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Delivery of Lymph Node-targeted Adriamycin by Gastric Submucosal Liposomal Injection in Rabbits

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We investigated the feasibility of specifically delivering adriamycin (ADR) to the regional lymph nodes via gastric submucosal injection of liposomal adriamycin (Lipo-ADR) in a rabbit model. We determined the tissue distribution of ADR for up to 7 days after the gastric submucosal injection of Lipo-ADR (0.4 mg/kg of ADR potency) and i.v. administration of an equal dose of free adriamycin (F-ADR). The area under the ADR concentration-time curve (AUC) of the regional lymph nodes was 85.4 μg·day/g after gastric submucosal injection of Lipo-ADR and 8.44 μg·day/g after i.v. administration of F-ADR. The targeting index of the regional lymph nodes, defined as the ratio of the AUC after gastric submucosal injection of Lipo-ADR to the AUC after i.v. administration of F-ADR, was 10.1. Gastric submucosal injection of Lipo-ADR enhanced lymph node-specific delivery of ADR compared with i.v. administration of F-ADR. The targeting index was 0.47 for the heart, 0.25 for the bone marrow, and 0.41 for the spleen, indicating that gastric submucosal injection of Lipo-ADR reduced delivery of ADR to these organs, as compared with i.v. administration of F-ADR. These data demonstrate that gastric submucosal injection of Lipo-ADR is well suited for specific delivery of ADR to the regional lymph nodes, suggesting that this method of administration may be useful in delivering preoperative adjuvant chemotherapy for preventing gastric cancer recurrence.

Key words: Lymph node-targeted chemotherapy — Gastric submucosal injection — Adriamycin — Liposome — Tissue distribution

Preoperative regional chemotherapy targeting lymph nodes is a promising approach for prevention of lymph node recurrence of gastric cancer following surgery.[1–4]

ADR[4] is commonly used to treat a variety of types of cancer.[5,6] Its anticancer efficacy may depend on the dose administered; *in vitro* studies have demonstrated that the cytocidal efficacy of ADR depends on its concentration and duration of exposure.[7] Despite its clinical efficacy, high-dose ADR administered intravenously is associated with severe manifestations of acute toxicity, such as bone marrow suppression and immunosuppression, as well as cumulative dose-limiting cardiotoxicity.[8] The ability to deliver ADR to regional lymph nodes via an appropriate route, thus increasing its therapeutic index, would enhance the drug's therapeutic efficacy and reduce the risk of toxic side effects.

The gastric submucosal injection of F-ADR makes it possible to target selectively the regional lymph nodes.[9] However, injection of F-ADR into the gastric submucosa causes severe ulceration because of local toxicity. An appropriate drug carrier is needed to minimize ADR's local toxicity before gastric submucosal injection can be an effective approach.

Liposomes, which have been studied extensively as a vehicle for improving the delivery of various therapeutic agents to a targeted organ, have the potential to reduce drug toxicity by reducing the accumulation of drug in organs susceptible to damage and to enhance therapeutic efficacy.[10–17] Liposomal ADR has been found to attenuate direct tissue toxicity, such as dermal necrosis resulting from the extravasation of F-ADR.[18] Furthermore, regional administration reportedly provides more efficient drug delivery to the targeted organ.[19]

We investigated the ability of gastric submucosal injection of Lipo-ADR to provide efficient and selective delivery of drug to the regional lymph nodes in a rabbit model and the potential clinical usefulness of this route of administration in preoperative adjuvant chemotherapy for prevention of recurrence of gastric cancer in lymph nodes.

MATERIALS AND METHODS

Materials and animals Adriamycin (Adriacin® Inj.) in lyophilized form, 10 mg potency/vial supplemented with

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[4] The abbreviations used are: ADR, adriamycin; Lipo-ADR, liposomal adriamycin; F-ADR, free adriamycin; AUC, the area under the concentration-time curve; MLVs, multilamellar vesicles; HPLC, high-performance liquid chromatography.

100 mg of lactose, was provided by Kyowa Hakko Kogyo, Tokyo. Purified egg lecithin (COATSOME NC-10, Nichiyu Liposome Co., Tokyo) was used as the pharmaceutical adjuvant for parenteral administration. Cholesterol was purchased from Wako Pure Chem. Co. Ltd., Osaka. Mannitol (20% w/v) was obtained from Nikken Kagaku Co. Ltd., Tokyo. Physiological saline was purchased from Otsuka Pharmaceutical Co., Osaka. Male Japanese white rabbits were obtained from Chubu Kagaku Shizai Co. Ltd., Nagoya and kept in a temperature-controlled room (25°C) with free access to water and a normal pelleted diet.

Preparation of lyophilized Lipo-ADR We used a system for reformulating pharmaceutical products officially approved for clinical practice into their corresponding liposomal forms that insured that all operations, from the liposome preparation to the closure of vials, were performed under aseptic conditions (T. Yotsuyanagi *et al.*, unpublished data). Adriamycin-containing liposomes were prepared by the reverse-phase evaporation method.[20] In brief, a water-in-oil emulsion consisting of purified egg lecithin and cholesterol (molar ratio, 2/1) and adriamycin solution was placed under negative pressure to remove the organic solvent (ether). The resulting liposomes were distributed into vials and freeze-dried. The vials were then closed under negative pressure. Each newly lyophilized liposome pellet measured 0.8 cm thick and 2 cm in diameter and contained 10 mg of ADR, 100 mg of egg lecithin, 24 mg of cholesterol, and 100 mg of lactose. This product was stored at −20°C; the drug prepared by this method is stable for at least 3 months.

Reconstitution of Lipo-ADR To reconstitute Lipo-ADR, 1 ml of sterile saline was added to each vial, and vials were vortexed for 10 min. Lipo-ADR was adjusted to a concentration of 1 mg/ml of ADR potency by the addition of another 8.7 ml of sterile saline. This reconstitution produced MLVs with particle sizes ranging from approximately 1 to 10 μm. The median diameter of particles was 3.85 μm and the mode diameter was 4.41 μm, measured with a laser diffraction particle size analyzer (SALD-1100, Shimadzu, Kyoto). The entrapment efficacy was determined by the following method. The MLVs were collected by centrifugation and resuspended in fresh sterile saline after removal of the supernatants. The MLVs were washed with sterile saline three times. The MLVs was dissolved in a mixture of butanol and toluene (1:1). The extract was evaporated *in vacuo*, and the residue was dissolved in a mixture of phosphate buffer (pH 3.0) and methanol (1:1). An aliquot of this solution was eluted with 1 *N* formic acid and methanol (55:45) and assayed by HPLC[21] with a fluorescence detector (Ex. 470 nm and Em. 585 nm). The total amount of ADR associated with the MLVs was determined by this method. The entrapment efficacy, defined as the ratio of the total amount of ADR associated with the MLVs to the total amount of ADR in the sample, was 41.7±3.2% (mean of 4 independent experiments±SD). The preparation retained this entrapment efficacy for 6 h at room temperature.

Tissue distribution studies Rabbits weighing 2.0 to 3.0 kg were anesthetized by i.v. administration of sodium pentobarbital. The animals underwent gastrotomy and 0.4 ml/kg Lipo-ADR (total ADR potency of 0.4 mg/kg) was injected into the gastric submucosa of the posterior wall in the antrum. An equal dose of F-ADR (1 mg/ml) was injected into the gastric submucosa or administered intravenously in control animals. For blood clearance kinetics studies, blood samples were taken from the right femoral artery over a 6-h period after drug administration. For tissue distribution studies, eighty-four rabbits were anesthetized and killed at 1 h, 6 h, 12 h, 1 day, 2 days, 4 days, and 7 days after drug administration. Various organs, including the regional lymph nodes of the stomach surrounding the portal vein, the heart, the bone marrow of the right femur, the spleen, and the liver, were excised immediately, rinsed in saline, weighed, and stored at −40°C. The ADR concentration of these tissues was measured by HPLC.[21] Drug distribution to the various organs was estimated in terms of the AUC of ADR concentrations from 1 h to 7 days after drug administration. In addition, the targeting index of the various organs, defined as the ratio of the AUC after the gastric submucosal administration of Lipo-ADR or F-ADR to the AUC after i.v. administration of F-ADR, was determined.

Statistical analysis The statistical significance of differences between experimental results was tested by using Student's *t* test. A P value of <0.05 was considered significant.

RESULTS

Regional toxicity to gastric mucosa The same dose of Lipo-ADR or F-ADR (0.4 ml/kg or total ADR potency of 0.4 mg/kg) was injected into the gastric submucosa of the posterior wall in the antrum. Minor ulcerations, limited to the submucosa, were noted at the Lipo-ADR-injected sites after day 4. Severe ulceration was noted at F-ADR sites after day 4, extending to the muscle layers. Liposomal encapsulation of ADR reduced local toxicity manifested by ulcer formation. Ulcers were not a serious complication in either group over the 7-day observation period.

Plasma ADR levels The i.v. administration of ADR resulted in a rapid clearance of the drug from the circulation; the drug was barely detectable in plasma after 30 min (Fig. 2). After the gastric submucosal injection of Lipo-ADR, no drug was detected in the plasma up to 30 min, and only a very low concentration at 60 min (Fig. 2).

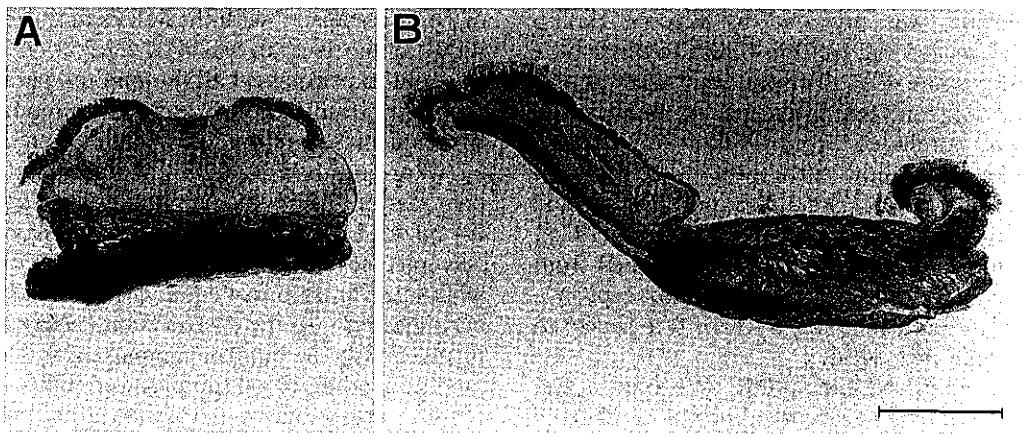

Fig. 1. Histological specimens of ADR-injected site. A: Minor ulcer, limited to the submucosa, was noted on the Lipo-ADR-injected site on day 7. B: Severe ulcer, extending to the muscle layer, was noted at the F-ADR site on day 7. Bar: 5 mm.

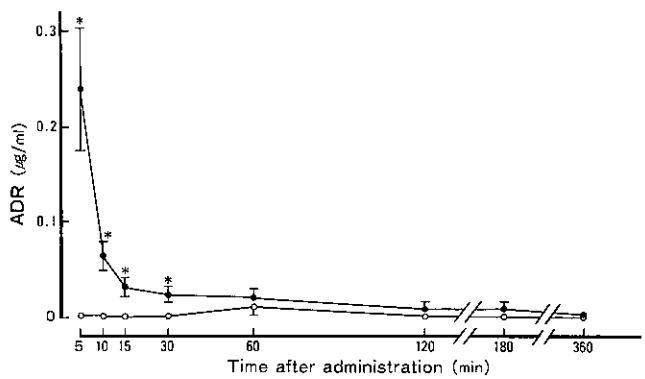

Fig. 2. Adriamycin plasma levels. Lipo-ADR (ADR potency of 0.4 mg/kg) was injected into the gastric submucosa (○). An equal dose of free ADR was administered i.v. (●). ADR plasma concentrations were determined by HPLC. Results are the mean values of 4 independent experiments; bars represent SD (where no bar appears, the SD was smaller than the symbol); $*P<0.05$ Lipo-ADR gastric submucosal injection group vs. F-ADR i.v. group.

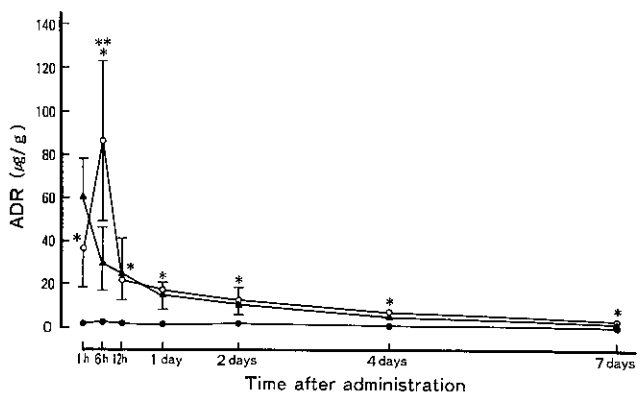

Fig. 3. Adriamycin levels in regional lymph nodes of the stomach. Lipo-ADR (○) or F-ADR (▲) (ADR potency of 0.4 mg/kg) was injected into the gastric submucosa. An equal dose of F-ADR was administered i.v. (●). At the indicated times, the regional lymph nodes of the stomach were collected and the tissue ADR concentrations were determined by HPLC. Circles and triangles represent the mean of 4 independent experiments; bars are SD (where no bar appears, the SD is smaller than the symbol); $*P<0.05$ Lipo-ADR gastric submucosal injection group vs. F-ADR i.v. group and $**P<0.05$ Lipo-ADR gastric submucosal injection group vs. F-ADR gastric submucosal injection group.

ADR levels in the regional lymph nodes of the stomach

After the gastric submucosal injection of Lipo-ADR or F-ADR, ADR concentrations in the regional lymph nodes of the stomach were significantly higher than after i.v. administration of F-ADR and remained high for up to 1 day (Fig. 3). The maximum ADR concentration in the regional lymph nodes after gastric submucosal injection of Lipo-ADR was 87.0 μg/g at 6 h, which was 24.8 times higher than the maximum concentration 6 h after i.v. administration of F-ADR. The maximum concentration of F-ADR after gastric submucosal injection was 52.1 μg/g at 1 h, ADR concentrations gradually decreased thereafter (Fig. 3). The difference between the gastric submucosal injection groups was significant only at 6 h. The area under the ADR concentration-time curve (AUC) was 85.4 μg/g·day after gastric submucosal injection of Lipo-ADR, 69.2 μg/g·day after gastric submucosal injection of F-ADR, and 8.44 μg/

Table I. AUC Values of ADR and the Targeting Indices

Organ	AUC (μg/g·day)[a]			Targeting index[c]
	Gastric submucosal injection[b]		i.v.[b]	
	Lipo-ADR	F-ADR	F-ADR	
Lymph nodes	85.4	69.2	8.44	10.1 8.20
Bone marrow	1.11		4.51	0.25
Heart	0.45		0.96	0.47
Spleen	2.60		6.27	0.41
Liver	1.04		1.19	0.87

a) AUC was estimated from data based on 7 days of observation.
b) The dose of ADR was 0.4 mg/kg.
c) Targeting index was defined as the ratio of the AUC after gastric submucosal injection to the AUC after i.v. administration.

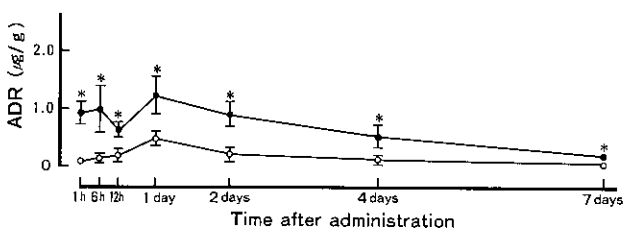

Fig. 4. Adriamycin levels in bone marrow. Lipo-ADR (ADR potency of 0.4 mg/kg) was injected into the gastric submucosa (○). An equal dose of F-ADR was administered i.v. (●). Tissue ADR concentrations were determined at the indicated times. Circles represent the mean values of 4 independent experiments; bars are SD (where no bar appears, the SD is smaller than the symbol); * $P<0.05$ Lipo-ADR gastric submucosal injection group vs. F-ADR i.v. group.

g·day after i.v. administration of F-ADR. The targeting index was 10.1 for gastric submucosal injection of Lipo-ADR, and 8.20 for gastric submucosal injection of F-ADR. Gastric submucosal administration enhanced delivery of drug to the regional lymph nodes compared with i.v. administration (Fig. 3, Table I).

ADR levels in bone marrow ADR concentrations in the bone marrow 1 h to 7 days after gastric submucosal injection of Lipo-ADR were lower as compared with levels after i.v. administration of F-ADR (Fig. 4). The differences between the two groups were significant ($P<0.05$) from 1 h to 7 days. The AUC value was 1.11 μg/g·day after gastric submucosal injection of Lipo-ADR and 4.51 μg/g·day after i.v. administration of F-ADR. The targeting index was 0.25 (Fig. 4, Table I).

AUC values of ADR and targeting indices in the heart, spleen, and liver AUC values in the heart and the spleen were low after the gastric submucosal injection of Lipo-ADR compared with values after i.v. administration of F-ADR (Table I). In the heart, the AUC value was 0.45 μg/g·day after gastric submucosal injection of Lipo-ADR and 0.96 μg/g·day after i.v. administration of F-ADR. The targeting index was 0.47. In the spleen, the AUC value was 2.60 μg/g·day after gastric submucosal administration of Lipo-ADR and 6.27 μg/g·day after i.v. administration of F-ADR. The targeting index was 0.41. In the liver, the AUC values were similar after gastric submucosal administration of Lipo-ADR (1.04 μg/g·day) and i.v. administration of F-ADR (1.19 μg/g·day). The targeting index was 0.87 (Table I).

DISCUSSION

Regional lymph nodes are common sites of gastric cancer recurrence after surgery.[1,2] To improve the prognosis, several investigators have attempted to target regional chemotherapy to the lymph nodes.[3,4,22-24] The endoscopic injection of Lipo-ADR into the gastric submucosa adjacent to the main tumor could reduce the risk of lymph node recurrence and improve prognosis.

Gastric submucosal injection of F-ADR is an effective method for specific delivery of ADR to the regional lymph nodes.[9] However, this type of regional chemotherapy is associated with local toxicity to the gastric wall, which manifests as an ulcer. This toxicity may be attenuated by encapsulation of ADR in liposomes (Fig. 1).[18]

The use of liposomes has been shown to enhance the selective delivery of various anticancer drugs, increase their anticancer efficacy, and reduce various toxic side effects.[10-17,19,25] However liposomes cannot be routinely used in clinical practice because of the need for multistep preparation and their physicochemical properties. The Lipo-ADR used in this study was prepared as a freeze-dried mixture in a commercially supplied vial of ADR. This ready-to-use freeze-dried form made long-term preservation of the lyophilized Lipo-ADR possible and ensured an accurate dose of ADR in the Lipo-ADR preparation. Thus, this form may facilitate clinical use of the drug. Our Lipo-ADR preparation contained 41.7% of liposome-entrapped ADR and 58.3% of free ADR. The entrapment efficacy was stable 24 h after the preparation of the liposome form because of the equilibrium between liposome-entrapped ADR and free ADR (data not shown).

To explore the usefulness of this method of targeting regional chemotherapy to the lymph nodes, we investigated ADR delivery to various organs in a rabbit model after gastric submucosal injection of Lipo-ADR, gastric submucosal injection of F-ADR, and i.v. administration of F-ADR. Tissue ADR levels were measured 1 h, 6 h, 12 h, 1 day, 2 days, 4 days, and 7 days after drug administration (Figs. 3 and 4). ADR delivery was expressed

as the area under the tissue ADR concentration-time curve (Table I). The AUC of tissue ADR concentration has been shown to correlate well with anticancer efficacy and toxic side effects.[26, 27] The AUC of the regional lymph nodes of the stomach after gastric submucosal injection of ADR was significantly higher than after i.v. administration of F-ADR. In addition, the AUC of the regional lymph nodes was significantly higher than that of other tissues after gastric submucosal injection. The targeting index for the regional lymph nodes was 10.1 after gastric submucosal injection of Lipo-ADR and 8.20 after gastric submucosal injection of F-ADR. Gastric submucosal injection of ADR significantly enhanced drug delivery to the regional lymph nodes as compared with i.v. administration. However, the injection of F-ADR into the gastric submucosa caused severe ulceration and is not appropriate for clinical use. Injection of Lipo-ADR reduced the local toxicity and enhanced lymph node-specific delivery of the drug. The targeting indices for the heart, bone marrow, and the spleen after gastric submucosal injection of Lipo-ADR were very low, indicating that the unfavorable ADR delivery to these organs was reduced. These data demonstrate that the gastric submucosal injection of Lipo-ADR is an effective means of targeting the regional lymph nodes. Therefore, the gastric submucosal injection of Lipo-ADR may reduce the risk of lymph node recurrence and improve the prognosis of patients with gastric cancer.

ADR injected into the gastric submucosa in the Lipo-ADR form may be delivered to various organs via two potential routes out of the gastric submucosal space: the lymphatic circulation and the blood capillary circulation. ADR was barely detected in plasma, except for a very small amount (0.01 μg/ml) at 60 min after injection of Lipo-ADR (Fig. 2). ADR is thought to be released slowly from the gastric submucosal space into the lymphatic circulation and the capillary circulation.[9] The liposome-entrapped ADR may be delivered to regional lymph nodes in the lymphatic flow[28–30] together with the free drug. Each fraction may be released from the gastric submucosal space and delivered to the regional lymph nodes at a different rate. Liposome-entrapped ADR is delivered more slowly than the free drug because of the slow movement of the micron-sized vesicles in the lymphatic flow. Therefore, a staggered delivery may result, leading to prolonged and higher concentrations. This concept seems reasonable based on our results showing that the drug concentrations in the regional lymph nodes continued to increase for up to 6 h after injection (Fig. 3). With the passage of time, the flow volume of the microcirculation in the gastric submucosa surrounding the injected site decreased as a result of the local toxicity of ADR. Consequently, the concentration of ADR in the regional lymph nodes decreased after 6 h. Some ADR may have been transported into the blood capillary circulation and a large fraction may have been trapped in the liver via the portal vein. ADR delivery to the liver with gastric submucosal injection of Lipo-ADR was as high as with i.v. administration of F-ADR (Table I). The kinetics of ADR injected as Lipo-ADR into the gastric submucosa seem to resemble those of cancer cell metastasis. Further studies are required to determine the optimal lipid composition of liposomes and the combination of liposome-entrapped ADR and free ADR that would be optimal for gastric submucosal administration.

In a preliminary clinical study, we administered an endoscopic gastric submucosal injection of Lipo-ADR to one patient with gastric cancer as preoperative adjuvant chemotherapy. Sufficient ADR was delivered to the resected lymph nodes to obtain anticancer efficacy against micrometastases involving the lymph nodes. No ulceration or other side effects were noted. In addition, clinical trials of this preparation will allow us to evaluate the risk of lymph node recurrence after surgery for gastric cancer and the effect on the prognosis.

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