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Enhancement of therapeutic efficacy of bleomycin by incorporation into biodegradable poly-d,l-lactic acid

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Abstract A new system for the delivery bleomycin (BLM) to target lesions was established by incorporating BLM into a small cylinder of a biodegradable polylactic acid (PLA) of low molecular weight. Cross-sectional analysis of the system (BLM-PLA) showed that BLM particles were uniformly enclosed in the PLA matrix. In vitro studies demonstrated that BLM was released continuously for more than 3 weeks from BLM-PLA immersed in saline. BLM-PLA was implanted subcutaneously into the backs of rats. A high concentration of BLM was maintained in the connective tissues near the implants for 2 weeks. In contrast, the level of BLM activity was low when a BLM solution (BLM-SOL) was administered subcutaneously by injection. The concentration of BLM in the abdominal lymph nodes was significantly higher following BLM-PLA implantation than following subcutaneous BLM-SOL injection. The inhibitory effects of BLM-PLA and BLM-SOL on tumor growth were compared with no treatment using a subcutaneously transplanted Yoshida sarcoma. The antitumor effect of BLM-PLA was significantly higher than that of BLM-SOL and no treatment. BLM-PLA also resulted in a more favorable distribution of BLM than BLM-SOL. Thus, BLM-PLA proved to be effective in controlling this experimentally transplanted tumor.

Key words Drug delivery system · Bleomycin · Polylactic acid · Locoregional chemotherapy

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Introduction

Water-soluble anticancer agents administered locally are rapidly absorbed into the circulation with little selective distribution. Accordingly, various systems of local or regional chemotherapy [3, 16, 17] have been developed to reduce the toxicity of these anticancer agents and to maintain a high and prolonged drug concentration in the lesion. Recent experimental and clinical studies have shown that anticancer agents adsorbed onto specific drug carriers can be effectively delivered to target lesions. Experimental studies and clinical trials [2, 9, 15] have shown certain biodegradable materials to be safe for use in living tissues.

In this study we determined the properties and efficacy in treating transplanted rat tumors of a previously described bleomycin (BLM) delivery system comprising a cylinder of polylactic acid (PLA) containing BLM [11].

Materials and methods

Preparation of drug delivery system

Poly-d,l-lactic acid (supplied by Taki Chemical Co., Kakogawa, Hyogo, Japan) is a low molecular weight polyester with an average molecular weight of 3500 Da as measured by gel-permeation chromatography using standard polystyrenes (homopoly DL-lactic acid 1500). It is synthesized by direct polycondensation of hydroxy acids in the absence of a catalyst [6]. The BLM (purchased from Nippon Kayaku Co., Tokyo, Japan) used as the anticancer agent in this study was incorporated into poly-d,l-lactic acid (BLM-PLA) by a melt-pressing technique [5] under sterile conditions. A powder containing BLM and PLA mixed at a ratio of 1:19 was inserted into a small cylinder and heated at 50 °C for 1.5 min in a water bath under a pressure of 100 kg/cm^2. The mixture was momentarily heated from the solid to the molten state, after which it was allowed to cool to 4 °C. The resulting solid mixture in the form of a small cylinder was then removed and crushed. The crushed mixture was inserted again into the same cylinder and similarly pressed at 50 °C to obtain a homogeneous mixture which on cooling to 4 °C formed a solid in the form of a small cylinder.

Electron microscopy of BLM-PLA

The cross-sectional appearance of the BLM particles, PLA and BLM-PLA was examined by scanning electron microscopy (JMS-840: Nippon Denshi Co., Tokyo, Japan). A thin gold coating was applied to the BLM-PLA prior to observation.

Release of BLM from BLM-PLA

Five BLM-PLA cylinders containing a total of 2.5 mg BLM were immersed in 10 ml of physiological saline and maintained at 37 °C for 5 weeks. On days 1, 3, 7, 14, 21, 28 and 35, 1 ml of fluid was collected for the determination of BLM concentration using a thin agar plate bioassay using *Bacillus subtilis* PCI-219. The solution of BLM (1 ml) was poured onto a round paper disc 6 mm in diameter. The paper discs were placed on thin agar plates and refrigerated for 2 h. The refrigeration allowed the drug to diffuse into the agar. The agar plates were then incubated at 37 °C for 16 h in an atmosphere containing 100% CO_2.

After incubation, the diameter of the zone of inhibition of growth formed around each disc on the agar plate was measured. BLM activity was calculated by referring to a graph that showed the relationship between the diameter of the zone of inhibition and known concentrations of BLM. The release of BLM from BLM-PLA is expressed as the mean ± SD of the release from five BLM-PLA cylinders. The lower limit of BLM activity detected by this assay was 0.05 μg/ml.

Animal experiments on the anticancer efficacy of BLM-PLA

Male Donryu strain rats (obtained from Kuroda Laboratory Animal Center, Kumamoto, Japan), were kept under a 12-h light/12-h dark schedule in a temperature-controlled room (22 °C) with free access to water and a normal pelleted diet. A rat Yoshida sarcoma cell line used as the experimental tumor was supplied frozen from the pharmaceutical research laboratories of Kyowa Hakko Kogyo Co. (Shizuoka, Japan). Once a week, the tumor was passaged as ascites cells by intraperitoneal (i.p.) transplantation into young adult rats of the Donryu strain.

A total of 84 male Donryu strain rats, 7 weeks old and weighing about 300 g, were divided into two groups: BLM-PLA group ($n = 48$) and BLM aqueous solution (BLM-SOL) group ($n = 36$). Rats in the BLM-PLA group were anesthetized with diethylether for drug administration. A cylinder of BLM-PLA (4.5 mm in diameter, 5 mm in length) containing 5 mg BLM was implanted subcutaneously via a small incision into the back of each rat. In the BLM-SOL group, 0.5 ml of BLM-SOL containing 5 mg BLM was injected at the same site into the subcutaneous tissue using a 27-gauge needle. On days 3, 7, 14, 21, 28 and 35 after drug administration, eight rats in the BLM-PLA group and six in the BLM-SOL group were sacrificed. The subcutaneous tissues near the site of drug administration as well as the lungs, liver, kidneys, abdominal lymph nodes and samples of blood were removed for measurement of BLM activity. Tissue sampled from each organ (1 g) was minced and suspended in a given amount of 7.5% trichloroacetic acid (TCA) solution. The tissue suspension was centrifuged at 10 000 rpm for 10 min. The concentration of BLM in the supernatant was determined by the method described above. To measure the serum concentration of BLM, 1 ml serum was suspended in 1 ml phosphate-buffered saline (PBS, pH 7.4).

A total of 63 male Donryu strain rats, 5 weeks old and weighing 150–180 g, were divided into three groups of 21 rats each: BLM-PLA, BLM-SOL and untreated. On day 0, all rats received a subcutaneous inoculation of 5×10^5 Yoshida sarcoma cells in 0.1 ml saline into the back. BLM-PLA and BLM-SOL were administered on day 5 after tumor inoculation when the tumor had grown to between 500 and 800 mm^3. Tumor volume was calculated from the formula: $V = A \times B^2/2$, where A and B are the longest and shortest axes, respectively, as measured with a caliper. Treatment protocols were as follows. In the first group, each rat was anesthetized with diethylether and a cylinder of BLM-PLA (4.5 mm in diameter, 2 mm in length) containing 2.5 mg BLM was implanted subcutaneously via a small incision in the skin near the tumor. In the second group, BLM-SOL containing 2.5 mg BLM was injected near the tumor using a 27-gauge needle. The last group received no treatment. Tumor volume and death rate were monitored for 60 days.

Statistical analysis

The statistical significance of differences between experimental results was tested using Student's *t*-test. A *P*-value below 0.05 was considered significant.

Results

Properties of BLM-PLA

Figure 1 shows the macroscopic appearance of BLM-free PLA and BLM-PLA (4.5 mm in diameter, 5 mm in length) containing 5 mg BLM. BLM-free PLA was transparent and BLM-PLA was opaque owing to the presence of BLM particles. A scanning electron micrograph of a section of the PLA matrix, BLM particles and BLM-PLA showed no impurities and so the PLA appeared to be homogeneous (Fig. 2a). BLM was present as irregularly shaped particles (Fig. 2b). Examination of a cross section of a BLM-PLA cylinder prepared by the melt-pressing technique showed that the BLM particles were uniformly distributed in the PLA matrix (Fig. 2c).

Release of BLM from BLM-PLA

Figure 3 shows the time course of BLM release from BLM-PLA preserved in physiological saline. The amount (μg/ml) of BLM released was 24.48 ± 6.13 after 1 day, 52.34 ± 14.83 after 3 days, 116.25 ± 33.16 after 7 days, 166.25 ± 44.19 after 14 days, 33.49 ± 8.09 after 21 days, 11.01 ± 3.10 after 28 days and 0.83 ± 0.79 after 35 days. Macroscopically, BLM-PLA preserved in saline gradually dissolved and disappeared completely

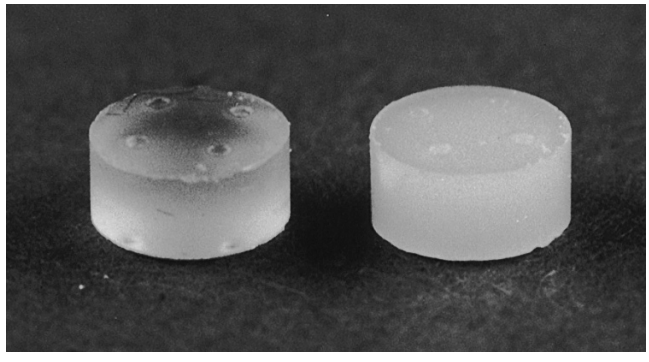

Fig. 1 Macroscopic appearance of BLM-free PLA (*left*) and BLM-PLA (*right*)

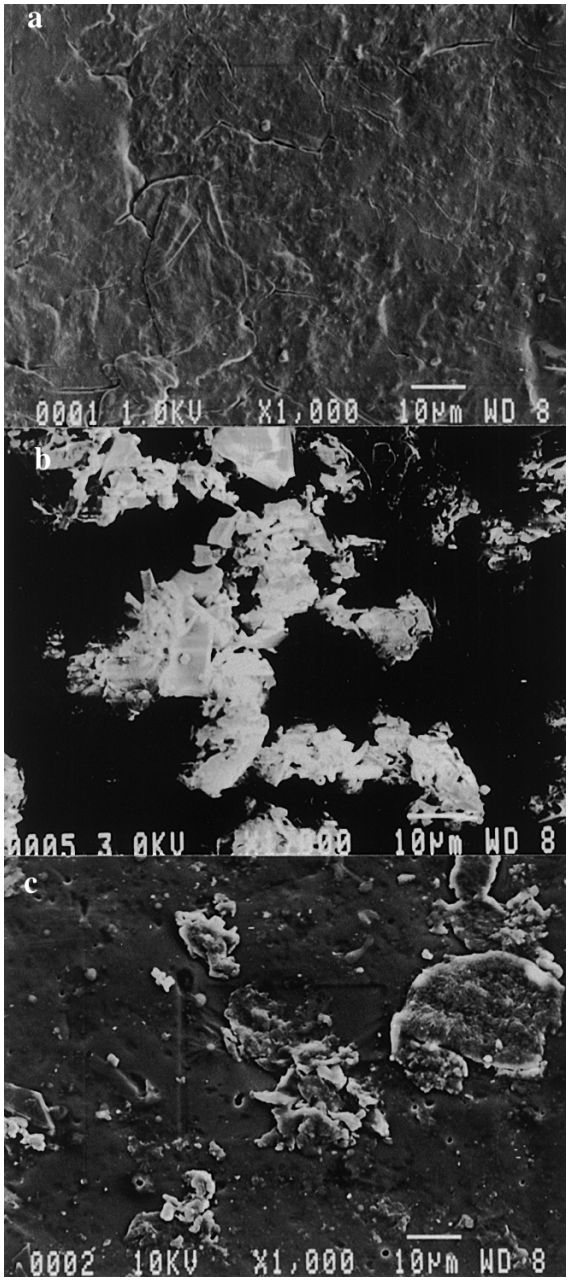

Fig. 2a–c Scanning electron photomicrographs (× 1000). **a** PLA matrix, **b** BLM particle, **c** BLM-PLA

after 35 days. Thus, BLM in BLM-PLA was released slowly over more than 3 weeks.

Drug distribution

The time course of BLM activity was compared between the subcutaneous tissue 2 cm around the site of implantation of BLM-PLA and in the tissue 2 cm around the site of injection of BLM-SOL (Fig. 4). High BLM activity was maintained in the subcutaneous tissue near the implants for at least 14 days. In contrast, the BLM-

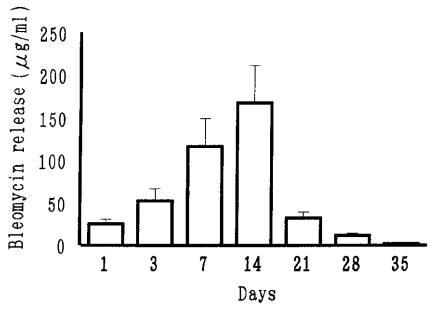

Fig. 3 The time course of BLM release from BLM-PLA in physiological saline

SOL group demonstrated no further BLM activity after 3 days. The concentration of BLM in the abdominal lymph nodes, lung, liver, kidney and blood are shown in Table 1. Significantly higher BLM activities in the abdominal lymph nodes were maintained in the BLM-PLA group between 7 and 35 days after drug administration compared with the BLM-SOL group. The distribution of BLM in other organs tended to be lower in the BLM-PLA group than in the BLM-SOL group.

Antitumor efficacy

In the BLM-PLA group, subcutaneous tumors disappeared macroscopically in 14 of 21 rats after treatment, and they survived for the entire 60 days. Tumor volumes in these rats began to decrease 4 days after drug administration, becoming undetectable by day 27. Since microscopic examination also revealed that there was no residual tumor in the subcutaneous tissue at the implantation site or in the lymph nodes, liver, lung and kidney, the effect of the drug on the tumor was judged to be a complete response. In the BLM-SOL group, a complete macroscopic and microscopic response was

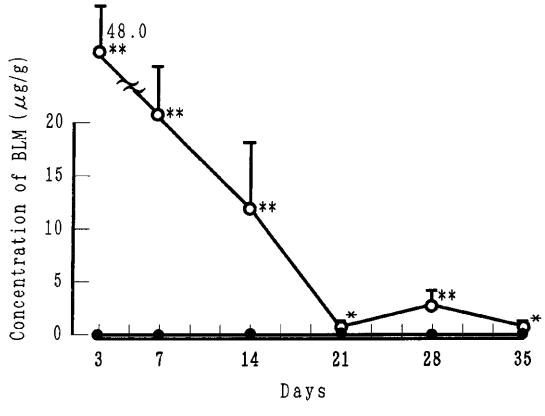

Fig. 4 The change in BLM concentration in the subcutaneous tissues near the site of drug administration (○ BLM-PLA group, $n = 8$; ● BLM-SOL group, $n = 6$). Error bars represent SD. $^{*}P < 0.05$, $^{**}P < 0.01$

Table 1 BLM concentration in the abdominal lymph nodes, lung, liver, kidney and blood. Values are mean BLM concentrations (μg/g) ± SD (BLM-PLA group, $n = 8$; BLM-SOL group, $n = 6$)

Organ	Group	Time after drug administration (days)					
		3	7	14	21	28	35
Lymph nodes	BLM-PLA	1.73 ± 0.9	1.69 ± 0.87 *	3.49 ± 1.75 **	4.07 ± 2.79 **	5.25 ± 3.72 **	1.11 ± 0.71 **
	BLM-SOL	0.92 ± 0.65	0.67 ± 0.4	0	0	0	0
Lung	BLM-PLA	1.22 ± 1.05	0.94 ± 0.79	0.80 ± 0.61	0.42 ± 0.38 **	0 **	0.53 ± 0.37 **
	BLM-SOL	2.12 ± 0.83	1.72 ± 1.25	1.31 ± 0.59	1.87 ± 0.51	2.07 ± 0.97	2.0 ± 0.9
Liver	BLM-PLA	2.39 ± 1.57 *	2.16 ± 0.78 *	0.69 ± 0.57 **	0.64 ± 0.61 **	0.7 ± 0.53 **	0.53 ± 0.36 **
	BLM-SOL	4.12 ± 0.91	3.95 ± 1.9	3.38 ± 1.78	3.74 ± 1.56	3.69 ± 1.14	3.92 ± 1.35
Kidney	BLM-PLA	2.77 ± 1.9 *	2.53 ± 0.69 *	0.79 ± 0.69 **	0.81 ± 0.73 **	0.69 ± 0.61 **	0.06 ± 0.05 **
	BLM-SOL	6.1 ± 2.69	6.03 ± 3.74	4.84 ± 2.41	4.94 ± 1.93	4.72 ± 1.02	4.36 ± 0.89
Blood	BLM-PLA	0.13 ± 0.09 **	0.1 ± 0.07 *	0.24 ± 0.18 **	0.17 ± 0.13 *	0.08 ± 0.08 **	0.11 ± 0.11 **
	BLM-SOL	0	0	0	0	0	0

$^{*}P < 0.05$, $^{**}P < 0.01$

seen in only 5 of 21 rats and these also survived for the entire 60 days. However, the remaining 16 rats died as a result of systemic tumor metastasis. In the untreated group, the tumor volume increased steadily after inoculation, and all 21 rats died within 15 days as a result of systemic tumor metastasis.

Discussion

Various drug delivery systems have been developed to increase the local chemotherapeutic effect of anticancer agents [1, 4, 13, 19]. In particular, regional chemotherapy by intracavity [12], intraarterial, intravenous [20] and intratumoral [7, 18] administration is reportedly an excellent means for improving the therapeutic index of certain anticancer agents. In most patients with advanced carcinoma, the disease has already progressed beyond curative treatment and curative surgical excision is often not feasible. We have been studying the development of a drug delivery system that can be placed at the site of residual tumor or at sites predisposed to tumor recurrence under direct intraoperative observation [14]. We report here the incorporation of the anticancer drug, BLM into a biodegradable PLA powder of low molecular weight formed into a small solid cylinder. To be efficacious, BLM must be administered frequently or continuously, rather than intermittently or via a bolus injection [8]. PLA is hydrolyzed both enzymatically and nonenzymatically at ester bonds in its main chains, finally producing water and carbon dioxide. The incorporation of anticancer agents into PLA is a simple procedure, and by scanning electron microscopy, we confirmed that BLM particles were uniformly distributed in the PLA matrix.

Our preliminary in vitro experiments showed that when BLM-PLA was preserved in saline, it released BLM for more than 3 weeks. When BLM-PLA was implanted subcutaneously into the back of rats, a high level of drug activity was maintained for 2 weeks in the connective tissues near the implants. In contrast, BLM-SOL injection resulted in a low activity of BLM. These results indicate that BLM was gradually dissolved and released from the subcutaneous BLM-PLA. High BLM activity was detected in the lymph nodes, particularly 2 to 3 weeks after the administration of BLM-PLA. The level of BLM activity was below the limit of detection 2 weeks after the administration of BLM-SOL. We conclude therefore that BLM is continuously released from BLM-PLA at the site of implantation, and that small oligomer particles containing BLM produced by degradation of the ester bonds of the main chains, are selectively drained by the lymphatic system to the regional lymph nodes. As a result of the higher level of BLM activity at both the site of implantation and the lymph nodes, the biological effect of BLM-PLA significantly exceeded that of BLM-SOL. The level of BLM was significantly lower in the lung, liver and kidney after

administration of BLM-PLA than after administration of BLM-SOL, suggesting that the local administration of BLM-PLA may avoid major systemic side effects.

The effects of BLM-PLA on a transplanted tumor in the back of rats were also studied. Yoshida sarcoma cells invade various organs to form metastatic tumors [10], particularly in regional and abdominal lymph nodes, liver and lung. The BLM-PLA-treated group had a greater number of survivors than the BLM-SOL-treated group. Tumor volume in the 60-day survivors gradually decreased in proportion to the release of BLM and eventually the tumors completely disappeared. This therapeutic effect of BLM-PLA was further supported by the tissue distribution study.

In conclusion, we demonstrated that BLM-PLA released BLM into the connective tissues near the implantation site and regional lymph nodes for long periods, and exhibited greater therapeutic efficacy than BLM-SOL. These results indicate that BLM-PLA could be a useful method for the administration of locoregional chemotherapy.

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