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Evaluation of in vivo release characteristics of protein-loaded biodegradable microspheres in rats and severe combined immunodeficiency disease mice

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Abstract

The in vivo release characteristics of protein-loaded biodegradable microspheres were examined in normal rats and severe combined immunodeficiency disease (SCID) mice. Bovine-derived superoxide dismutase (bSOD), encapsulated into microspheres comprised of poly(D,L-lactic-co-glycolic acid) and poly(D,L-lactic acid), was used as a model protein. Three types of bSOD-loaded microspheres with different release profiles were subcutaneously administered to normal rats. Anti-bSOD antibodies were first detected at day 9 after the administration of these microspheres, which was independent of their release profiles in vitro. A typical formulation with a sigmoidal release profile was subcutaneously administered to SCID mice. No immunoreaction was observed. The plasma bSOD concentration–time profile, determined by an enzyme-linked immunosorbent assay, well reflected the in vitro release profile of the formulation. The disappearance profile of active bSOD from the administration site also partly corresponded to both profiles. However, the relative bioavailability calculated with a profile on the subcutaneous injection of bSOD solution was 40.7%. These results indicated possible instability of bSOD released gradually at the administration site. The results of the present study using SCID mice would be suitable for discussing the in vitro–in vivo correlation of protein release from microspheres, and useful for designing long-term release formulations of protein drugs.

Keywords: In vitro–in vivo correlation; Bovine superoxide dismutase; Biodegradable microspheres; Release kinetics; Immunogenicity; SCID mouse

1. Introduction

With the recent progress in biotechnology, many therapeutic proteins have emerged as an important class of drugs for the treatment of cancer, chronic diseases and infectious diseases [1]. In the pharmaceutical field, the development of suitable delivery systems for protein drugs with high molecular weights and short half-lives is of current interest. One such system, biodegradable microspheres of poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA), has been studied extensively [2–4].

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To date, various technologies have been proposed to fabricate protein-loaded microspheres [5–9]. Many have adopted a classical multiple (water-in-oil-in-water) emulsion method [5,8], while others are new, including a solid-in-oil-in-water (S/O/W) method [6]. Our recent studies have featured a novel protein encapsulation methodology that utilizes the S/O/W emulsion process [10,11], and demonstrated some release modifications of bovine-derived superoxide dismutase (bSOD), as a model protein, from reservoir-type PLGA/PLA microspheres [10].

Generally, release profiles of long-term releasing formulations must be designed on the basis of the relationship between the pharmacokinetics of the released drug and its therapeutic effects on the body. Concerning protein-loaded microspheres using PLA or PLGA, researchers have tried to attain excellent release kinetics in vitro, such as a zero-order release, and a pulsed release [10,12]. However, the release characteristics in vivo of these long-term protein delivery systems have yet to be fully investigated. In other words, it is still unclear whether the ideal release characteristics of protein designed in vitro can be realized in vivo.

Crucial to the in vivo study of prolonged release formulations of proteins is the selection of a laboratory animal. This is because, except for the development of vaccines, immunogenicities against the encapsulated protein affect the evaluation of pharmacokinetics and pharmacodynamics [13]. In this sense, Johnson et al. used rats immunosuppressed by cyclosporin in screening formulations of human growth hormone (hGH)-loaded microspheres [4]. They also used transgenic mice to investigate immunogenicities in the administration of hGH-microspheres [14]. However, these models are not easy to prepare and their usage is restricted. Thus, a more convenient in vivo evaluation system with wide applicability to long-term protein delivery systems is needed. Until now, no attempts have been made to examine the in vitro–in vivo correlation of such a protein formulation in congenitally immunodeficient animals.

Our main purpose was to provide some useful guidelines for studying the release characteristics in vivo of protein-loaded microspheres. In this paper, bSOD-loaded microspheres, prepared using recently established technology [10], were administered to severe combined immunodeficiency disease (SCID) mice [15] as well as to normal animals, for discussing the in vitro–in vivo correlation of bSOD-loaded microspheres.

2. Materials and methods

2.1. Materials

Superoxide dismutase from bovine erythrocyte (bSOD) was purchased from Nacalai tesque (Kyoto, Japan). All the biodegradable polymers and poly(ethylene glycol)s (PEG) used for preparing microspheres (see Section 2.2) were purchased from Wako Pure Chemical (Osaka, Japan). SOD Test Wako and 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) were obtained from Wako Pure Chemical. Micro-BCA protein assay reagent was obtained from PIERCE Chemical (Rockford, IL). Reagents used in enzyme-linked immunosorbent assay (ELISA) for the determination of bSOD concentration in plasma and anti-bSOD antibody titer are described in Sections 2.5 and 2.6, respectively. All other reagents used were of reagent grade.

2.2. bSOD-loaded microspheres

Three kinds of bovine SOD-loaded microspheres comprised of poly(D,L-lactic acid) (PLA), poly(D,L-lactic-co-glycolic acid) (PLGA), and PEG were prepared according to the S/O/W emulsion–solvent evaporation method reported in our previous paper [10]. Formulation A was made of PLGA (lactic acid/glycolic acid (L/G) ratio of 50/50) with a MW of 20 000 (PLGA5020) and PLA with a MW of 20 000 (PLA0020) (mixing ratio of 1:4), loaded with 2.5% PEG with a MW of 6000. Formulation B was made of PLGA5020 and PLA0020 (mixing ratio of 1:4), loaded with 10% PEG with a MW of 6000. Formulation C was made of PLGA (L/G ratio of 50/50) with a MW of 10 000 (PLGA5010) and PLA0020 (mixing ratio of 1:4), loaded with 2.5% PEG with a MW of 70 000. Methods for the measurement of active bSOD contents in microspheres were also described earlier [10].

2.3. In vitro release study

In vitro release study for bSOD-loaded microspheres was performed as previously described [10]. Briefly, microspheres (50 mg) were placed in test tubes and suspended in 10 ml of phosphate buffered saline (PBS; pH 7.4) containing 0.02% sodium azide. Incubation was conducted at 37°C by rotating the test tube at 25 rpm. At predetermined intervals, each test tube was centrifuged at 2000 rpm for 5 min, and 9 ml of supernatant were withdrawn. After the addition of 9 ml of fresh medium, the test tube was continuously incubated. The supernatant was filtered through a 0.5-μm membrane filter, and assayed for protein release by a micro-BCA protein assay.

2.4. In vivo experiments

Six-week-old male Wistar rats weighing about 200 g were purchased from Japan SLC (Kyoto, Japan). Six-week-old male severe combined immunodeficiency disease (SCID; C.B-17/Icr-scidJcl) mice weighing 20~25 g and ICR (Jcl/Icr) mice weighing 30~35 g were obtained from Crea Japan, Inc. (Tokyo, Japan). Animals were housed in a specific pathogen-free environment. All the in vivo experiments were conducted with the approval of the ethical committee for the use of laboratory animals at our company.

2.4.1. Administration to rats

Seven rats per group (one group was used for one formulation) were subcutaneously injected with bSOD-loaded microspheres (10 mg of bSOD/kg body weight). Injection volumes were 10 ml/kg body weight in a dispersion vehicle, i.e. saline containing 0.5% (w/v) sodium carboxymethylcellulose and 0.1% (v/v) Tween 80®. Serum samples were collected 5, 7, 9, 11, 14, 17, and 21 days after administration of the microspheres, and analyzed for an anti-bSOD antibody titer by ELISA.

2.4.2. Administration to mice

Four mice per group (one group was used for each time point) were subcutaneously injected with bSOD-loaded microspheres (10 mg (as bSOD)/kg body weight). Injection volumes were 10 ml/kg body weight in sterile dispersion vehicle. SCID mice were sacrificed at 1 h, and day 1, 3, 7, 10, 14, 21, and 35 after administration of the microspheres. ICR mice were sacrificed only at days 14 and 35. Microspheres at the injection sites were recovered and analyzed for SOD activity as described in Section 2.6. Plasma samples were also collected and analyzed for bSOD concentration and an anti-bSOD antibody titer by ELISA. Placebo microspheres were also administered to mice in order to measure the SOD activity of the blank tissue. In a separate experiment, bSOD in a saline solution was injected subcutaneously to SCID mice at a dose of 1 mg/kg body weight.

2.5. Determination of bSOD concentration in mouse plasma

A specific sandwich-type of ELISA was established in our laboratory to determine the concentration of bSOD in mouse plasma. Briefly, 96-well polystyrene microtiter plates (Costar-Corning, New York, NY) were coated with 100 μl (per well) of rabbit anti-bSOD antiserum (Polyscience, Warrington, PA) diluted 1/2500 in PBS (pH 7.4) for 30 min at room temperature. After being rinsed with a washing buffer, i.e. PBS containing 0.05% (v/v) Tween 20® (PBS/T20), the plates were blocked for 1 h at room temperature with 200 μl of diluted Block Ace (Snow Brand Milk Products, Japan) to prevent non-specific binding. After another wash, plasma samples (100 μl) diluted 1/10 in PBS/T20 containing 10% (v/v) Block Ace (PBS/T20/BA) were added to the wells and the plates were incubated for 3 h at room temperature. After further washing, 100 μl of peroxidase conjugated rabbit anti-bSOD antiserum (Rockland, Gilbertsville, PA) diluted 1/2500 in PBS/T20/BA was added to each well, and incubation continued for 1 h at room temperature. After a thorough wash, 100 μl of ABTS solution (150 μg/ml in citrate buffer, pH 4.0) was added to each well and the plates were incubated at room temperature for 15 min. The reaction was stopped with 100 μl of 1.5% oxalic acid solution per well, and plates were read at 405 nm in a microplate reader (Model 3550; Bio-Rad Laboratories, Hercules, CA).

2.6. Anti-bSOD antibody measurement

A specific anti-bSOD antibody titer in rat or mouse serum was also determined by another ELISA method. First, 96-well polystyrene microtiter plates were coated with 50 μl of bSOD in PBS (50 μg/ml) and left overnight at 4°C. The next morning, they were washed with the washing buffer, and non-specific binding was blocked with 200 μl of diluted Block Ace for 1 h at room temperature. The plates were washed again, and after 50 μl of serum sample at four separate dilutions from 1/10 to 1/10 000 in PBS/T20/BA had been added to each well, incubated for 3 h at room temperature. After further washing, 100 μl of peroxidase conjugated anti-rat IgG (Carbiochem-Novabiochem Corp., La Jolla, CA) diluted 1/1000 in PBS or peroxidase conjugated anti-mouse IgG (Chemicon International Inc., Temecula, CA) diluted 1/1000 in PBS was added to each well and the plates were incubated for 1 h at room temperature. After extensive washing, the activities of peroxidase bound by the antigen–antibody reaction were determined in the manner described in Section 2.5.

2.7. Determination of the active bSOD remaining in the recovered microspheres

The active bSOD in the microspheres recovered from the site of administration in mice was determined as follows. The recovered microspheres were fully homogenized with a polytron homogenizer at 16 000 rpm in acetone. After centrifugation at 12 000 rpm for 5 min, the supernatant was removed. Precipitates were redispersed with freshly added acetone. This treatment was repeated twice. After vacuum-drying for 2 h, the collected fraction was dissolved in PBS at 37°C for 1 h for the determination of SOD activity using SOD Test Wako. Results, i.e. total SOD activities remaining at the administration site, were presented as percentages of the administered dose (initial level).

3. Results

3.1. Studies in normal rats

To identify the limitations of in vivo studies of protein-loaded microspheres in normal animals, bSOD-loaded microspheres with different release profiles were administered to normal rats. Typical release profiles of the bSOD-loaded microspheres are shown in Fig. 1. The first formulation (A) exhibited an initial burst release of 12%, followed by a sigmoidal release profile with a lag time of about 5 days. The second formulation (B) had an initial rapid release, followed by an extended slow release of protein. The third formulation (C) showed a reduced initial burst and a short lag time, followed by a gradual release profile for 3 weeks. Because in primary structure, bSOD is different from rat SOD [16], immune responses to bSOD would be anticipated in the rat body. In Table 1, anti-bSOD titers after the administration of bSOD-loaded microspheres are compared between the three different release profiles. Anti-bSOD antibodies in the rat serum first appeared 9 days after the administration for all formulations, and gradually increased until day 21. There was little difference in the mean titer values induced by these three formulations. Because the anti-bSOD antibodies in blood may affect the systemic clearance of bSOD and/or inhibit the detection of bSOD in plasma by ELISA, it was clear that the pharmacokinetic analysis in rats should last no more than 1 week.

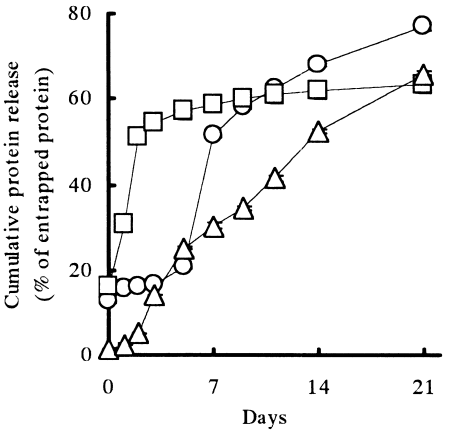


Fig. 1. In vitro release profiles of bSOD-loaded microspheres used in the experiment in normal rats. Open circle represents formulation A, open square represents formulation B, and open triangle represents formulation C. The initial content of active bSOD in microspheres was 2.28% for formulation A, 2.00% for formulation B, and 2.48% for formulation C.

Table 1
The incidence of anti-bSOD antibody in rat serum after subcutaneous administration of the three kinds of bSOD microspheres (dose of 10 mg (as bSOD)/kg body weight) shown in Fig. 1

		Days after administration of bSOD to normal rats				
		7 days	9 days	11 days	14 days	21 days
Formulation A	Incidence (n^+/N)	0/7	3/7	7/7	7/7	7/7
	Mean titer	(N.A.[a])	(1.3)	(1.5)	(2.6)	(3.1)
Formulation B	Incidence (n^+/N)	0/7	4/7	6/7	7/7	7/7
	Mean titer	(N.A.[a])	(1.3)	(1.5)	(2.0)	(2.9)
Formulation C	Incidence (n^+/N)	0/7	6/7	7/7	7/7	7/7
	Mean titer	(N.A.[a])	(1.3)	(1.7)	(2.7)	(3.6)

[a] N.A.=not applicable.

3.2. Studies in SCID mice and normal mice

Bovine SOD-loaded microspheres with a reduced initial burst and a typical sigmoidal release profile as shown in Fig. 2(a) were subcutaneously administered to SCID mice and normal (ICR) mice. At first, in order to confirm a lack of immune functions in SCID mice, immunogenicities against bSOD in two different species of mice were compared in Table 2. In normal mice, antibodies against bSOD were observed at day 14 after the administration of bSOD-

Table 2
Comparison of the incidence of anti-bSOD antibody (titer) after subcutaneous administration of the bSOD-loaded microspheres (dose of 10 mg (as bSOD)/kg body weight) shown in Fig. 2(a) (formulation C) in SCID mice and ICR mice[a]

Mouse	At 14 days		At 35 days	
	n^+/N	mean titer	n^+/N	mean titer
SCID	0/4	N.A.	0/4	N.A.
ICR	4/4	3.0±0.6	4/4	3.0±0.6

[a] Data are shown as an average±SD (n=4). N.A.=not applicable.

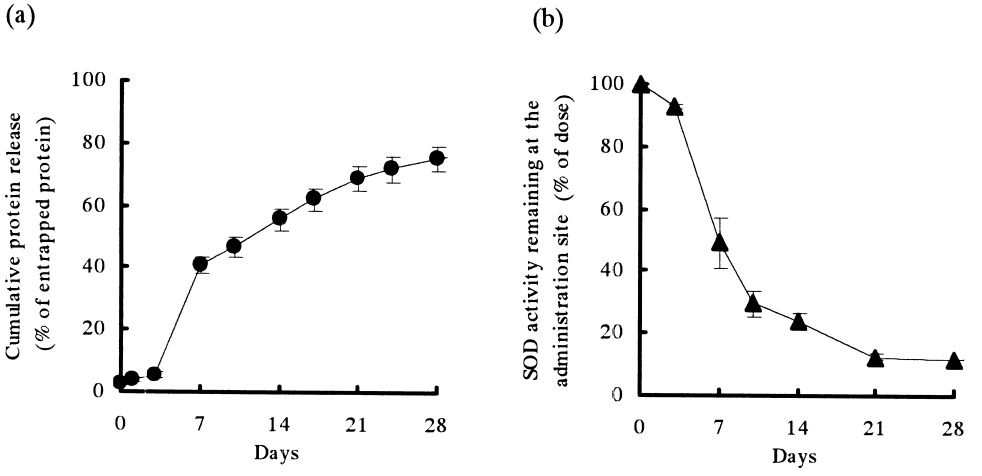


Fig. 2. The in vitro release profile of the bSOD-loaded microspheres used in the experiment in mice (formulation C) (a) and the in vivo disappearance profile of active bSOD at the administration sites in SCID mice (b). (a) The initial content of active bSOD in microspheres was 2.05%. Data are shown as an average±S.D. (n=3). (b) Data are given as the average±S.D. (n=4).

loaded microspheres in all animals. By contrast, no specific antibodies were produced in SCID mice.

The in vivo release of encapsulated bSOD must be related to the decrease in unreleased bSOD in microspheres. The profile of the disappearance of active bSOD from the site of administration of the microspheres in SCID mice is shown in Fig. 2(b). A lag period with little decrease in active bSOD was observed until day 3, and was followed by a gradual decrease in active bSOD for about 2 weeks. The levels of active bSOD remaining at day 14 and 35 are shown in Table 3 and compared with the results in normal mice, showing no significant difference in the two species.

The plasma bSOD concentration–time profiles, evaluated by the ELISA method, after the administration of the bSOD-loaded microspheres to SCID mice are shown in Fig. 3. The plasma concentration at 1 h was 3.7±1.7 ng/ml, which corresponded to an initial release of 0.08% of dose, estimated from the results of subcutaneous injection of bSOD solution (inset in Fig. 3). Therefore, the reduced initial burst attained in vitro was realized in vivo. The plasma concentrations at day 1 through day 3 were under the detection limit (lower than 1.5 ng/ml). At day 7, the plasma level rose above 50 ng/ml, and was followed by a moderate decline for the next 14 days. The lag until day 3 and the peak of the plasma concentration at day 7 well corresponded to the second burst-like release observed in the in vitro release study (Fig. 2(a)). The value of the area under the plasma concentration–time curve (AUC), calculated by the trapezoidal rule, for the administration of the bSOD-loaded microspheres (10 mg/kg dose) and the bSOD solution (1 mg/kg dose), was 273.3 and 67.2 ng/day/ml, respectively, which gave a relative bioavailability of 40.7%.

Table 3
Comparison of active bSOD remaining at the administration site in SCID mice and in ICR mice after subcutaneous administration of the bSOD-loaded microspheres shown in Fig. 2(a) (formulation C)[a]

Mouse	Remaining active bSOD (% of dose)	
	At 14 day	At 35 day
SCID	23.9±2.5	13.8±1.3
ICR	22.6±3.9	13.2±1.2

[a] Data are given as an average±S.D. (n=4).

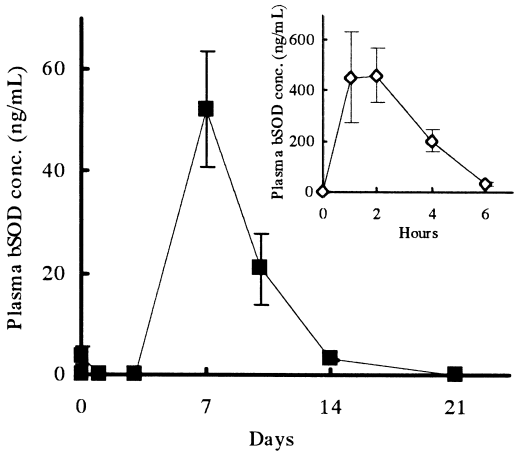
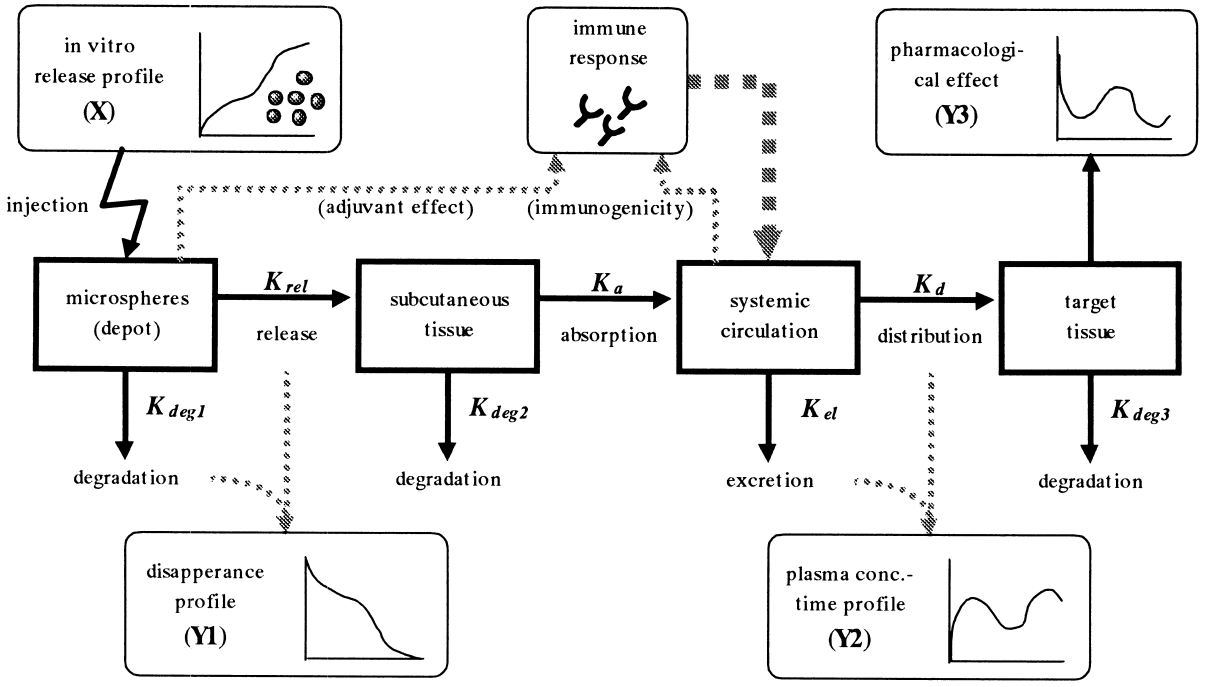


Fig. 3. The plasma concentration–time profile after the subcutaneous administration of bSOD-loaded microspheres shown in Fig. 2(a) (formulation C) (dose of 10 mg (as bSOD)/kg body weight) in SCID mice. Data are shown as an average±S.D. (n=4). (Inset) The plasma concentration–time profile after the subcutaneous injection of bSOD solution (dose of 1 mg/kg body weight) in SCID mice. Data are given as the average±S.D. (n=4).

4. Discussion

Over the last few decades, several methods for preparing protein-loaded microspheres using biodegradable polymers have been proposed [5–11]. In our previous paper, a new technology for preparing protein-loaded microspheres, without any loss of activity, via a S/O/W emulsion process using PEG as a protein micronization adjuvant, was described [10]. Once a new formulation technology has been established, release profiles should be designed in vitro. However, it is still unclear whether release characteristics designed in vitro reflect the pharmacokinetics of the released protein in vivo. In the present study, the in vitro–in vivo correlation of protein-loaded microspheres prepared with the newly developed technology was examined.

Scheme 1 summarizes general considerations for studying the in vivo release characteristics of protein-loaded microspheres, including a simple kinetic model. Briefly, the microspheres administered



Scheme 1. General considerations for the in vitro–in vivo correlation of protein-loaded microspheres. Details of all rate constants in the kinetic model (K_{rel}, K_a, and so on) were explained in the text.

to the body should function as a drug reservoir (depot). Protein released at a rate K_{rel} (in vivo release) will be absorbed in relation to the rate K_a dominated by the diffusibility of the protein through subcutaneous tissue. Protein absorbed into the systemic circulation will be eliminated by kidney or liver (K_{el}) while distributing to various tissues (K_d).Usually, a disappearance profile of drug from the administration site (**Y1**) and/or a plasma concentration–time profile (**Y2**), as output functions, should be compared with the in vitro release profile of the formulation (**X**) as an input function. Further, in many cases, pharmacological effects (**Y3**) should be intimately related to the pharmacokinetics of drug (**Y2**).

Generally, most human-derived proteins differ from proteins of animal species in amino acid arrangements. In preformulation studies using laboratory animals, this difference of molecular structure can lead to problems of antigenicity, making it difficult to evaluate the pharmacokinetics of protein drugs especially repeated administrations. Long-term formulations such as PLGA microspheres have the same problem, as shown by a thick dotted line in Scheme 1, while the adjuvant effects of PLGA microspheres have been applied to the development of vaccines. In our studies using bovine-derived SOD, antibodies against bSOD first appeared on day 9 in normal rats (Table 1), which was independent of the release profiles of microspheres. These results suggest that, because specific antibodies could affect the evaluation of the released protein, the in vivo pharmacokinetic study of microspheres loaded with a protein from a different animal species must be made within 1 week in normal animals.

Thus, to exclude the effect of specific antibodies, immunodeficient animals should be used for the study of the in vitro–in vivo correlation of protein release from microspheres. Johnson et al. reported an extended release of hGH from microspheres in rats immunosuppressed by cyclosporin [4]. However, the preparation of such a model is highly complex and sometimes not reproducible. Additionally, immunosuppressing agents like cyclosporin might have renal

toxicity, which could change the clearance of protein in kidney [17]. Thus, in the present study, the application of SCID mice as congenitally immunodeficient animals was tried.

SCID mice are known as a model animal with deficiencies for immune functions mediated by T and B lymphocytes [15]. In fact, no anti-bSOD activities were observed in SCID mice in our study, while specific antibodies appeared in normal mice (Table 2). Antibodies in normal mice seemed to have almost no effect on the remaining activity of the unreleased bSOD in microspheres (Table 3). However, the plasma bSOD concentration at day 14 was 'undetectable' in normal mice, while that was 3.1 ± 0.2 ng/ml in SCID mice. Although a detailed plasma concentration–time profile was not examined in normal mice, this difference may suggest that antibodies in normal mice alter the clearance rate of bSOD, or inhibit the detection of bSOD in plasma by ELISA based on an antigen–antibody reaction. Therefore, the plasma concentration–time profile in SCID mice (Fig. 3) would reflect the in vivo release of the bSOD-loaded microspheres.

The profile of the disappearance of active bSOD from the administration site (Fig. 2(b)) seemed to be closely related with the in vitro release profile, signifying a correlation between the lag-period until day 3 and the subsequent burst-like release.

The reason for the low relative bioavailability (40.7%) on administration of bSOD-loaded microspheres must be discussed. Protein administered to the body would be enzymatically degraded at the site of administration (K_{deg2}), in blood (K_{el}), and/or in various tissues (K_{deg3}) (Scheme 1). In the case of microsphere formulations, the stability of the protein in the depot (microspheres) after the administration (K_{deg1}) needs to be considered. Hence, comparison of the in vivo release profiles with the in vitro release profile would provide useful information. In Fig. 4, the in vivo release properties of bSOD calculated from the disappearance profile in Fig. 2(b) (profile (b) in Fig. 4), and estimated from the plasma concentration–time profile in Fig. 3 (profile (c) in Fig. 4) were plotted as well as the in vitro release profile (profile (a) in Fig. 4). Three profiles well corresponded in terms of the initial burst release, the lag-period until day 3, and the subsequent second burst-like release at day 7. However, the gradual

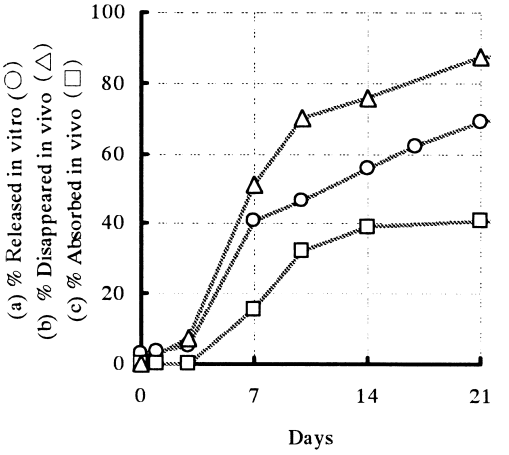


Fig. 4. Comparison of the estimated bSOD release profiles in vivo with the in vitro release profile of the bSOD-loaded microspheres. Open circle shows the in vitro release profile in Fig. 2(a). Open triangle shows the disappearance profile of active bSOD calculated from the remaining active bSOD in Fig. 2(b). Open square shows the estimated release profile, in which the % released at day 21 is 40.7%, calculated from the plasma concentration–time profile in Fig. 3.

release rates after day 7 were obviously different. The difference between profile (a) and (b) would be due to the enhanced release in vivo or the instability of bSOD in the depot. The activity of unreleased bSOD in microspheres was almost completely preserved in the in vitro release study (data not shown). However, the conditions affecting the protein stability in microspheres in vivo might be different from those in vitro. The difference between profile (b) and (c) would mainly result from degradation of released bSOD in subcutaneous tissue (K_{deg2}). Jadot et al. reviewed the pharmacokinetics of bSOD, in which the AUC of bSOD administered intramuscularly to humans showed good linearity with dose [18]. Although details of the susceptibility of bSOD to enzymatic degradation in mice are not known, it would be natural to assume that bSOD gradually released from microspheres is more susceptible to degradation, before being absorbed into the systemic circulation, than when administered by a single injection of solution. Alternatively, the systemic clearance of protein might be altered by continuous infusion. Further investigation is needed to clarify this point.

These results indicate the importance of the

stability of the encapsulated protein in vivo as well as the possibility of applying our encapsulation technology to the design of formulations for protein-loaded microspheres. Although bSOD was used as a model protein, the results are relevant to discussions on the in vitro–in vivo correlation of long-term formulations containing other proteins.

5. Conclusions

In this study, the in vivo release performance of protein-loaded microspheres was examined in SCID mice, using bovine-derived SOD as a model protein. Release profiles of protein-loaded microspheres, or any other long-term release formulations, should be designed based on pharmacokinetics and pharmacodynamics. The results of the present study should prove useful for designing long-term release formulations of protein drugs.

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