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The effect of combination therapy of radiation and Z-100, an arabinomannan on tumor growth in mice

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Abstract

The effect of radiation combined with intraperitoneal administration of Z-100, an immunomodulatory arabinomannan extracted from *Mycobacterium tuberculosis*, was studied using Meth A fibrosarcoma in BALB/c mice and metastasis of Lewis lung carcinoma, 3LL, in C57BL/6 mice.

In mice bearing Meth A fibrosarcoma, a moderate degree of growth inhibition was observed in the group of single therapy with Z-100 or radiation (10 Gy). When radiation was combined with Z-100, the tumor growth was significantly inhibited. In mice bearing 3LL, slight inhibition of pulmonary metastasis was observed in the group of single therapy, while significant degrees of inhibition of primary tumor growth and pulmonary metastasis were observed in the combination group. This suggests the usefulness of combined use of Z-100 in radiation therapy.

Abbreviations: 3LL: Lewis lung carcinoma; IL-3: Interleukin-3; BRM(s): Biological response modifier(s); N-CWS: Nocardia-Cell wall skeleton; CSF: Colony stimulating factor

Introduction

Nonspecific immunopotentiators have been widely used in the clinical and experimental treatments of cancers. However, therapeutic effects with immunotherapy alone have been shown to be limited. Radiotherapy as well as surgical therapy are important and commonly used local cancer therapies. However, radiotherapy is known to induce immunosuppression as a side effect and cause opportunistic infections. So, it may be desirable to combine the immunotherapy and radiotherapy for a good therapeutic performance.

Z-100 is a polysaccharide-rich preparation extracted from *Mycobacterium tuberculosis* [1]. Z-100 has been shown to have various immunopotentiating activities including enhancement of granulopoietic activity of the reticuloendothelial system [2], protective effect against leukopenia induced by X-irradiation [3], protective effect against *Pseudomonas aeruginosa* infection in immunosuppressed mice [4], and induction of cytokines such as interleukin-1 [5], interleukin-3 [6], mitogenic factor [7] and interferon γ [8]. Furthermore, Z-100 has been reported to regulate the generation of suppressor cells in tumor-bearing hosts [9, 10]. As to the antitumor activity, Z-100 has been demonstrated to show an antitumor activity *in vivo* on several syngeneic tumors in mice [6, 11].

The antitumor effect of Z-100 has been considered to be mediated by the effector mechanism of the host such as cytotoxic T lymphocytes and activated macrophages [11]. Recently, Sasaki *et al.* [12] reported the importance of tumor infil-

trating lymphocytes stimulated by IL-3 which were produced from Lyt 1 + T cells by Z-100 stimulation.

In the present study, the effects of combination therapy with Z-100 and local irradiation on tumor growth of Meth A fibrosarcoma (in BALB/c mice) and metastasis of Lewis lung carcinoma (in C57BL/6 mice) were investigated.

Materials and methods

Z-100

Z-100, supplied by Zeria Pharmaceutical Co., Ltd., Tokyo, Japan, was dissolved in saline at a concentration of 2 mg/ml just before experimental use.

Animals and tumors

Female eight-week-old BALB/c and C57BL/6 mice, purchased from Japan SLC Co., Shizuoka, Japan, were used. Meth A tumor was serially maintained in ascites form in BALB/c mice by i.p. inoculation of 1×10^6 cells/mouse. To study the antitumor activity 10^6 cells were inoculated subcutaneously. Lewis lung carcinoma (3LL) was serially maintained by s.c. inoculation in the abdomen in C57BL/6 mice. Tumor tissues of Lewis lung carcinoma were removed aseptically and minced. The tumor fragments were stirred in PBS containing 0.1% collagenase and 50 U/ml DNAse at 37°C. The cell suspension was filtered through a stainless mesh. The tumor cells were collected by centrifugation (1.000 rpm, 5 min) and suspended in PBS. Viable tumor cells were counted using a hemocytometer by trypan blue dye exclusion method. A suspension of 5×10^5 viable cells was inoculated s.c. into the footpad of C57BL/6 mice.

Combination therapy of Z-100 and radiation

Meth A tumor and 3LL were inoculated into BALB/c and C57BL/6 mice on day 0. On day 14, the local irradiation of 10 Gy (Meth A tumor) or 20 Gy (3LL) was delivered by a 70 KVp electron beam. Z-100 was administered intraperitoneally at a dose of 10 mg/kg every day for 18 days from 3 days after tumor inoculation.

In vivo *antitumor activity*

Tumor sizes were measured across the perpendicular dimeters by a microcaliper twice a week. Tumor sizes were expressed as the product of the largest diameter (a) and the smallest diameter (b) of the solid tumors, i.e., $a \times b\ mm^2$. The antitumor effects in experimental groups were evaluated on the basis of the inhibition percentage of tumor growth calculated utilizing the following formula:

$$\% \text{ of inhibition} = \left(1 - \frac{\text{mean tumor size of treated group}}{\text{mean tumor size of control group}}\right) \times 100$$

Then the mice were killed on day 24, and tumors were removed and weighed. Inhibition ratio of Z-100 was calculated using the following formula:

$$\% \text{ of inhibition} = \left(1 - \frac{\text{mean tumor weight of treated group}}{\text{mean tumor weight of control group}}\right) \times 100$$

Lung metastasis

The mice bearing 3LL were killed and the lungs removed on day 21, rinsed in PBS containing heparin (1 unit/ml), and fixed overnight in Bouin's solution. The number of metastatic foci was determined by counting the number of surface colonies.

Statistic analysis

The experimental results were analyzed for their statistical significance by student's t-test. A p value less than 0.05 was judged significant.

Results

Effect of combination therapy with Z-100 and radiation on Meth A tumor

The effect of combination of Z-100 and radiation (combination group) on the growth of Meth A tumor was investigated. Z-100 at 10 mg/kg was intraperitoneally administered 18 times from day 3 after tumor inoculation. Irradiation at 10 Gy was performed once on day 14. On day 25, tumor size was measured using a microcaliper. As shown in Fig. 1, tumor growth was inhibited

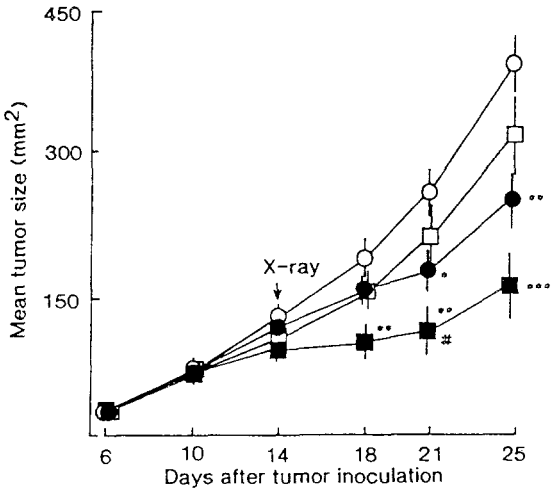

Fig. 1. Effect of combination therapy with Z-100 and irradiation on Meth A sarcoma in BALB/c mice. BALB/c mice were inoculated s.c. with Meth A tumor cells (1×10^6) on day 0. Local irradiation of 10 Gy was delivered on day 14. Z-100 was administered i.p. at a dose of 10 mg/kg 18 times on every day from day 3. ○—○ Untreated control: n = 11; □—□ Z-100 control: n = 9; ●—● Irradiated control: n = 9; ■—■ Irradiation + Z-100: n = 8; $*; p < 0.05$, $**; p < 0.01$, $***; p < 0.001$, *compared with tumor control.* $\#; p < 0.05$, *compared with X-ray irradiated group.*

Table 1. Effects of combination therapy with Z-100 and irradiation on the weight of Meth A solid tumor in BALB/c mice

Experimental group	Tumor weight(g) mean ± S.E.	Inhibition (%)	Cured mice
Untreated	2.93 ± 0.94	–	0/11
Z-100 alone	2.42 ± 0.54	17.3	0/9
Irrad. alone	1.69 ± 0.29	42.1	0/9
Z-100 + Irrad.	0.93 ± 0.34*	68.1	0/8

*$p < 0.05$, Student's *t* test.

BALB/c mice were inoculated s.c. with Meth A tumor cells (1×10^6) on day 0. Local irradiation of 10 Gy was delivered on day 14. Z-100 was administered i.p. at a dose of 10 mg/kg every day from 3 to 21 days after tumor inoculation. Tumors were weighed on day 25.

by 20% in the Z-100 group and by 37% in radiation group. When Z-100 was combined with radiation, the tumor growth was inhibited by 60%. As shown in Table 1, the inhibition of tumor weight at the time of autopsy on day 25 (11 days after irradiation) was found to be 17.3% in the Z-100 group and 42.1% in the radiation group (10 Gy). Inhibition rate in the combination group was 68.1%, which was statistically significant compared with the radiation group ($p < 0.05$).

Effect of single therapy with Z-100 on Lewis lung carcinoma

Three days after tumor inoculation (3LL) (day 3), C57BL/6 mice were treated intraperitoneally with 1 to 30 mg/kg dose of Z-100. On 28 day, tumor weight and lung metastasis were examined. As shown in Table 2, single therapy with Z-100 demonstrated no effect on primary tumor weight or incidence of lung metastasis. However, the number of pulmonary metastatic foci per animal was reduced by 28% in the group treated with Z-100 at 10 mg/kg, and by 32% in the group treated at 30 mg/kg (Table 3). Although no statistically significant differences were observed. There were no differences in lung weight between the non-treated tumor-bearing group and any of the groups treated with Z-100.

Effect of combination therapy with Z-100 and radiation on Lewis lung carcinoma

Z-100 at doses, ranging from 0.1 to 30 mg/kg, was intraperitoneally administered every day for 18 days from day 3 after tumor inoculation. Irradiation at 20 Gy was performed on the site of inoculated tumor once on day 14. Mice were killed and autopsied on day 21, and lung weight

Table 2. Antitumor effects of Z-100 on primary tumor and pulmonary metastasis of 3LL in C57BL/6 mice

Dose of Z-100 (mg/kg)	Primary tumor weight(g) mean ± S.E.	Inhibition (%)	Incidence of lung metastases (%)
Untreated	2.86 ± 0.31	–	100
1	2.83 ± 0.22	1.0	100
3	2.87 ± 0.27	0.0	100
10	2.83 ± 0.26	1.0	100
30	2.85 ± 0.32	0.3	100

C57BL/6 mice were inoculated s.c. with 3LL cells (5×10^5) into the left footpad. Z-100 was administered i.p. at a dose of 10 mg/kg daily from 3 to 21 days after tumor inoculation. Tumors were weighed on day 21.

Table 3. Effects of Z-100 on the number of metastatic foci and the weight of lung in 3LL bearing mice

Dose of Z-100 (mg/kg)	Weight of lung (g)		No. of metastasis	
	mean ± S.E.	Inhibition (%)	mean ± S.E.	Inhibition (%)
Untreated	0.71 ± 0.09	–	24.5 ± 1.6	–
1	0.70 ± 0.06	1.3	23.4 ± 2.2	4.6
3	0.65 ± 0.05	7.9	21.4 ± 3.2	12.7
10	0.56 ± 0.07	21.8	17.6 ± 1.8	28.0
30	0.54 ± 0.12	24.6	16.7 ± 2.0	32.0

C57BL/6 mice were inoculated s.c. with 3LL cells (5×10^5) into the left footpad. Z-100 was administered i.p. at a dose of 10 mg/kg daily from 3 to 21 days after tumor inoculation. The lung of C57BL/6 mice was removed and weighed on day 21. The metastatic foci of lung was counted on day 21, as described in text.

and numbers of metastatic foci per animal measured. As shown in Table 4, lung weight was reduced by 24.3% in the group treated with radiation alone (radiation group), as compared with the non-treated tumor-bearing group. When Z-100 at 10 mg/kg was combined with radiation, lung weight was reduced at 51.4% compared to untreated group, showing significant synergy. However, there were no statistically significant differences between the radiation group and the groups combined with Z-100 at other doses.

The number of pulmonary metastatic foci was reduced by 15% in the radiation group as compared with the non-treated tumor-bearing group, and the numbers of foci were reduced in the combination groups by 32.6% at 1 mg/kg, by 60.7% at 10 mg/kg ($p < 0.001$ compared to the non-treated tumor-bearing group and $p < 0.01$ compared to the radiation group) and by 49.9% at 30 mg/kg ($p < 0.01$ compared to the non-treated tumor-bearing group and $p < 0.05$ compared to the radiation group).

The inhibitory effect of combination therapy on the growth of primary tumor is shown in Fig. 2. The growth of primary tumor was not inhibited by single therapy with Z-100 or radiation alone. On the other hand, when Z-100 was combined with radiation, the growth of primary tumor was significantly inhibited by 38% as compared with the nontreated tumor-bearing group ($p < 0.05$). However, there was no significant difference in growth inhibition between the combination group and the radiation group.

Life prolongation effect of combination therapy with Z-100 and radiation on Lewis lung carcinoma

The life prolongation effect of combination therapy with Z-100 and radiation on 3LL-bearing mice was investigated under the experimental condition that provided significant inhibition on lung metastasis. As shown in Table 5, all of the mice in three groups including the radiation, the Z-100 and the combination group died by lung metastasis within 30 days.

Table 4. Effect of combination therapy with Z-100 and irradiation on pulmonary metastasis in 3LL bearing mice

Experimental group	Weight of lung (g)		No. of pulmonary metastases	
	mean ± S.E.	Inhibition %	mean ± S.E.	Inhibition %
Untreated	0.70 ± 0.15	–	34.1 ± 4.4	–
Irrad. alone	0.53 ± 0.06	24.3	29.0 ± 3.9	15.0
Irrad. + Z-100				
0.1 mg/kg	0.51 ± 0.07	27.1	27.7 ± 4.2	18.8
1 mg/kg	0.53 ± 0.10	24.3	23.0 ± 4.1	32.6
10 mg/kg	0.34 ± 0.10	51.4	13.4 ± 1.7	60.7
30 mg/kg	0.50 ± 0.14	28.6	17.1 ± 3.6	49.9

$*p < 0.05$, $**p < 0.01$, @$p < 0.001$, Student's t test.

C57BL/6 mice were inoculated s.c. with 3LL cells (5×10^5) into the left footpad. Local irradiation of 20 Gy was delivered on day 14. Z-100 was administered i.p. at a dose of 10 mg/kg every day for from 3 to 21 days after tumor inoculation. The weight of lung and the number of pulmonary metastases were counted on day 21.

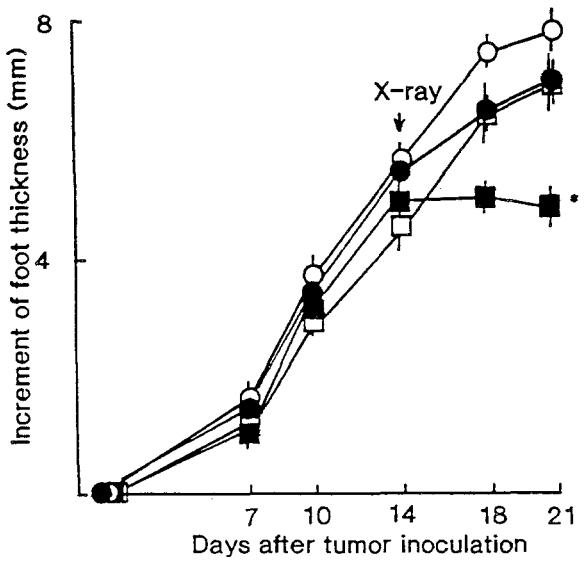

Fig. 2. Effect of combination therapy with Z-100 and irradiation on the growth of Lewis lung carcinoma in mice. C57BL/6 mice were inoculated s.c. with 3LL cells (5×10^5) into the left footpad. Local irradiation of 20 Gy was delivered on day 14. Z-100 was administered i.p. at a dose of 10 mg/kg every day for 18 treatment 3 days after tumor inoculation. ○—○ Untreated control: n = 10; □—□ Z-100 control: n = 10; ●—● Irradiated control: n = 10; ■—■ Irradiation + Z-100: n = 10

*; $p < 0.05$, *compared with tumor control.*

Table 5. Effect of combination therapy with Z-100 and irradiation on the survival time of 3LL-bearing mice

Experimental group	Mean survival time (days)	Cured mice
Untreated	26.3 ± 1.32 (24 ~ 30)	0/8
Z-100 alone	27.6 ± 1.65 (24 ~ 30)	0/8
Irrad. alone	29.1 ± 2.15 (24 ~ 33)	0/8
Z-100 + Irrad.	28.5 ± 2.88 (24 ~ 37).	0/8

Local irradiation of 20 Gy was delivered on day 14. Z-100 was administered i.p. at a dose of 10 mg/kg every day from 3 to 21 days after tumor inoculation. The survival was monitored for 40 days.

Discussion

It has been reported that local irradiation of tumor foci enhances the activity of tumor specific immune killer cells in mice and rats [13, 14]. One of possible mechanisms of this effect is that the tumor specific immune response is enhanced through an increase in antigenicity of tumor cells exposed to irradiation. Studies on the combination of irradiation and biological response modifier (BRM) have been performed in an attempt to induce enhanced direct inhibitory effects on the growth of tumors through augmented infiltration of cytotoxic lymphocytes into tumor tissues and to reduce the immunosuppression caused by radiation [15, 16]. The results have varied according to (1) the difference in irradiation conditions, and (2) the difference in pharmacological properties of BRM used and (3) the difference in sensitivities of tumors to irradiation or immune attack. In the presence study, we showed the usefulness of combination of Z-100 and radiation in murine model systems with nonmetastatic Meth-A tumor and metastatic Lewis lung carcinoma (3LL).

At present, primary cancer lesions can be controlled by several methods, but such improved therapeutic managements do not always promise life prolongation because of recurrence and/or metastasis from remaining small lesions. Thus we first irradiated a primary lesion of 3LL and then evaluated the effect of Z-100 on metastasis and life prolongation. It was revealed that not only the growth of primary lesions, but also the metastatic pulmonary lesions in number were significantly reduced in the combination group, compared to those in the group receiving radiation therapy alone. Despite such favorable effects of combined therapy, the duration of survivals in 3LL-bearing mice was not prolonged. Inhibiting effect on metastatic foci may be temporary even in the combination group.

Inhibitory effects of BRM on metastasis, including N-CWS [17], *C. parvum* [18] and sizofiran [19], have been studied using 3LL, their effects has been found to depend largely upon administration methods. When the administration is not timed BRMs exert less effect, but also facilitate the growth of primary lesion and metastasis and recurrence. It has been reported that Z-100 induces cytokines such as CSF and IL-3 [6, 21], and facilitates hematopoiesis, leading to the amelioration of decreases in peritoneal cell infiltration and peripheral leukocytes caused by irradiation [3]. Also IL-3 and CSF have been reported to activate cytotoxic macrophages [22]. Sone et al. (20) postulated that activation of alveolar macrophages is important in the inhibition of metastasis of 3LL to the lung. So we

assume that alveolar macrophages are activated by cytokines in response to the stimulation with Z-100, giving rise to the significant inhibition on 3LL observed in this study.

These results suggest that the inhibitory effect of irradiation on tumor growth is enhanced by Z-100, even if irradiation fails to induce complete tumor regression, and that the combination of Z-100 and irradiation of primary lesion is effective for the control of the growth of distinct metastatics lesions.

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