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Study of biodegradable copoly(L-lactic acid/glycolic acid) formulations with controlled release of Z-100 for application in radiation therapy

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Abstract

Z-100, *Mycobacterium tuberculosis* strain Aoyama B, which suppresses the growth of various experimental tumor cell lines, was incorporated into biodegradable copoly(L-lactic acid/glycolic acid) (70:30 mol%) copolymer with a number-average molecular weight of 2900, in order to evaluate the controlled release function, prolongation of survival duration, and hematopoietic functions such as endogenous colony forming unit of spleen (endogenous CFU-S) and colony forming unit of culture (CFU-C) in mice irradiated with a lethal dose of 8.5 Gy (γ-rays from a ^{60}Co source). In this study, the Z-100 formulation was implanted subcutaneously in the back of mice immediately after irradiation. All of the control mice without the use of the Z-100 formulation died within 19 days, as compared to only 30% mortality throughout the experimental period of 35 days for the experimental mice treated with the Z-100 formulation. Such prolongation of survival time in mice promoted recoveries of stem cells or granulocytic and macrophagic precursor cells.

Keywords: *Mycobacterium tuberculosis* strain Aoyama B; Biodegradable copoly(L-lactic acid/glycolic acid); Controlled drug delivery; Mouse; Radiation effect

1. Introduction

Z-100, which is prepared by purification of a hot water extract of *Mycobacterium tuberculosis* strain Aoyama B, contains polysaccharides such as arabinomannan and mannan as major constituents (Kobatake et al., 1981), in which arabinomannan consisting of an arabinan core and a mannan core coupled alternately has an average molecular weight of approx. 12000 and mannan with many side chains has an average molecular

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weight of approx. 5000. This compound suppresses the growth of various experimental tumor cell lines (Suzuki et al., 1986) and also has various biological activities, e.g., stimulation of circulating interferon-γ production (Hayashi et al., 1981), promotion of interleukin-1 production (Hayashi et al., 1990a), and enhancement of activities of human lymphocytes (Yanagi et al., 1980) and neutrophils (Niwa et al., 1985). Other immunopotentiating anticancer agents extracted from bacteria such as BCG (Pouillart et al., 1976; Singer et al., 1978) and *C. parvum*, (Gordon et al., 1977; Eliopoulos et al., 1980) have also been reported to enhance hematopoietic function and to increase peripheral leukocytes.

Recently, Hayashi et al. (1990a,b) reported that the subcutaneous administration of Z-100 twice a week starting immediately after supralethal whole-body irradiation of mice led to the prolongation of survival time, indicating that the treatment with Z-100 promoted colony formation in the spleen of the treated mice, endogenous colony forming unit of spleen (CFU-S) in the femoral bone marrow, and colony forming unit of culture (CFU-C). A single subcutaneous dose of Z-100 immediately after irradiation inhibited the reduction in the total number of nucleated cells in the femoral bone marrow of the treated mice. Greater efficacy of this drug may be expected by extending the duration of action of drug activity in the blood. We attempted to prepare biodegradable polymer formulations for the controlled release of Z-100 over a prolonged time. In the present study, a copoly(L-lactic acid/glycolic acid) (70:30 mol%) copolymer with a number-average molecular weight (M_n) of 2900, which completely degraded in vivo after 56 days implantation, was chosen as a biodegradable polymer matrix. It is necessary to maintain the efficacy of Z-100 over periods of 30 days or more, in close relation to the death of mice (approx. 20 days). Z-100 was incorporated into this matrix, using a melt-pressing technique. We describe the effect of possible prolongation of survival time by the administration of controlled Z-100 release polymer formulations on the hematopoietic function of mice irradiated with a lethal dose of γ-rays from a ^{60}Co source.

2. Materials and methods

2.1. Materials

L-Lactic acid (LA) monomer with an optical purity of 99%, as measured by enzymatic methods (Holl, 1974), was purchased from C.V. Chemie Combinatie (Amsterdam, The Netherlands). Glycolic acid (GA) was obtained from Tokyo Kasei Kogyo Co., Ltd (Japan).

Z-100, obtained by purification of a hot water extract of *M. tuberculosis* strain Aoyama B, which contains polysaccharides such as arabinomannan and mannan as major constituents, is a clear colorless or slightly yellowish liquid preparation before dilution. The liquid preparation (containing 20 mg D-arabinose basis/ml) manufactured by Zeria Pharmaceutical Co., Ltd (Japan) was lyophilized to obtain the powdered product.

2.2. Animals

9–10-week-old female C_3H/HeN mice (Charles River Japan, Inc., Japan) were used to evaluate the in vivo characteristics of biodegradable copoly(LA/GA) formulations with controlled release of Z-100 implanted subcutaneously in the back of mice after 8.5 Gy whole-body γ-irradiation. The 10 mice housed in a plastic cage were bred in an air-conditioned facility (EBAC-5, Clea Japan, Inc., Japan) kept at 24 ± 1°C with a humidity of 55 ± 5%. The animals had free accesss to a solid diet (CRF-1, Oriental Yeast Co., Ltd, Japan) and tap water. At fixed time intervals, the spleen and bone marrow cells were separately excised from animals killed under ether anesthesia.

2.3. Synthesis of biodegradable copoly(LA / GA)

The biodegradable copoly(LA/GA) (70:30 mol%) copolymer with $M_n = 2900$ was prepared by direct copolycondensation of LA and GA in the absence of a catalyst. Thus, a mixture (100 g) of 70 mol% LA and 30 mol% GA was charged into a 30 mm inner diameter glass ampoule and nitrogen gas was bubbled into the mixture at a flow rate of 200 ml/min. This ampoule was then

immersed in an oil bath maintained at 200°C for 16 h, to obtain the solid copolymer. This product, which was ground to a powder, was used without further purification.

The glass transition temperature (T_g) of the copolymer was determined with a Seiko differential scanning calorimeter (DSC), Model DSC-10, at a heating rate of 5°C/min. Copoly(LA/GA) (70:30 mol%) with $M_n = 2900$ exhibited only T_g (42°C), resulting in the formation of amorphous copolymer.

The molecular weight of copoly(LA/GA) was determined by high-performance liquid chromatography (HPLC, Waters Model ALC-2400) at 25°C at a flow rate of 1 ml/min through 10, 10^2, 10^3, 10^4, and 10^5 nm Ultrastyragel columns (Waters, U.S.A.) in tetrahydrofuran (Asano et al., 1989), as shown in Fig. 1. The number-average molecular weight (M_n), weight-average molecular weight (M_w), and molecular weight distribution of the copolymer (M_w/M_n), calibrated by the use of standard polystyrene, were found to be 2900, 8600, and 2.39, respectively.

On the other hand, the ratio of LA to GA components in the copolymer was determined by employing ^{1}H-NMR spectrometric analysis, the LA/GA ratio in the copolymer being found to be 71:29 mol%, although LA and GA were charged in 70:30 molar ratio in synthesis. Henceforth, the copolymer of 71:29 molar ratio is represented by copoly(LA/GA) (70:30 mol%).

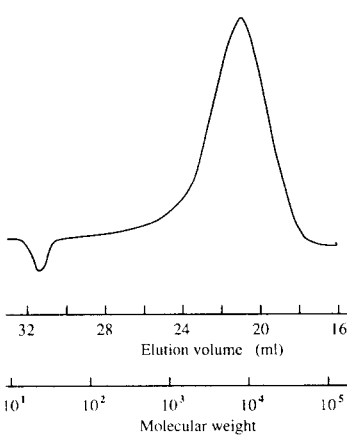

Fig. 1. HPLC curve of a copoly(LA/GA) (70:30 mol%) with $M_n = 2900$ ($M_w = 8600$, $M_w/M_n = 2.39$).

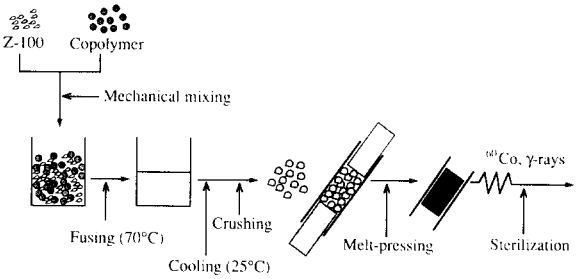

Fig. 2. Schematic diagram for the preparation of a small cylindrical formulation of copoly(LA/GA) (70:30 mol%) with controlled release of Z-100.

2.4. Preparation of small cylindrical formulations consisting of copoly(LA / GA) and Z-100

A schematic diagram for the preparation of a small cylindrical formulations consisting of copoly(LA/GA) (70:30 mol%) and Z-100 is shown in Fig. 2. The powder copolymer was mechanically mixed with Z-100. This blended powder was melted at 70°C to obtain a heterogeneous specimen, then allowed to cool to 25°C, and crushed. 50 mg of the crushed mixture, which was composed of 49.2 mg of the copolymer and 0.8 mg of Z-100, was charged into a polytetrafluoroethylene tube of 2 mm inner diameter and the piston rods from both sides of the tube were inserted under a pressure of 150 kg/cm^2 at 40°C, to obtain the melt-pressed formulation in a small cylindrical form. This tube containing a small cylindrical polymer-drug formulation was sterilized by irradiation up to 30 kGy at −78°C (dry ice temperature) with γ-rays from a ^{60}Co source.

2.5. In vivo characteristics of small cylindrical copoly(LA / GA) formulations with Z-100

9-week-old female C_3H/HeN mice were exposed to single whole-body γ-irradiation of 8.5 Gy (0.6 Gy/min). The small cylindrical copolymer formulation with controlled release of Z-100 was inserted subcutaneously in the back of mice immediately after irradiation (one sample per

mouse; 10 mice per group). At the prescribed time intervals, the implants were excised from killed mice, pooled after being freed of surrounding connective tissues, lyophilized, and weighed.

The degree of in vivo degradation of the formulation was estimated as a percentage of the original.

The in vivo cumulative amount of Z-100 released from implants was measured as follows. The formulation after the above implantation treatment was dissolved in chloroform (10 ml) and water (10 ml) was added in order to extract the water-soluble Z-100 from the chloroform solution. The amount of Z-100, which was defined as the amount of polysaccharides converted on a D-arabinose basis, was measured according to the phenol-sulfuric acid method (Hodger et al., 1962).

2.6. Endogenous colony formation unit of spleen (endogenous CFU-S)

This experiment was conducted according to the method described by March et al. (1967). Test animals were exposed to 8.5 Gy whole-body γ-irradiation. The small cylindrical copolymer formulations with and without Z-100 were inserted subcutaneously in the back of mice immediately after irradiation (one formulation per mouse; 10 mice per group). After 10 days, the spleen was removed from the treated mice and fixed in Bouin.

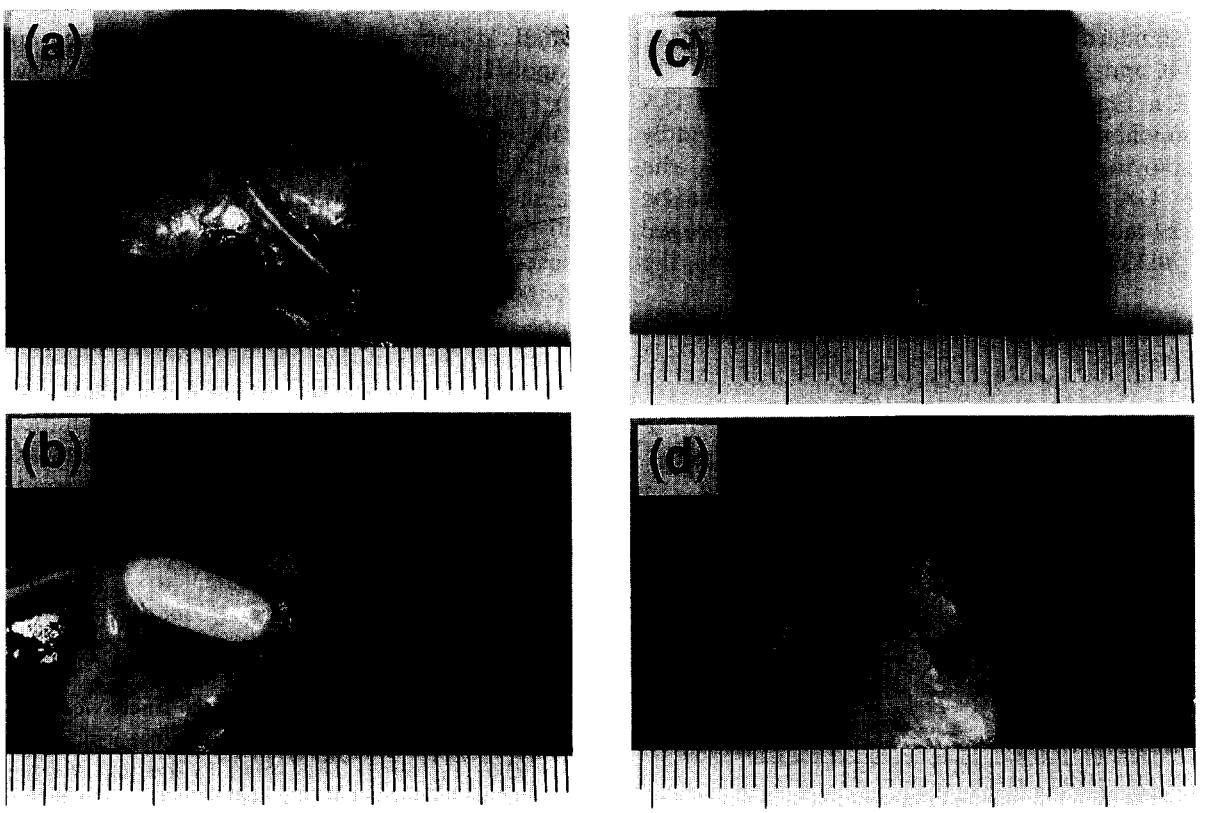

Fig. 3. Appearances of a small cylindrical copoly(LA/GA) (70:30 mol%) formulation with M_n = 2900 implanted for periods of (a) 0, (b) 14, (c) 21, and (d) 56 days.

After depigmentation with 50% ethanol, colonies formed on the surface of the capsula were counted under a stereoscopic microscope.

2.7. Colony forming unit of culture (CFU-C) in the femoral bone marrow

This procedure was carried out as described by Metcalf et al. (1975) and Chen et al. (1970). Mice were entirely irradiated with 8.5 Gy of γ-rays. After irradiation, the small cylindrical copolymer formulations with and without Z-100 were implanted subcutaneously in the back of mice (one formulation per mouse; 10 mice per group).

Marrow cells were obtained from the femoral bone using 2 ml of α-MEM medium as the washout solution. The marrow cell suspension was passed through stainless-steel mesh (200 mesh), and the filtrate was put into a petri dish. The dish was incubated for about 1 h at 37°C in CO_2 to remove adhering cells. Subsequently, a cell suspension was prepared at a concentration of 2×10^6 cells/ml with α-MEM medium containing 20% fetal calf serum (FCS). To a 0.6 ml aliquot of the suspension, 1.2 ml of FCS, 0.6 ml of GM-CSF (mouse-recombinant GM-CSF: Genzyme), 0.6 ml of 2-fold concentration of α-MEM and 3.0 ml of 0.3% agar were added and mixed well to make a cell suspension diluted to $3–4 \times 10^5$ cells/ml. This suspension was poured into five petri dishes (35 × 10 mm, w/2 mm grid tissue culture dish: LUX5217) so that each contained 2×10^5 cells. These dishes were incubated for 7 days at 37°C in 5% CO_2, and colony counts were determined under an inverted microscope by regarding a cell population of about 30 cells or more as a colony. Incubation was performed 0, 3, 5, 10, 15, 20, 25, and 30 days after irradiation to determine the number of colonies.

3. Results and discussion

3.1. In vivo characteristics of small cylindrical formulation with Z-100

The cylindrical formulation, which is composed of copoly(LA/GA) (70:30 mol%) with M_n = 2900 and Z-100, was implanted subcutaneously in the back of mice. The appearances of small cylindrical formulations during the in vivo degradation are shown in Fig. 3. The cylindrical solid formulation was deformed markedly after 14 days implantation owing to the low T_g and in vivo degradation (Fig. 3b), followed by complete disappearance of the formulation, at the 56th day from the start of implantation (Fig. 3d). The degree of in vivo degradation of this formulation is demonstrated in Fig. 4, in which it exhibits a typical S-type degradation pattern, characterized by the existence of a lag time during the initial stage.

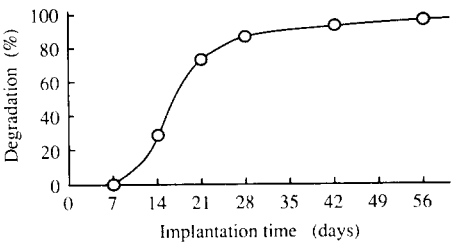

Fig. 4. In vivo degradation profile of a small cylindrical copoly(LA/GA) (70:30 mol%) formulation with M_n = 2900.

The cumulative amount of Z-100 released in vivo from a small cylindrical copoly(LA/GA) formulation was estimated from the amount of drug remaining in the formulation which was excised from killed mice at the prescribed time intervals. This is clearly shown in Fig. 5 which depicts the in vivo cumulative amount of Z-100 released from the formulation during the in vivo degradation. The drug release also showed an S-type pattern, suggesting that the initial burst release of drug

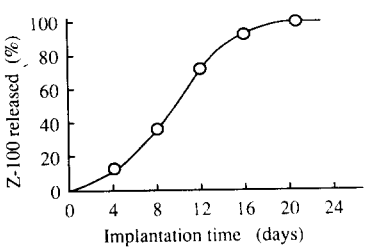

Fig. 5. In vivo cumulative amount of Z-100 released from a small cylindrical copoly(LA/GA) (70:30 mol%) formulation with M_n = 2900.

can be retarded by using the polymer matrix with a characteristic S-type degradation pattern. The resulting in vivo release of Z-100 was complete after 21 days implantation. However, the duration of release of Z-100 is much shorter than that of degradation of the copolymer formulation. The cause of this is not clear at present, but may be related to the high hydrophilicity of the drug.

3.2. Prolongation of survival duration

A biodegradable copoly(LA/GA) (70:30 mol%) formulation with controlled release of Z-100 was implanted subcutaneously in the back of mice immediately after irradiation with a lethal dose of 8.5 Gy, to evaluate the prolongation of survival time. The results are shown in Fig. 6. In the control group, about 10 days after implantation, piloerection decreased spontaneous activity and weight loss appeared. The control mice led to progressive hyposthenia and showed hemorrhaging and symptoms of infection. The major cause of death is ascribed to hematopoietic failure, as all of the control mice died within 19 days after implantation. Similar symptoms were observed in the case of mice with a Z-100 formulation, although the duration of survival was prolonged, compared with the controls, in which the mice in this group showed an improvement of symptoms and recovery of body weight onward from about 12 days after implantation. The occurrence of death was observed slightly after 9 and 12 days implantation. No deaths occurred from 19 days after implantation and 70% (7/10) of the mice survived until the completion of the study.

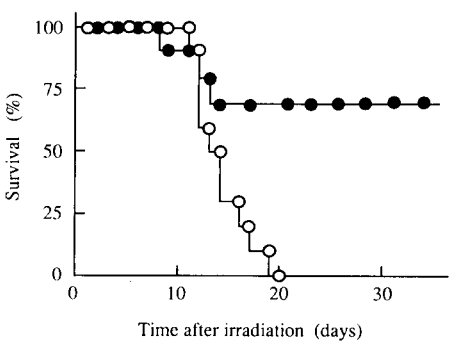

Fig. 6. The survival of mice treated (●) with and (○) without controlled Z-100 release formulation after irradiation up to 8.5 Gy.

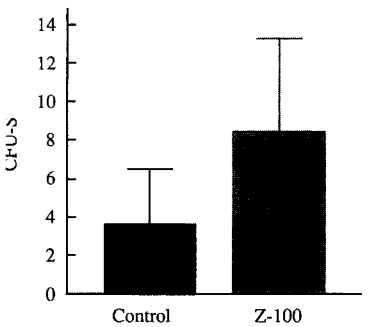

Fig. 7. Recovery effect of controlled release formulation of Z-100 on the CFU-S (endogenous) level reduced in mice after irradiation up to 8.5 Gy. The polymer formulations with and without Z-100 were implanted subcutaneously in the back of mice immediately after irradiation and the mice were then killed at the 10th day from the start of implantation.

3.3. Promotion of recovery of hematopoietic functions

The recovery effect of the controlled release formulation of Z-100 on the CFU-S level (endogenous) was reduced in mice after irradiation up to 8.5 Gy as shown in Fig. 7. On day 10, the mice were killed and the CFU-S level was examined. The colonies in the spleen of C_3H/HeN mice treated with Z-100 showed a significant increase (means $\pm$ SD, $P < 0.05$). Therefore, it is obvious that the Z-100 formulation results in the promotion of recovery of stem cells from impairment by higher irradiation dose. On the other hand, the colony stimulation of CFU-C in the femoral bone marrow treated with the controlled release formulation of Z-100 after 8.5 Gy whole-body γ-irradiation was also examined. The number of colonies was counted and the CFU-C levels are shown in Fig. 8. The CFU-C levels in the femoral bone marrow obtained from mice were significantly higher than the respective levels for the control mice, resulting in the promotion of recovery of granulocytic and macrophagic precursor cells.

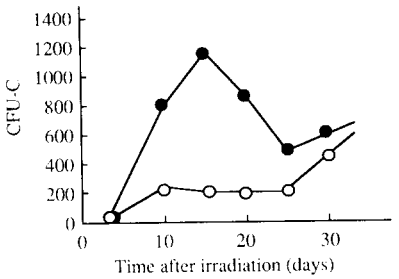

Fig. 8. Recovery effect of controlled release formulation of Z-100 on bone marrow CFU-C per femur after irradiation up to 8.5 Gy. The polymer formulations (●) with and (○) without Z-100 were implanted subcutaneously in the back of mice immediately after irradiation. At 3, 10, 15, 20, 25, and 30 days after implantation, the marrow cells were collected from the mice of each group and were cultured (2×10^5 cells/dish) with GM-CSF for 7 days.

In conclusion, Z-100 was incorporated into a biodegradable copolymer of LA/GA to give controlled drug release function. The use of this formulation prolonged the survival time in mice irradiated with a lethal dose of γ-rays and also faciliated the recovery of various hematopoietic precursor cell lines and marrow cells by promoting the reqeneration and proliferation of stem cells remaining in the bone marrow impaired by irradiation. Therefore, it can be expected that a combination of the Z-100 formulation and radiotherapy has the potential of improving the treatment of cancer in irradiation therapy.

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