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Preparation and Evaluation *in Vitro* and *in Vivo* of Polylactic Acid Microspheres Containing Doxorubicin

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Polylactic acid microspheres containing doxorubicin were prepared by a solvent evaporation process and release patterns of the drug from the microspheres were examined *in vitro*. Microspheres with greater drug contents released more drug. The release rate of the drug after the initial burst was found to be small, so that prolonged release was obtainable. It was also found that the wettability of the microspheres might influence the release rate. Venous plasma levels of the drug following intra-arterial administration into dog liver were considerably lower than those following administration of the drug solution. Microangiography revealed embolization due to the microspheres in peripheral arteries in the liver.

Keywords——polylactic acid; microsphere; doxorubicin; intra-arterial infusion; embolization; liver; microangiography

Selective delivery of anticancer drugs to the target tumor seems to be the most promising approach to enhance the therapeutic effect and to minimize severe side effects in cancer chemotherapy. From this viewpoint, many attempts have been made to improve the effectiveness of drugs, including chemical modification of drug molecules,[1] and the development of dosage forms such as liposomes,[2] emulsions,[3] microcapsules,[4-8] and polymer matrices.[9-11] Among these, the use of microcapsules in intra-arterial chemoembolization[6] is a reasonable approach to the treatment of solid tumors in organs. Long-term release of drugs from such systems as microcapsules and polymer matrices can be achieved by selecting a suitable polymeric material. In this case, biodegradable polymers which do not cause any toxic reaction within the body are preferable to non-biodegradable polymers which remain in the body even after all of the drug is released.

Natural polymers such as gelatin[12] and albumin,[4,5,8] and synthetic polymers such as polylactic acid[7,9] and polyglycolic acid[11] have been investigated for use in drug delivery systems for anticancer agents. We have also reported on the possible use of polycarbonates in sustained delivery of anticancer drugs.[13]

In the present report, the preparation, release characteristics *in vitro*, and evaluation *in vivo* of polylactic acid (PLA) microspheres containing an anticancer antibiotic, doxorubicin (adriamycin), are described.

Experimental

Materials——Doxorubicin hydrochloride was generously supplied by Kyowa Hakko Kogyo Co., Tokyo. Poly-L-lactic acid (PLA) was purchased from Polysciences, Warrington, Pennsylvania, and its average molecular weight was calculated to be 45000[14] based on the intrinsic viscosity of 1.36 in chloroform determined at 20 °C using a viscometer (Low-Shear 30, Contraves AG, Switzerland). Gelatin, alkaline processed, 200 bloom, was a gift from

Nitta Gelatin Co., Yao, Osaka. Methylene chloride and other chemicals of reagent grade were used without further purification.

Preparation of PLA Microspheres——PLA microspheres were prepared by a solvent-evaporation process similar to that reported previously.[15] Doxorubicin hydrochloride powder (5 or 10 mg) was added to 1 ml of 3 or 5% (w/v) PLA solution in methylene chloride, and the suspension was sonicated (Sonicator UR-20P, Tomy Seiko Co.) for 30 s to effect better dispersion. Subsequently the suspension was poured into a beaker containing 30 ml of 1 or 2% (w/v) gelatin solution (a nonsolvent) buffered at pH 7.4 with NaH_2PO_4–Na_2HPO_4 under stirring at a rate of 850 rpm by means of a magnetic stirrer. The stirring was continued for 1 h at room temperature to evaporate off methylene chloride. Then the microspheres were collected by filtration through a sintered glass disk, washed with distilled water to remove gelatin, and dried at room temperature *in vacuo*.

Optical and Scanning Electron Microscopic Observation of Microspheres——Microspheres were observed under an optical microscope (Olympus BH-2) to determine their size and shape. Diameters of randomly sampled microspheres were measured and their mean diameter was calculated.

The microspheres were also observed under a scanning electron microscope (MINI-SEM®, MSM-102, Hitachi-Akashi) to check their surface characteristics after Au-coating of the microspheres using an ion coater (Model 1B-3, Eiko Engineering Co.).

Release Studies *in Vitro*——Approximately 10 mg of microspheres was suspended in a flask containing 25 ml of normal saline solution. The flask was placed in a shaker bath maintained at 37 °C and shaken horizontally. At predetermined intervals, 5 ml of the solution was sampled and 5 ml of fresh medium was added.

Administration of Doxorubicin to Dogs——Doxorubicin hydrochloride corresponding to a dose of 1.5 mg/kg was administered into the hepatic artery of a dog as a sline solution or a suspension of the microspheres in saline solution. A dog weighing about 10 kg was anesthetized by pentobarbital injection. After surgical catheterization from the gastroduodenal artery into the proper hepatic artery, 5 ml of saline solution containing the drug in solution or the microsphere suspension was infused slowly in about 3 min through the catheter from a syringe. Thereafter, blood samples (3 ml) were drawn from the jugular vein at predetermined time intervals. The blood samples were heparinized and centrifuged at 3000 rpm for 10 min immediately after the sampling. Plasma samples were kept frozen until analysis.

Determination of Doxorubicin——The concentration of doxorubicin in the release medium was determined fluorometrically; the excitation wavelength was 470 nm, and the emission wavelength was 585 nm. The content of doxorubicin in the microspheres was determined spectrophotometrically at 480 nm after the following extraction process. First, 0.5 ml of methylene chloride was added to weighed amount of the microspheres to dissolved the polymer, then 5 ml of saline solution was further added to the organic solution, and the mixture was shaken for 30 min to extract the drug into the aqueous layer, which was used for the spectrophotometric determination.

The plasma concentration of doxorubicin was determined by high-pressure liquid chromatography. The extraction procedure was similar to that reported by Baurain *et al.*[16] with minor modifications. To 100 μl of the plasma was added 50 μl of daunorubicin hydrochloride (an internal standard) solution (1.6 μg/ml), and the drug was extracted with 3 ml of a chloroform–methanol mixture (4 : 1 by volume). After sonication for 1 min, the mixture was centrifuged at 3000 rpm for 10 min. The organic layer (2 ml) was taken and the solvent was evaporated off under reduced pressure at room temperature. The residue was dissolved in 125 μl of methanol and 100 μl of the solution was injected into the chromatograph. When the plasma concentration was below 0.1 μg/ml, 1 ml of the plasma, 11 ml of the chloroform–methanol mixture, and 10 ml of the organic layer after extraction were used instead to obtain sufficient sensitivity.

The chromatographic conditions used were similar to those reported by Haneke *et al.*[17] A high-pressure liquid chromatograph equipped with a spectrofluorometric detector (LC-5A and RF-530, Shimadzu Manufacturing Co., Kyoto) was used for the analysis. Excitation and emmision wavelengths were set at 480 and 560 nm, respectively. A 25 cm × 4.6 mm i.d. stainless steel column, packed with C_{18} reversed-phase packing material (Zorbax ODS®, Shimadzu-du Pont, Kyoto), was used. The mobile phase consisted of 650 ml of methanol and 350 ml of 0.01 M $NH_4H_2PO_4$ solution containing 5 ml of acetic acid. The flow rate was 1.4 ml/min.

Microangiography of Livers——Livers were resected from dogs 24 h after the administration of the microspheres or the saline solution containing the drug. As a reference, an intact liver was also resected from an untreated dog. Microangiography was performed by injecting 5 to 8 ml of 50% barium sulfate suspension into the proper hepatic artery.

Results and Discussion

Sizes and Drug Contents of Microspheres

Sizes and drug contents of microspheres prepared by using two loading levels of doxorubicin, two concentrations of PLA, and two concentrations of gelatin are shown in Table I. Preparations A, B, C, D and E were prepared without the drug. When the

TABLE I. Effect of Loading of the Drug, Concentration of PLA and Nonsolvent at Preparation on the Sizes and Drug Contents of Microspheres

Preparation	Drug (mg)	PLA concn. (%)	Gelatin concn. (%)	Diameter (μm) Mean ± SEM ($n=100$)	Drug content (%)[a]
A	—	3	1	62.2 ± 2.2	—
B	—	3	2	55.4 ± 2.0	—
C	—	3	2[b]	76.8 ± 2.5	—
D	—	5	1	75.6 ± 2.5	—
E	—	5	2	64.8 ± 1.8	—
F	5	5	2	99.3 ± 3.5	5.2
G	5	3	2	73.6 ± 2.4	8.7
H	10	3	2	101.9 ± 2.8	18.0

a) Mean of two preparations.
b) Autoclaved at 121 C for 20 min.

concentration of PLA was higher, larger microspheres were obtained (preparations D *vs.* A and E *vs.* B). In contrast, when the concentration of gelatin was higher, smaller microspheres were prepared (preparations B *vs.* A and E *vs.* D). It seems that higher viscosity of PLA solution due to higher concentration of PLA effected the formation of larger droplets in the gelatin solution (a nonsolvent). Alternatively, lower emulsifying capacity of the nonsolvent due to a lower concentration of gelatin resulted in the larger microspheres. When a gelatin solution was autoclaved for use in a sterile preparation, the viscosity of the solution was apparently decreased and larger microspheres were obtained (preparation C). Therefore the size of microspheres can be controlled to some extent by using a suitable concentration of PLA and/or gelatin.

When microspheres containing the drug were prepared, a similar effect of the concentration of PLA on the size was observed (preparations F *vs.* G). In addition, larger microspheres were obtained when a larger amount of the drug was used at preparation, possibly because of increased viscosity of the organic phase (preparations H *vs.* G). As for drug contents, increasing the drug/PLA ratio at preparation resulted in an increased content of the drug (preparations F < G < H).

Good reproducibility in the preparation of microspheres could be achieved by using the same experimental setups and by carefully adopting the same conditions such as the stirring rate, evaporation time, room temperature (25 ± 2 °C), washing of microspheres, and drying time at every preparation.

Figure 1 shows a scanning electron photomicrograph of PLA microspheres, preparation H. Many small pores, which might have been formed during the evaporation process, were observed on the surface of the spheres. Other microspheres with different drug contents looked almost the same as these.

The preparation of microspheres under sterile conditions was also attempted. PLA solution in methylene chloride was filtered through a 0.2 μm polytetrafluoroethylene membrane which had been autoclaved, and gelatin solution was also autoclaved. Microspheres were prepared from these materials in a clean bench. The microspheres thus prepared were confirmed to be sterile according to the JP X Sterility Test.

Release Patterns

Figure 2 shows the release patterns of doxorubicin in saline solution from microspheres of preparations F, G, and H, having drug contents of 5.2, 8.7, and 18.0%, respectively.

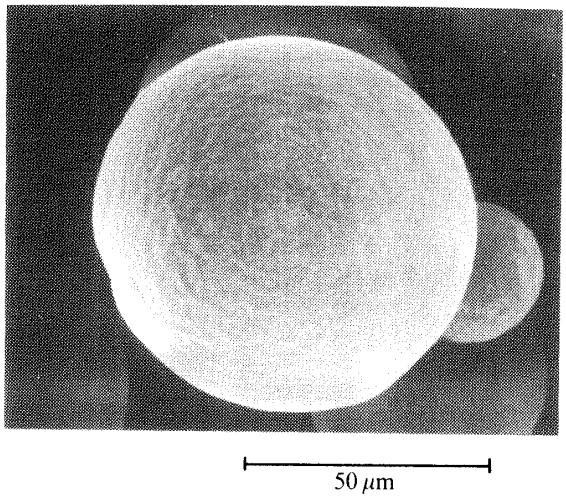

Fig. 1. Scanning Electron Photomicrograph of PLA Microspheres Containing 18.0% Doxorubicin (Preparation H)

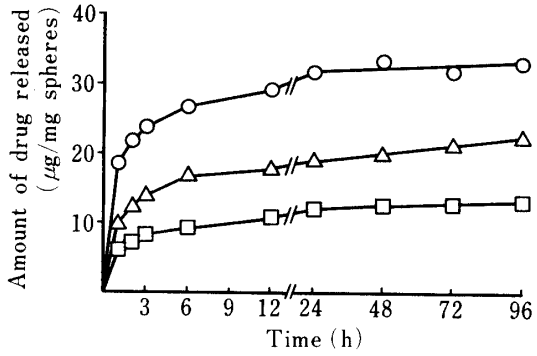

Fig. 2. Release Patterns of Doxorubicin in Saline Solution from Microspheres Containing 5.2% (F: □), 8.7% (G: △), and 18.0% (H: ○) Doxorubicin

Average of two experiments.

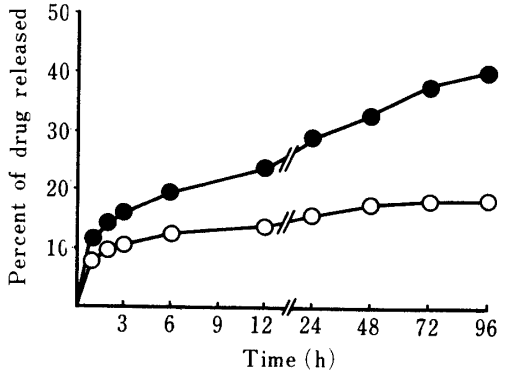

Fig. 3. Release Patterns of Doxorubicin from Microspheres Containing 18.0% Doxorubicin (Preparation H) in Saline Solution (○) and Saline Solution Containing 0.1% Polysorbate 80 (●)

Average of two experiments.

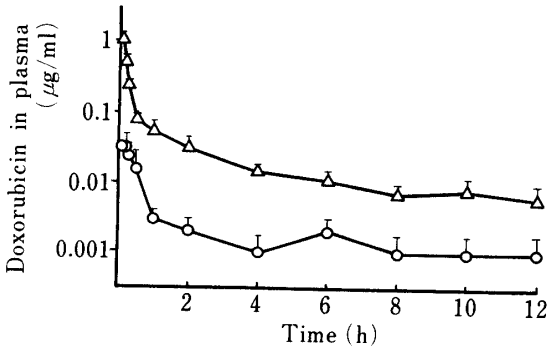

Fig. 4. Plasma Levels of Doxorubicin Following Administrations of Doxorubicin Hydrochloride (1.5 mg/kg) in Saline Solution (△) and in Microspheres (○) into the Hepatic Artery of Dogs

Each value represents the mean+SEM of three experiments.

Although these three preparations were somewhat different in size, microspheres with greater drug contents released more drug. In all cases, after the initial burst, the release rate of the drug was very small. This suggests that diffusion of doxorubicin molecules in the PLA matrix is very slow. In addition to the diffusion in the polymer matrix, diffusion of the drug in aqueous medium which has penetrated through pores into the microspheres may also contribute to the overall release rate, because many small pores were observed on the surface as can be seen in Fig. 1. Wettability of the microspheres may influence the penetration of aqueous medium into the matrix and, hence, affect the release rate of the drug, because PLA microspheres are hydrophobic in nature. When a surfactant, polysorbate 80, was added to the release medium, a higher release rate of the drug was observed (Fig. 3). Such an increase in release rate may be attributable to better wetting of the microspheres, promoting the penetration of the aqueous medium through the pores. In biological fluids such as blood, proteins or other surface-active materials are expected to wet the surface of the microspheres effectively. Considering the better wetting and possible degradation of PLA, doxorubicin

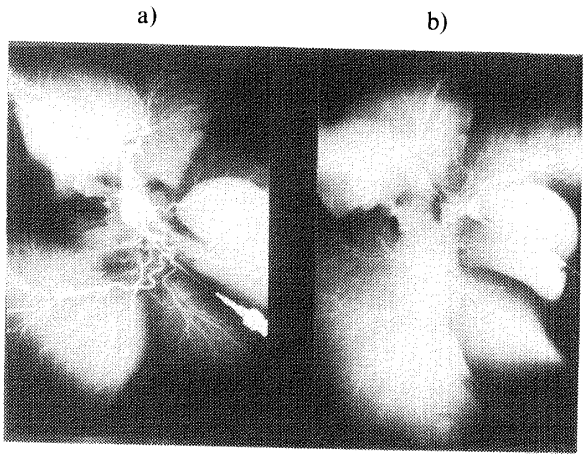

Fig. 5. Microangiograms of Dog Liver

a) Control (intact) liver. b) 24 h after administration of microspheres (Preparation H) corresponding to a dose of 1.5 mg/kg of doxorubicin.

might be released faster in blood than in saline solution.

Plasma Levels of Doxorubicin Following Administration of Solutions and Microspheres into the Hepatic Artery in Dogs

Figure 4 shows plasma levels of doxorubicin after hepatic intra-arterial infusion of saline solutions and microspheres containing the drug at a loading level of 1.5 mg/kg in dogs. When the saline solution of the drug was administered, plasma levels exhibited biphasic elimination profiles: namely, a rapid decrease in the initial period and a much slower decrease thereafter. Even at 12 h after administration, the plasma level was still about 0.01 μg/ml. On the other hand, plasma levels were much lower when the microspheres were administered. Calculation of the area under the plasma level–time curves for the two cases showed that less than 10% of the drug appeared in venous blood following administration of the microspheres in comparison with administration of the solution up to 12 h. Such a suppressed efflux of the drug into the general circulation is desirable from the viewpoint of reducing unwanted side effects.

Microangiography of the Liver

Antitumor effects of intra-arterially infused microspheres containing an antitumor drug would be obtained by embolization in capillaries of the organ as well as by direct action of the drug locally released from the microspheres.[6] In order to examine the lodging of the microspheres in the liver, microangiography was performed and the angiograms are shown in Fig. 5. In contrast to the control (intact) liver, much lower vascularity, especially in the peripheral region, was observed in the treated liver indicating embolization with the microspheres in peripheral arteries. In these experiments, nearly 3×10^5 microspheres with an average diameter of approximately 100 μm were administered. When a higher degree of embolization is required, microspheres in larger amount or of larger size can be employed.

Conclusions

In the present study, PLA microspheres containing doxorubicin were prepared and the release patterns of the drug *in vitro* were examined. It was shown that the size and drug content of microspheres can be modified by changing the loading level of the drug, concentration of PLA, and concentration of gelatin in a nonsolvent. The release rate of doxorubicin from the microspheres was found to be small after the initial burst, indicating that prolonged release occurs. Reflecting the slow release *in vitro*, plasma levels of doxorubicin following intra-arterial administration of microspheres containing the drug into the dog liver were considerably lower than those following administration of the drug solution.

Microangiography indicated the occurrence of embolization due to the microspheres in peripheral arteries in the liver. Further studies on the degradation characteristics of PLA *in vivo*, antitumor effect in animals and appropriate selection of size, drug content, and duration of drug release of microspheres are now being undertaken.

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