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Intravascular Tumour Targeting of Aclarubicin-loaded Gelatin Microspheres. Preparation, biocompatibility and biodegradability

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Abstract □ This study is to evaluate the potential use of aclarubicin-loaded gelatin microspheres as an intravascular biodegradable drug delivery system for the regional cancer therapy. The diameter of the microspheres prepared by water in oil emulsion polymerization could be controlled by adjusting the stirring rate in the range of 10-50 μm: D(in μm) = −73.8 log(rpm) + 262.7. The addition of proteolytic enzyme increased the in vitro aclarubicin release, but it did not change the amount of the initial burst release which reached about 45%. Microspheres injected intravenously into the mouse tail vein embolized only to the lung when observed by fluorescence microscopy. From histological examination following injection of gelatin microspheres into mouse femoral muscle, mild inflammation was observed from the appearance of neutrophils after 2 days and rapid repair process was confirmed thereafter. Biodegradation process of gelatin microspheres lodged on the pulmonary capillary bed was followed up by microscopic observation; degradation was taking place by about 36 hrs, followed by severe damage on the spherical shape and microspheres was no longer found 10 days after injection.

Keywords □ Gelatin microspheres, Aclarubicin, Biocompatibility, Biodegradability, Targeting, Drug delivery system.

There has been a growing interest in the use of targetable drug delivery system for cancer chemotherapy to achieve localization of cytotoxic drugs at the specific tumour site[1], because general cytotoxic drugs have a low therapeutic ratio due to systemic toxicity.

Among the approaches for the drug targeting delivery systems including drug-macromolecule conjugates[2], monoclonal antibody[3] and liposome[4], microspheres have been suggested as a noble means of attaining high local concentration of drug in specific tissues due to its applicability for both passive and active drug targeting[5]. Contrary to the active drug targeting by alternation of the nature of microspheres by both surface modification[6] and extracorporeal magnetic guidance[7-9], passive drug targeting by means of microspheres almost depends on the size of microspheres[10].

There are a number of possible biodegradable polymers that could be used for this type of drug carrier, microspheres. Natural polymers, *e.g.* albumin[11], gelatin[12] and starch[13] and synthetic polymers, *e.g.*, polyalkylcyanoacrylate[14], polylactic acid[15] and ethylcellulose[16] have been used previously as a way of altering the systemic distribution of cytotoxic drugs within the body. In this study, gelatin microspheres containing cytotoxic antibiotic, aclarubicin (aclacinomycin A) were prepared and their biocompatibility and biodegradability were assessed by microscopic observation of muscle and lung tissue of mouse following intramuscular injection into femoral muscle and intravenous injection into tail vein, respectively. And in vivo distribution and in vitro drug release experiments with and without proteolytic enzyme were also performed.

EXPERIMENTAL METHODS

Preparation of aclarubicin-loaded gelatin microspheres

The principle involved in microsphere formation is to form a water in oil emulsion and to stabilize the dispersed aqueous phase with cross-linked gelatin.

Thirty milliliters of cotton seed oil (Hanawa Chemicals, Japan) was added to a round-bottomed glass beaker (diameter 37 mm, height 125 mm). After pre-stirring the oil with a motor-driven two-bladed impeller (rod diameter 10 mm, blader 9×

27 mm) for 10 min at the desired speed and temperature, 75℃, 2 m*l* of aqueous solution containing 200 mg of gelatin (Nakarai Chemicals, Japan) and 20 mg of aclarubicin hydrochloride (Sanraku-Ocean, Japan) was added dropwise from a syringe to the oil. After stirring the resulting water in oil emulsion for an additional period of time, it was cooled on the ice bath and 1 m*l* of 0.5 or 5% aqueous glutaraldehyde (Tokyo Kasei Chemicals, Japan) was added. Stirring was continued for 30 min at 1500 rpm, and then the microspheres were isolated by centrifugation (Minor, MSE, England) for 5 min at 1500 rpm followed by decantation of the supernate. After resuspending the microspheres in diethyl ether and collecting them by centrifugation, residual oil was removed by further washing with acetone. The microspheres were vacuum dried and stored in air-tight vials at 4℃ until they were used.

Particle size measurement

Microspheres were sized using a scanning electron microscope (JCM-35C, Jeol, Japan). About 1 mg of microspheres was sputter-coated (JFC-1100, Jeol, Japan) with a 30 nm layer of gold. The diameter of the microspheres were manually measured from the resultant photomicrographs. In addition, some microspheres were also sized with a light microscope (Vickers, England) equipped with a calibrated micrometer.

Drug release experiment

Ten milliliters of microspheres containing aclarubicin was suspended in 4 m*l* of phosphate buffer solution (pH 7.4) or in the same buffer solution containing 40 units of protease. The suspension was continuously shaked at 50 rev/min and incubated at 37℃ in a thermostatted air bath. At various intervals, a sample was taken, centrifuged at 12000 rpm (HC-30, Kokusan, Japan) and the amount of aclarubicin released from the microspheres was spectrophotometrically determined at 425 nm (Lamuda 5, Perkin Elmer, USA).

In vivo distribution and biodegradability test [17,18]

Each male mouse (about 25g) was injected via tail vein under diethyl ether anesthesia with five milligram of microspheres dispersed in 0.5 m*l* of saline solution. Three mice were sacrificed at given intervals following injection of microspheres and lungs, liver, spleen and kidneys were isolated and fixed with formalin for overnight. The formalin-fixed organs were frozen cut (Cryostat OTF/AS/EC/D, Bright, England) in 15 μm, and unstained samples were mounted using canada balsam for fluorescence microscopy. Sections were examined with a incident light fluorescence microscope equipped with a camera (Model 2071 M, American Optical, USA) at the emission filter of 515 nm after excitation with filter of 436 nm.

Blood samples were also observed by fluorescence microscopy with the same filter combination.

For the biodegradability test, another sections of lungs were stained with hematoxylin-eosin[37] and observed with a light microscope equipped with a camera (M17, Vickers, England).

Evaluation of biocompatibility

Two milligrams of microspheres dispersed in 0.2 m*l* of saline solution was injected into the femoral muscle of each male mouse. Saline solution was used as a control. Within each group, two mice were killed at given intervals following injection of microspheres and the femoral muscle of mice was isolated and fixed with formalin. Then 15 μm sections of formalin-fixed muscle were frozen cut and stained with hematoxylin-eosin for light microscopy to evaluate the signs of inflammation on the tissue around the microspheres and repair process of injured tissue.

Lung sections stained for biodegradability test were also observed in terms of inflammatory characteristics.

RESULTS

Preparative conditions and characteristics of microspheres

The design of mix-cell and impeller have been found to be critical for producing reproducibly uniform microspheres. The use of horizontal type impeller made the microspheres having a broad size range. With the cell and impeller designed in this study, the mean diameter of the microspheres

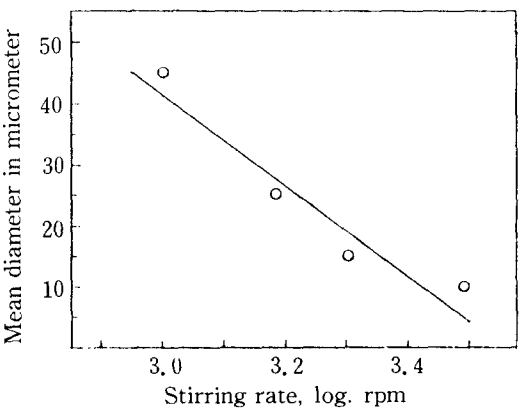

Fig.1. Effect of stirring rate on mean size of aclarubicin-loaded gelatin microspheres prepared with 5% glutaraldehyde.

produced could be plotted as function of the rate of stirring in the range of 10-50 μm, as shown in Fig.1.

It was also important to adjust the center of stirring rod in the cell and to use a syringe for the dropping of aqueous phase into the oil phase to get well-defined microspheres. Incorporation of non-ionic surfactant into the emulsion led to microspheres having a narrower size range, but it resulted in a great decreasing of the trapping yield of aclarubicin into gelatin microspheres probably due to the increasing of solubility of aqueous phase into oil phase. With the procedure described above, the incorporated aclarubicin in the gelatin microspheres was between 5.1-5.4% by weight of the formed microspheres. Stirring speed made no difference on the incorporation of drug.

In vitro aclarubicin release

Release profiles of aclarubicin from microspheres cross-linked with 0.5 and 5% of glutaraldehyde are shown in Fig.2. In the plots of the percentage of aclarubicin released against time, rapid initial release was observed in both 0.5 and 5% glutaraldehyde treated microspheres and more than 85% and 45% of the initial amount of aclarubicin were recovered in the medium, 20 min after the incubation. Microspheres prepared with 0.5% glutaraldehyde released more than 90% of drug within 30 min. On the contrary, drug released within 1 hr from those prepared with 5% glutaraldehyde was 55% and then remained almost constant in the next 4 hrs. Proteolytic enzyme, protease led spontaneous release of aclarubicin from the gelatin microspheres but made no difference on the amount of the initial burst release. After 16 hrs, no microsphere was observed in the medium contaning protease, whereas only mild physical changes were observed on the microspheres in simple phosphate buffer solution.

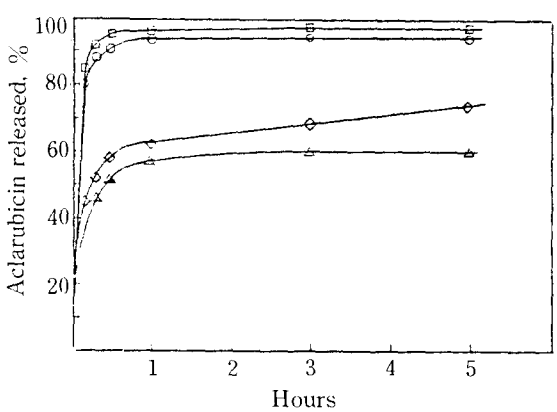

Fig.2. Effect of concentration of glutaraldeyde and proteolytic enzyme on in vitro release of aclarubicin.

○-○ ; 0.5% glutaraldehyde, □-□ ; 0.5% glutaraldehyde with protease, ◇-◇ ; 5% glutaraldehyde, △-△ ; 5% glutaraldehyde with protease.

Fig.3. Photomicrograph of lung section 10 min after injection of aclarubicin-loaded gelatin microspheres via tail vein of mouse.

Obtained with unstain and fluorescence microscopy(mag×80). White circles stands for microspheres appeared on the fluorescence microscopy yellow green.

In vivo distribution of microspheres following systemic injection

The intrinsic fluorescence of aclarubicin was adopted as a tracer to investigate the in vivo distribution of aclarubicin-loaded gelatin microspheres having mean diameter of 21.6 μm.

Gelatin microspheres containing aclarubicin appeared as yellow green fluorescent circles could be readily distinguished from surrounding organ tissue which made no fluorescence in this filter combination. The pulmonary distribution of microspheres after 10 min is illustrated in Fig.3. Immediately after injection, microspheres had already reached the lung and lodged in precapillary arterioles and capillaries. No gelatin microspheres was found within 24 hrs in blood samples and other organs tested, *e.g.*, liver, kidney and spleen.

Biocompatibility of microspheres

The histological observations for the femoral muscle of mice following injection of aclarubicin-loaded gelatin microspheres at intervals were performed as shown in Fig.4. No evidence of untoward reaction was seen in case of saline injection after either 2 or 5 days except the slight disruption of muscle tissue at the injection area.

Injection of microspheres caused damage on the muscle structure of injected site but no adverse tissue reaction associated with microspheres appeared within 12 hrs. Signs of inflammation

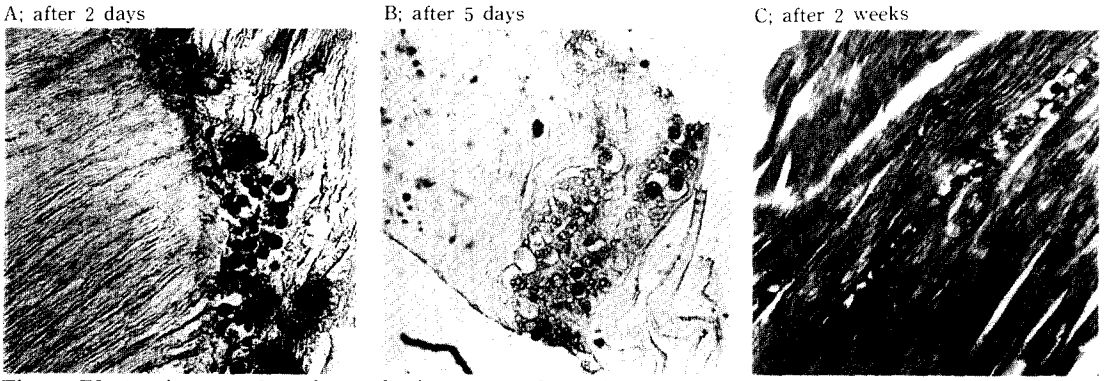

Fig.4. Photomicrographs of muscle tissue samples lodged with aclarubicin-loaded gelatin microspheres.
Obtained with hematoxylin-eosin stain and light microscopy(mag×25).

were observed after 2 days; appearance and accumulation of neutrophilic leukocytes on the tissue surrounding microspheres by cellular infiltration, which could be recognized by their segmented or lobulated nucleous stained blue with hematoxylin-eosin. At the 5th day, the number of neutrophils decreased dramatically, whereas regeneration of connective tissue increased and fibrous capsule formation for the microspheres seldom appeared. And also phenomenon of perforation on the surface of gelatin microspheres was seen.

The neutrophils completely disappeared from the tissue 2 weeks after injection and injected area was characterized by a tightly embedding gelatin microspheres between almost healed up muscle tissue. The microsphere-tissue interface could not be identified clearly and minute reduction in size along with the deformation in shape were also observed.

From the photomicrographs of Fig.5, slight engorgement on the lung tissue around the lodged microspheres was seen at 36 hrs but this phenomenon was no longer visible 3 days after injection.

Biodegradation of microspheres

Clearcut observation of the shape of microspheres among the tissue was possible by the staining of lung sections with hematoxylin-eosin due to the differences of stained color. Aclarubicin-loaded gelatin microspheres lodged in capillaries appeared deep red while the connective tissue pale scarlet.

Fig.5 shows photomicrographs of the lung at 1, 36 hrs and 3 days after intravenous injection of the aclarubicin-loaded gelatin microspheres. Gelatin microspheres tightly occupied the capillary

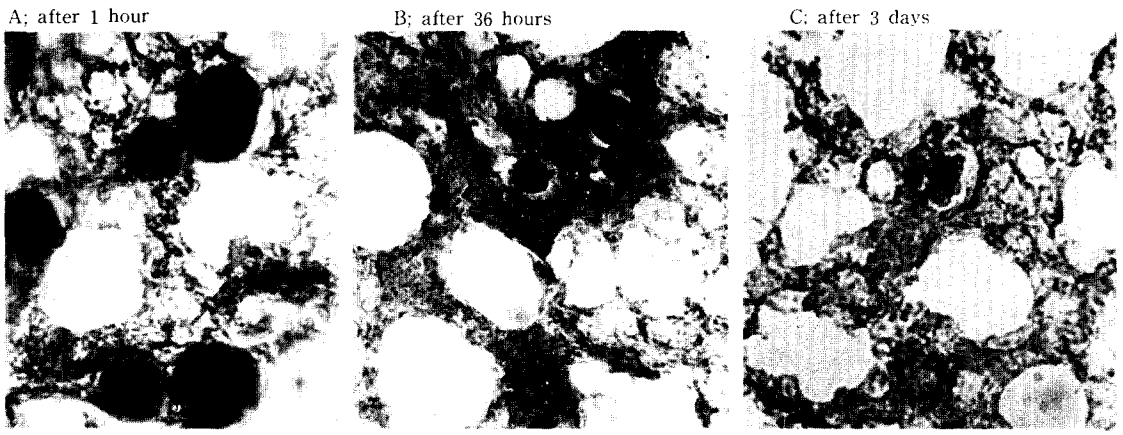

Fig.5. Photomicrographs of lung tissue samples lodged with aclarubicin-loaded gelatin microspheres.
Obtained with hematoxylin-eosin stain and light microscopy (mag×100).

lumen of lung and maintained their spherical shape. After 36 hrs the size of gelatin microspheres was reduced and some space between microspheres and capillary wall appeared, but there was no damage on their spherical shape. On this stage, it could be observed that cavities are formed on the microspheres which were also detected on the microspheres in the muscle tissue of the 5th day after injection as shown in Fig.4.

Severe damage on the spherical appearance of microspheres was detected after 3 days and no trace of microspheres was found after 10 days. Thus, Fig.5 shows the gradual digestion process of gelatin microspheres on the lung tissue.

DISCUSSION

With increasing interest in the targetable drug delivery system for the cancer chemotherapy, a number of attempts have been reported[21,22] and some of them have been applied in the clinical study[23].

The use of microspheres out of them has received much attention as a noble carrier for both active and passive drug targeting[5]. There have been three types of use of microspheres for drug targeting in terms of injection routes, size of microspheres and targeting tissue[10]. First, intravenous, intra-arterial or intraperitoneal injection of microspheres of less than 2 μm to lead uptake by polymorphonuclear system. Second, intravenous injection of microspheres of above 7-12 μm and between 3-12 μm, the former for lung and the latter for lung, liver and spleen. And third, intra-arterial injection of microspheres greater than 12 μm for liver, kidney, spleen of tumor-bearing organs.

From this point, we can easily figure out that the microspheres greater than 12 μm could be successfully applied as a targeting carrier for the treatment of solid tumors in organs.

Various production methods have successfully adapted for the preparation of gelatin microspheres. Simple coacervation method[24] has generally yielded gelatin microspheres of submicron and Madan *et al.*[25] proposed capillary method to get microspheres above 185 μm. Jizomoto[26] and Kaiser-Liard[27] prepared gelatin microspheres in the range of 50-400 μm by phase separation and emulsion-induction method, respectively. But it was found that the procedures based on those described in the above literature were inadequate to obtain gelatin microspheres suitable for intravascular injection. The relatively large particle size of microspheres may hamper the injection through the narrow gauge needle which are preferred for clinical use.

As shown in Fig.1, we could prepare aclarubicin-loaded gelatin microspheres suitable for intravascular injection from simple water in oil emulsion system including only vegetable oil and aqueous gelatin solution containing aclarubicin hydrochloride with the mix-cell and two-bladed impeller as described above. Microspheres injected intravenously into the mouse tail vein only embolized to the lung in a somewhat uniform distribution and any trace of microspheres was not found in other organs tested, *e.g.*, liver, kidney and spleen. This is postulated probably due to their first-pass mechanical entrapment by the lungs prior to the distribution to the whole body.

The technique adopted for the distribution of microspheres in the body, consisting of fluorescence microscopic observation followed by frozen section was simple and enough to distinguish the microspheres from other macromolecules in the biological milieu in relatively short time comparing with time-consuming paraffin embedding procedure and techniques employing labelled compounds. But this fluorescence microscopic observation of intrinsic fluorescence of aclarubicin was inadequate to show clearcut boundary characteristics of microspheres because of their emitted fluorescent light. Therefore, hematoxylin-eosin staining technique was chosen for further studies, *e.g.*, biocompatibility and biodegradability test.

The ideal drug carriers should be biocompatible and biodegradable in vivo so that they do not make any inflammation and accumulate indefinitely in the tissue.

Gelatin has been recommended as a noble material for the encapsulation of cells to avoid immune rejection of transplanted cells, as gelatin has been known to be biodegradable and less antigenic[28]. But apparently few attemps have been made on the evaluation of the tissue reaction and biodegradability of the cross-linked gelatin microspheres in vivo conditions.

To data no adverse tissue reaction has been reported following systemic administration of cross-linked gelatin microspheres but a mild inflammation following intra-articular injection into rabbit knee joints[29]. On the contrary, microspheres of polylactic acid and polybutylcyanoacrylate injected into the same site revealed to cause severe inflammation of the synovial membrane and underlying tissues.

In our mouse femoral muscle studies, a mild adverse reaction was observed as described in

results.

When microspheres are introduced into a living organism, the organism initiates an immune reaction, in the form of an inflammation, to the foreign body. On the soft tissue implantation of microspheres, in general, during the first stage of the inflammatory response, neutrophils are the major group of phagocytic cells present[33], which were also found in Fig.4.

This inflammation is supposed to be due to residual glutaraldehyde on the surface of gelatin microspheres, but this adverse tissue reaction might be decreased by removal of glutaraldehyde by treatment with a metabisulfite[30] or by quenching the aldehyde group on the surface of microspheres with 2-aminoethanol[31].

Whereas in mouse lung studies, no inflammatory response was observed except the slight engorgement of the lung tissue on the basis of light microscopic observation from which good biocompatibility of cross-linked gelatin microsphere could be ascertained even on the residence of about 3 days in the capillary beds.

The biodegradability of cross-linked or denatured carrier materials is still not fully established[32], regardless whether they are natural or synthetic in their origin.

Typically, the degradation of a polymer introduced into an organism is effected by water, salts, and the pH of the environment, as well as by enzymes[17]. Especially enzymes found inside cells or released from the various types of leukocytes which are accumulated by infiltration around the damaged or destroyed tissue as a inflammation responce[20] may contribute considerably to the degradation of natural polymer in a living body. For example, neutrophils and mast cells or macrophages appeared in the first and the succeeding stage of inflammation, respectively, release several enzymes including the proteases: collagenase, elastase, and the cathepsins[33].

On the gelatin-like biomaterial, the degradation of catgut suture, whose basic material is collagen, has been reported to be highly influenced by the enzymes[34]. And their kinetic rules and mechanism of its destruction process in an organism have come under intensive study recently. This leads to recommend catgut and reconstituted collagen as noble absorbable suture materials[35].

In vitro biodegradability test of cross-linked gelatin nanospheres in phosphate buffer containing 1 mg percent of either trypsin or collagenase was reported, from which they postulated that the nanospheres could be a partially reconstituted collagen/cross linked gelatin mixture and their degradation could be dependent on enzyme[36].

Based on the evidence presented in this report, the degradation of gelatin microspheres proved to be greatly influenced by digesting lysosomal enzymes in circulating system and considerably rapid degradation of gelatin microspheres in pulmonary capillary beds was also indicated.

The cavities appeared on the gelatin microspheres as shown in Fig.4 and 5 were found at the 5th day in femoral muscle and in lung tissue at earlier time, 36 hrs after injection and are probably caused by the attack of enzymes. These results may explain the variable enzyme activity required for degradation of microspheres at different sites of the body. This could be also confirmed on the comparison of disappearing time of microspheres in both muscle and lung tissue. The microspheres in muscle, which digestion might only take place by enzyme released from leukocyte cells, were found 2 weeks after injection with little deformed shape and smaller size than the initial, on the contrary the microspheres in lung tissue were no longer traced after 10 days probably due to digestion by rich lysosomal enzymes in circulating system.

In summary, these results suggest that intravenous injection of gelatin microspheres containing aclarubicin can localize the drug in lung tissue, providing a noble method of drug delivery for regional cancer chemotherapy while minimizing adverse tissue reaction and offering prolonged drug release on the specific site.

The relative higher localization of cytotoxic drug-loaded conjugates or microspheres at the tumour-site than in non-tumour tissue does not prove directly that the tumour-suppressive effects obtained are only due to targeting[3]. Moreover the therapeutic index of a drug has been dealing with plasma or blood concentration rather than the drug concentration in the target tissue[38] and little is known regarding the specific organ or tissue drug levels necessary for therapeutic effects[39]. Therefore, further work with this type of cytotoxic drug-loaded microspheres will concentrate on the pharmacokinetic variability of intravascularly injected microspheres and also on demonstrating suppression of tumour growth in animal models.

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