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Fibrinogen Microspheres as Novel Drug Delivery Systems for Antitumor Drugs[1)]

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Fibrinogen microspheres containing 5-fluorouracil (5-FU) were prepared and evaluated as a novel drug delivery system. The shape of the microspheres was invariably spherical. The average diameters were 3.9 and 1.1 μm and the drug contents in the microspheres were 16.1 and 6.1%, for the microspheres prepared at 90 and 160 °C, respectively. Release of the drug from fibrinogen microspheres was slow after the initial burst, and the drug release continued over 5 d. These results indicated that the drug-loaded fibrinogen microspheres could be prepared providing a sustained release property *in vitro*. The antitumor activity of fibrinogen microspheres containing 5-FU was evaluated against Ehrlich ascites carcinoma in mice. The mean survival time of the group receiving the microspheres was larger than that of the control group, though essentially the same as that of the free 5-FU-treated group. Fibrinogen microspheres show good biocompatibility and should be useful as a novel drug carrier in injectable delivery systems for antitumor drugs.

Keywords——fibrinogen microsphere; novel drug carrier; drug delivery system; 5-fluorouracil; sustained release; Ehrlich ascites carcinoma; mouse

In the field of cancer chemotherapy, it is desirable that antitumor drugs are delivered to the tumor site in a sufficient amount for a long period of time. Many attempts have been made to deliver the antitumor drugs to target sites within the tumor by means of various drug delivery systems.[2-4)] The use of microspheres as sustained-release targeting agents for antitumor drugs has received much attention during recent years, and albumin[5,6)] gelatin,[7)] and polylactic acid[8)] microspheres have been investigated.

The possible use of fibrin (a bioplastic prepared from human plasma) as a new biodegradable carrier for drug delivery systems has also been examined.[9,10)] In addition, the drug-carrier properties of fibrinogen also appeared to be of interest to us. In the present study, therefore, the potential use of fibrinogen microspheres as a novel injectable system for antitumor drugs was investigated. Fibrinogen is the precursor of fibrin, the blood clot substance. It is a large, asymmetric molecule which is highly elongated. The molecular weight is approximately 340000.[11)] Fibrinogen has commonly been used as a coagulant in the medical field, but little is known regarding its suitability as a carrier of antitumor drugs.[12)] Injectable microspheres prepared from fibrinogen should yield a novel biodegradable system for drug delivery.

This report describes the preparation and characterization of fibrinogen microspheres and their usefulness as a drug carrier. 5-Fluorouracil (5-FU) was used as a model drug in this examination.

Experimental

Materials——5-FU and fibrinogen from bovine blood (Type I-S) were obtained from Sigma Chemical Co. and

used without further purification. Pronase E® (Kaken Kagaku Co., 70000 PUK/g) was used as a protease.

Preparation of Fibrinogen Microspheres——Fibrinogen microspheres containing the antitumor agent 5-FU were prepared based on the principle developed for the preparation of albumin microspheres.[13] 5-FU (100 mg) was dissolved in 2 ml of 0.7 M tris(hydroxymethyl)aminomethane. Bovine blood fibrinogen (250 mg) was then dissolved in the solution. The resulting solution was mixed with 100 ml of 10% Span 85 in cottonseed oil, and homogenized (Nihon Seiki Seisakusho, type HB) for 10 min at 4500 rpm. The emulsion was added to an additional 100 ml of cottonseed oil preheated to a desired temperature (90 or 160 °C) and, after standing for 30 min with constant stirring, the mixture was cooled to room temperature. The microspheres were washed free of oil by adding 200 ml of ether, centrifuging for 10 min at 3500 rpm, and decanting the supernate. After the third wash, the microsphere was allowed to dry in a desiccator.

Microscopic Characterization of Microspheres——The dried microspheres were observed under a scanning electron microscope (Hitachi Seisakusho, type X-650). The photograph was taken after metal coating of the microspheres with an ion-coater (Eiko Seiki, model IB-3).

Drug Contents in Microspheres——The quantity of drug entrapped was determined by digesting the microspheres (50 mg) in pH 7.4 phosphate buffer (4 ml) containing 0.2 mg of the protease. After filtration with a Millipore filter (0.45 μm), the amount of 5-FU in the supernatant was determined spectrophotometrically.

***In Vitro* Drug Release**——Drug release from the fibrinogen microspheres was determined as described previously[9] using plastic dialysis cells with a cellulose membrane (Visking Co., type 36/32). The capacity of each half-cell was 4 ml and the surface area of the membranes was 3.14 cm^2. A 50 mg sample of fibrinogen microspheres containing 5-FU was suspended in 4 ml of phosphate buffer (1/15 M, pH 7.4). The suspension was placed in the donor compartment and an equal volume of the phosphate buffer was put in the receptor compartment. The assembled cell was shaken horizontally at the rate of 100 storokes/min in an incubator (Taiyo Kagaku, M-I^N) maintained at 37 °C. The total volume of the receptor solution was removed at certain intervals and replaced with 4 ml of fresh buffer. The drug concentration of the samples was determined at 265 nm with a spectrophotometer (Hitachi Seisakusho, model 100-20). The effect of the protease on the *in vitro* drug release was determined by adding 0.2 mg of the protease to 4 ml of microsphere suspension. Data shown in Fig. 2 are averages of three experimental runs; the results were satisfactorily reproducible.

Animal Experiments——Male ddY mice, 30—37 g, were used. For evaluating the antitumor effect of the drug-loaded fibrinogen microspheres, ddY mice were inoculated intraperitoneally with 2×10^6 Ehrlich ascites carcinoma (EAC) cells. One day after inoculation of the cells, the mice were injected with 0.3 ml of suspension of the fibrinogen microspheres containing 5-FU or 5-FU suspension (free 5-FU) in 0.9% NaCl containing 0.2% (v/v) Tween 80. The survival time of the tumor-bearing mice was recorded in each case.

Results

Characteristics and Drug Content of Fibrinogen Microspheres

Figure 1 shows a scanning electron micrograph of bovine fibrinogen microspheres containing 5-FU prepared at 90 (A) and 160 °C (B). The fibrinogen microspheres were invariably spherical with a smooth surface. The average diameters of the microspheres prepared at 90 and 160 °C were 3.9 ± 0.2 and 1.1 ± 0.1 μm (mean $\pm$ S.E., $n = 100$), respectively, as measured in micrographs. The quantity of drug entrapped was determined by digesting the microspheres in pH 7.4 phosphate buffer containing the protease. When the fibrinogen microspheres were prepared at 90 and 160 °C, 16.1 ± 0.1 (mean $\pm$ S.E., $n = 4$) and 6.1 ± 0.1 ($n = 3$)% of the 5-FU present in the orieginal emulsion were associated with the microspheres, respectively.

In Vitro Release of 5-FU from Fibrinogen Microspheres

The amount of 5-FU which can be released from fibrinogen microspheres into pH 7.4 phosphate buffer was determined with a dialysis cell as described earlier.[9] Figure 2 shows plots of the data, expressed as the cumulative percent of the drug released, *versus* time. In contrast with the rapid release of free 5-FU from cellulose membranes, the release of the drug from the microspheres through the membrane was slow and continued over 5 d. In the latter case, an initial burst was seen, probably owing to the release of surface 5-FU from the microspheres. After the initial period of rapid release, a gradual release (presumably of the drug entrapped in the microspheres) was observed. The dependency of the drug release profile on the temperature of microsphere preparation is also clear from Fig. 2. An increase in the

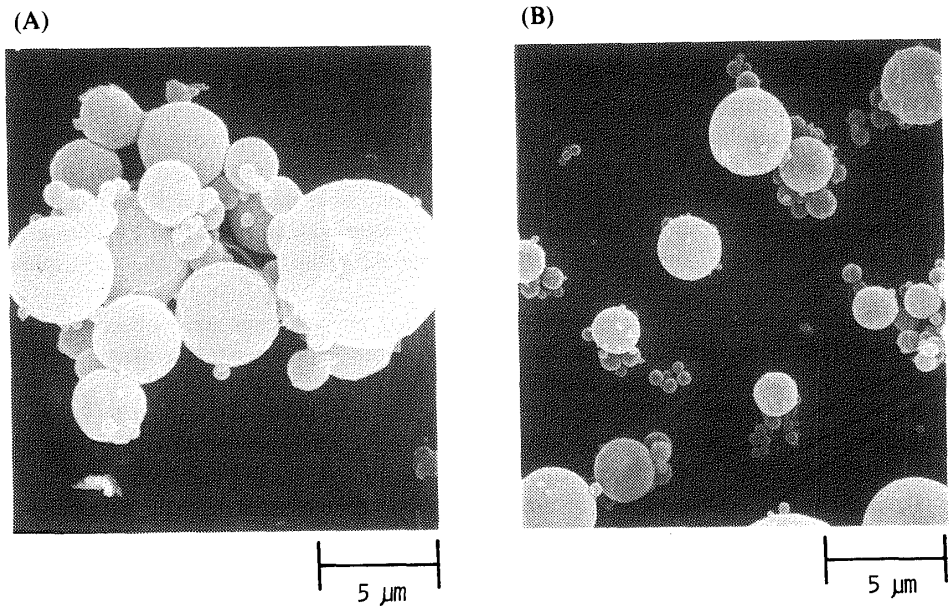

Fig. 1. Scanning Electron Micrographs of Fibrinogen Microspheres Containing 5-FU

The temperature of microsphere preparation was 90 (A) or 160 °C (B).

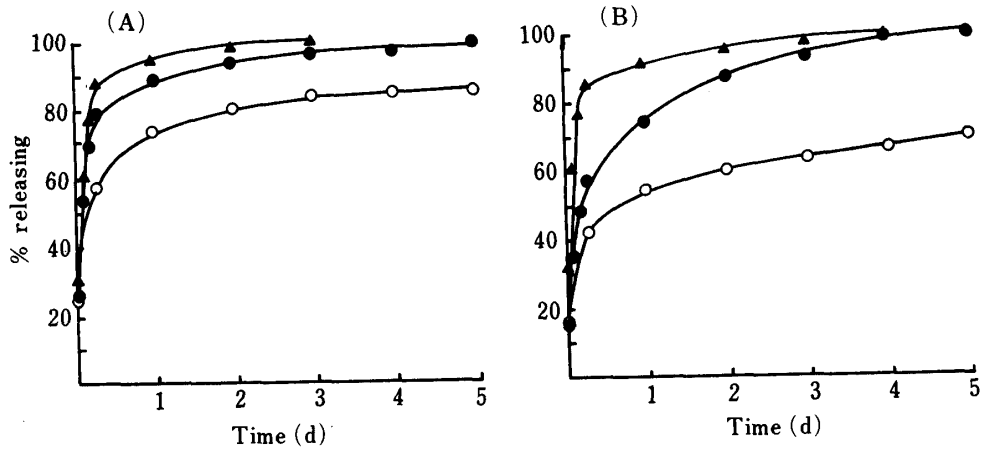

Fig. 2. *In Vitro* Release of 5-FU from Fibrinogen Microspheres at 37 °C

▲, free 5-FU; ○, microspheres; ●, microspheres treated with protease.
The temperature of microsphere preparation was 90 (A) or 160 °C (B). Each value represents the mean ± S.E. of 3 experiments.

temperature increases the hardness of the microspheres, leading to decreased drug release.

Scanning electron micrographs showing changes in the surface characteristics of microspheres with time are shown in Fig. 3. The surface of the microspheres before release was round and very smooth (Fig. 1), but became very rough after release for 5 d. In addition, the microspheres formed aggregates. It seems likely that the release mechanism of 5-FU from the microspheres is primarily diffusion.

The effect of a hydrolytic enzyme on the release of 5-FU from microspheres is also shown in Fig. 2. The addition of a protease (Pronase E®) to a microsphere suspension resulted in a large increase in the amount of drug released. This increase is presumably due to digestion of fibrinogen microspheres by the hydrolytic enzyme.

Scanning electron micrographs of the microspheres after release for 5 d showed that the microspheres had disintegrated in the presence of the protease (not shown). It is likely that the

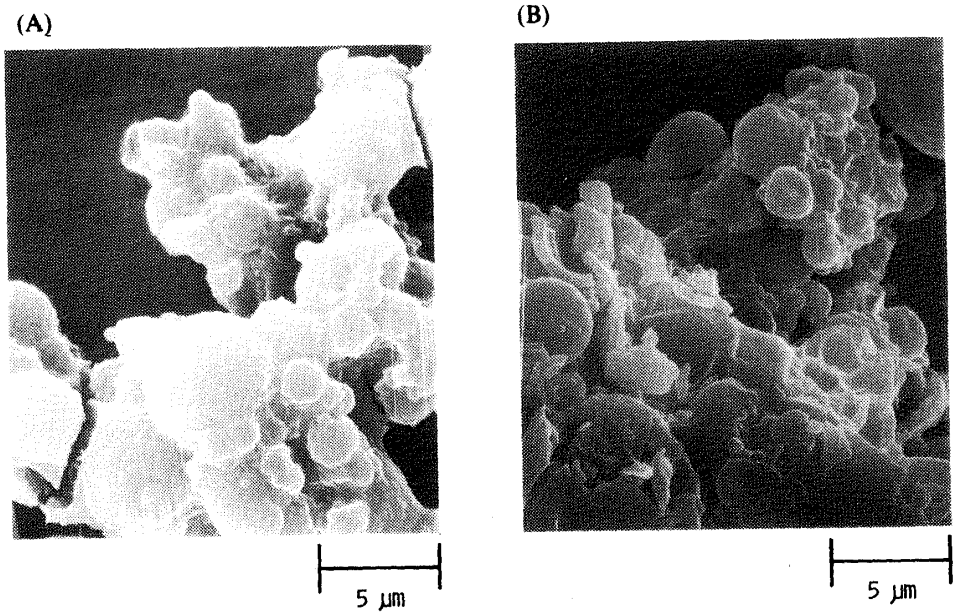

Fig. 3. Scanning Electron Micrographs of Fibrinogen Microspheres Containing 5-FU after Release for 5 d

The temperature of microsphere preparation was 90 (A) or 160 °C (B).

TABLE I. Effects of Fibrinogen Microspheres Containing 5-FU (Prepared at 90 °C) and of Free 5-FU on Survival Time of Mice Bearing Ehrlich Ascites Carcinoma

Compound	Dose (mg per mouse)	Survival time (d)[a]	T/C[b] (%)	Survivors at 60 d
Control	—	20.8±2.2	100.0	0/8
Free 5-FU				
	1	24.1±2.2	115.9	0/7
	2	39.4±6.7	189.4	0/7
Fibrinogen microsphere containing 5-FU				
	1	28.9±4.6	138.9	0/7
	2	29.3±5.5	140.9	0/6

a) Mean±S.E. *b*) Mean survival time of treated mice/mean survival time of control.

rapidity of drug release from the microspheres was due to both the disintegration of the microspheres by the proteolytic enzyme and the diffusion of 5-FU from the microspheres.

Antitumor Activity of the Fibrinogen Microspheres Containing 5-FU against Ehrlich Ascites Carcinoma

The antitumor activity of fibrinogen microspheres containing 5-FU was evaluated against EAC in mice. Tumor cell injections were done on day 0 and microsphere injections on day 1, both intraperitoneally.

Table I summarizes the antitumor activity of the fibrinogen microspheres containing 5-FU prepared at 90 °C in comparison with that of free 5-FU. All activities were evaluated as T/C%, calculated as the ratio of the mean survival time of the treated group (T) divided by that of the control group (C). Injection of fibrinogen microspheres containing 1 and 2 mg/mouse of 5-FU increased the T/C% values to 138.9 and 140.9%, respectively. Fibrinogen microspheres had slightly better activity than free 5-FU at a dose of 1 mg, whereas at a dose of 2 mg the free drug was superior to the microspheres.

TABLE II. Effects of Fibrinogen Microspheres Containing 5-FU (Prepared at 160 °C) and Free 5-FU on Survival Time of Mice Bearing Ehrlich Ascites Carcinoma

Compound	Dose (mg per mouse)	Survival time (d)[a]	T/C[b] (%)	Survivors at 60 d
Control	—	18.5±0.4	100.0	0/6
Free 5-FU				
	0.3	18.0±2.9	97.3	0/6
	1	22.5±2.4	121.6	0/6
Fibrinogen microsphere containing 5-FU				
	0.3	21.8±1.1	117.8	0/6
	1	23.8±1.6	128.6	0/6

a) Mean±S.E. *b*) Mean survival time of treated mice/mean survival time of control.

Similar results were noted after the intraperitoneal injection of the fibrinogen microspheres prepared at 160 °C into EAC-bearing mice (Table II). When fibrinogen microspheres containing 0.3 and 1 mg/mouse of 5-FU were injected into the mice, the T/C values were 117.8 and 128.6%, respectively. These values are larger than that of the control group ($p<0.025$) but are essentially the same as those obtained with free 5-FU, so that the antitumor activity of 5-FU remained mostly intact even after incorporation into these preparations.

Biocompatibility of Fibrinogen Microspheres

Fibrinogen microspheres without drug were prepared at 90 and 160 °C and evaluated in terms of the number of survivors and evidence of rejection at 30 d after intraperitoneal injection into normal ddY mice. None of the 6 mice receiving the high dosage (50 mg of microspheres per mouse) died and no localized inflammation or foreign reaction in the peritoneum was observed. The changes in body weight after injection of the pure fibrinogen microspheres were similar to those of normal untreated mice. Thus, fibrinogen microspheres showed excellent biocompatibility as an injectable carrier for drug delivery.

Discussion

Based on the evidence presented in this report, drug-loaded fibrinogen microspheres could be prepared. Fibrinogen microspheres show good biocompatibility and should be useful as a novel drug carrier for injected delivery systems of antitumor drugs.

The *in vitro* release of 5-FU from the fibrinogen microspheres was found to be slow after the initial burst (Fig. 2), indicating that sustained release occurs. However, treatment of EAC in mice with 5-FU–fibrinogen microspheres did not produce a significant prolongation of lifespan (Tables I and II). The mean survival time of the group receiving the microspheres was larger than that of the control group, but was essentially the same as that of the free 5-FU-treated group. It should be possible to improve the efficacy of an antitumor drug entrapped in fibrinogen microspheres by improvement of the *in vitro* drug release characteristics. Further work is necessary to produce microspheres with a greater range of release characteristics for evaluation in animal tumor models.

It is well known that albumin microspheres are useful as a tissue-specific drug carrier in chemotherapy. For successful application, however, it is desirable to produce microspheres with a higher drug content.[14] When the present fibrinogen microspheres are compared with albumin microspheres in terms of the drug content, the former seems to contain more drug (5-FU) than the latter. 5-FU contents in the fibrinogen microspheres were about 16 and 6% for those prepared at 90 and 160 °C, respectively; these values are higher than the 5-FU content

(about 4%) in albumin microspheres.[6] The level of drug content in microspheres might depend on differences of physicochemical interactions of the drugs with albumin and fibrinogen. These considerations suggest that with these systems, a wide spectrum of drug content as well as of release rate can be achieved by selecting a suitable drug carrier; albumin or fibrinogen.

Microspheres have been suggested as a means of attaining high local concentrations of a drug in specific tissues following introduction into the vascular system, by virtue of their diameter. Microspheres larger than 10 μm are deposited in the lung, while those of about 1 μm in size are taken up by the liver.[15] It has been reported that 5-FU entrapped in bovine serum albumin microspheres (0.4—1.0 μm in size) was localized in the liver after intravenous injection into mice.[6] It is possible that preferential uptake of fibrinogen microspheres (1—4 μm) into liver might be achieved and thus the required total doses as well as systemic side effects of antitumor drugs might be reduced. Further studies are in progress to determine the fate of intravenously administered fibrinogen microspheres containing 5-FU, as well as the degradation characteristics of the microspheres *in vivo*.

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