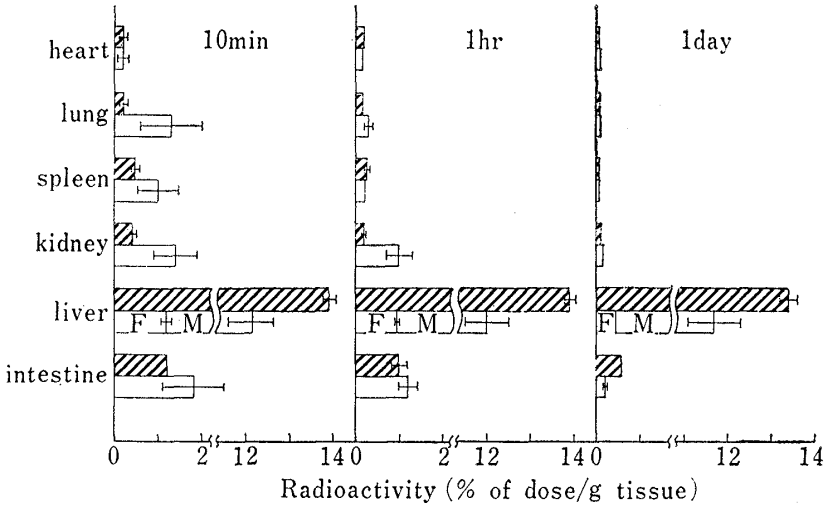

Fig. 3. Tissue Distributions of ^{125}I- and ^{3}H-Radioactivities at 10 min, 1 hr, and 1 day after Intraportal Injection of ^{125}I-Microspheres or Non-labeled Microspheres containing ^{3}H(G)-Adriamycin

▨; ^{125}I-microspheres,
□; microspheres containing ^{3}H(G)-adriamycin, F and M; free and microsphere fraction (see the text).
Each column represents the mean value of 4—5 rats. Vertical bars indicate S.E.M.

^{3}H- and ^{125}I-radioactivities even at 1 day after intraportal injection of microspheres were very high. At 10 min after intravenous injection of ^{125}I-microspheres, 85.3% of the dose was incorporated in the whole liver, and even at 1 day, 79.7% of the microspheres remained in the liver. These results indicate that the microspheres preferentially distribute into the liver, and that a high level is maintained in the liver. Radioactivity in the liver after intraportal injection of ^{125}I-microsphers was slightly higher than that after intravenous injection. This result might be explained by the difference in the amount of microspheres first-passed in the liver.

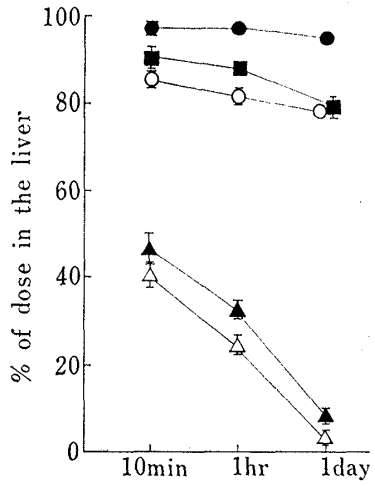

Fig. 4. Tissue Distributions of ^{125}I- and ^{3}H-Radioactivities in the Liver after Injection of Microspheres or Adriamycin

○; ^{125}I-microspheres after intravenous injection,
●; ^{125}I-microspheres after intraportal injection,
■; microspheres containing ^{3}H(G)-adriamycin after intraportal injection,
△; free ^{3}H(G)-adriamycin after intravenous injection,
▲; free ^{3}H(G)-adriamycin after intraportal injection.
Each point represents the mean value of 4—7 rats.
Vertical bars indicate S.E.M.

Figure 5 and Table I compare the antitumor effects of free and microsphere-entrapped adriamycin, and microspheres without entrapped drug against AH 7974 liver metastasis. When 10^{6} cells of AH 7974 ascites were inoculated into the portal vein in rats, the animals died between 10 and 16 days later due to the metastasis (control in Fig. 5), and the median

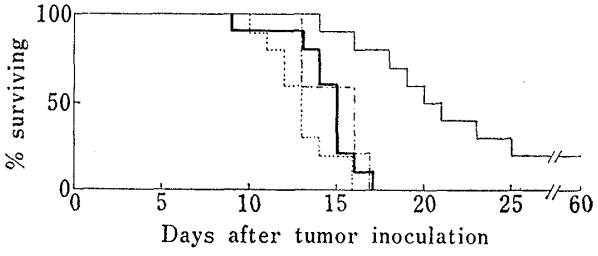

Fig. 5. Effect of Intraportally Administered Adriamycin or Microspheres on AH 7974 Liver Metastasis in Rats

--------; control,
——————; free adriamycin,
———————; microspheres containing adriamycin,
—·—·—; microspheres without drug.

Table I. Effect of Intraportally Administered Adriamycin or Microspheres on AH 7974 Liver Metastasis in Rats

	Survival[a] days	T/C %	Number of rats survived/treated
Control	13.0±0.6	—	0/10
Free adriamycin	14.0±0.7	110	0/10
Microspheres containing adriamycin	19.5±1.3[b] (27.6±5.3)[c]	150[b] (212)[c]	2/10
Microspheres without drug	15.0±0.8	115	0/5

a) Mean value±S.E.M.
b) Calculated for 8 rats that survived for less than 60 days.
c) Calculated for 10 rats.

survival time was 13.0 days. Injection of free adriamycin or microspheres without entrapped drug did not give any significant difference in survival from the control. In contrast, 2 out of 10 rats survived over 60 days after administration of microspheres containing adriamycin; the median survival time of the other 8 rats was 19.5 days and T/C % was 150.

Discussion

The development of a suitable method for the preparation of drug-containing albumin microspheres requires a knowledge of the physicochemical properties of the agents. The stability of drug-albumin aqueous solution in cottonseed oil emulsions and the particle size of albumin microspheres might be influenced by the viscosity of the mixed aqueous solution at the first step of the preparation of microspheres. The adriamycin content in the present microspheres was 0.15 mg/mg, which was higher than the 5-fluorouracil content (0.033 mg/mg) in microspheres reported elsewhere.[1,7] The difference of drug contents in microspheres might be due to a difference of drug leakage from microspheres at the washing stage of microspheres in the preparation, and this in turn might depend on differences of physicochemical interactions between albumin and drug, and/or change of the molecular mobility in the microspheres due to the difference of molecular size (130.08 molecular weight for 5-fluorouracil and 579.98 for adriamycin). As shown in Figure 1, the microspheres containing adriamycin exhibited sustained drug release. Since the burst effect[12] as observed in the case of *in vitro* 5-fluorouracil release from microspheres[7] was not observed in the case of adriamycin release, the efficiency of adriamycin entrapment in the microspheres appears to be higher than that of 5-fluorouracil. The release mechanism of drugs from microspheres is very complex, and further study is needed in order to understand the release characteristics of albumin microspheres containing various chemotherapeutic agents. We plan to study the effect of solidification temperature during preparation on the *in vitro* release of adriamycin.

After intravenous or intraportal injection, albumin microspheres were mainly distributed in the liver (Figs. 3 and 4). Microspheres after intravenous injection also distributed in the lung, but this was not the case after intraportal injection. If the drug or microspheres themselves caused side effects, especially in the lung, intraportal injection would be better than intravenous injection. Further if tumor cells and microspheres were released through the same route, they might disperse to the same sites in the body. Therefore, intraportal injection might be more useful than intravenous input against metastasis from gastric cancer to the liver through the portal vein.

From the results of Figure 5 and Table I, adriamycin entrapped in the microspheres shows a greater effect on liver metastasis than free adriamycin or non-entrapped microspheres, or the control. T/C % for rats treated with microspheres without entrapped drug was 115, suggesting that the microspheres physically interacted the tumor cells. The number of the tumor cells

inoculated in rats in this experiments was 10^6 cells per rat, whereas the amount of microspheres was 2 mg weight (2×10^8 particles).[13] Thus, a metastatic tumor cell might be surrounded by scores of microspheres. The microspheres which were approaching the tumor cell might be phagocytized in the cell, or might release the entrapped adriamycin. Since microspheres containing adriamycin show sustained release (Fig. 1), an effective level of adriamycin may be maintained around the tumor cell.

In conclusion, the present results that albumin microspheres show sustained release of adriamycin, preferential distribution in the liver after intraportal injection, and preventive effects on AH 7974 liver metastasis demonstrate the superiority of intraportal injection of microspheres in adjuvant chemotherapy of liver metastasis. It should be possible to improve the efficacy of antitumor agents entrapped in microspheres by the improvement of the *in vitro* drug release characteristics.

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References and Notes

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