Z-100, an Immunomodulatory Extract of Mycobacterium tuberculosis Strain Aoyama B, Prevents Spontaneous Lymphatic Metastasis of B16-BL6 Melanoma

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Lymphatic metastasis is common in advanced-stage carcinoma and is associated with a poor prognosis. However, few effective treatments to inhibit it are available. Z-100 is an immunomodulatory extract of Mycobacterium tuberculosis strain Aoyama B that contains polysaccharides such as arabinomannan and mannan. Here, we investigated the inhibitory effect of Z-100 on spontaneous lymphatic metastasis. C57BL/6N mice injected subcutaneously with B16-BL6 melanoma cells in the right hind footpad were administered Z-100 subcutaneously in the right inguinal region on a daily basis. On day twenty-one after the injection, the right inguinal lymph nodes were excised, and the extent of metastasis, the number of immune cells, and the amount of granzyme B protein in the lymph nodes were examined. We also investigated the combined effect of Z-100 and irradiation in this model. Results showed that Z-100 reduced the number of animals with metastasis, with respective metastasis rates of 85.7%, 42.9%, 7.1% and 0.0% in saline, 0.1 mg/kg Z-100, 1 mg/kg Z-100 and 10 mg/kg Z-100 group. Further, mice that had been given Z-100 were found to have more immune cells and granzyme B protein in the lymph nodes than control mice. The combination of low dose Z-100 and irradiation also inhibited spontaneous lymph node metastases. These findings suggest that Z-100 may be beneficial in preventing lymphatic metastasis by enhancing the immune response.

Key words lymph node metastasis; B16-BL6 melanoma cell; non-specific immunotherapy; radiation

Metastasis is the main cause of death in advanced-stage cancer patients and occurs through three major routes: hematogenous, lymphogenous, and transcoelomic spread. Lymphatic metastasis in particular is common in advanced-stage carcinoma and is generally associated with a poor prognosis. Although the influence of lymphatic metastasis on prognosis is thoroughly understood, effective treatments to inhibit it remain lacking.1,4)

Lymph node dissection is frequently performed to treat or prevent (or both) lymph node metastasis in several types of cancer. These procedures are limited to early-stage cancer patients, however, and their efficacy has been questioned.5,6) Chemotherapy and lymphangiogenesis inhibitors are administered in lieu of surgery to inhibit lymphatic metastasis,7,9) but these drugs have limited efficacy due to their toxicity and poor bioavailability. Specifically, conventional chemotherapy cannot be delivered to the lymphatic system effectively without dose-limiting toxicity, requiring drug delivery systems such as a liposome-based delivery system. 9,10) Therefore, novel therapeutics are needed for the use in treating cancer patients with lymphatic metastasis.

Immunotherapeutic approaches have been shown to inhibit lymphatic metastasis in animal models, indicating the potential of immunotherapy as treatment for lymphatic metastasis in humans.11–13) Z-100 is a hot-water extract of Mycobacterium tuberculosis strain Aoyama B and contains polysaccharides such as arabinomannan and mannan.14) Z-100 has been shown to enhance hematopoiesis by activating colony-stimulating factors and is marketed under the brand name “Ancer® S.C.”

Injection 20 μg” for irradiation-induced leukopenia. Z-100 has immunomodulatory effects and anti-tumor effects in experimental tumor models.15–17) In addition, a previous study reported that the induction of interleukin (IL)-12 and interferon (IFN)-γ by Z-100 led to a shift in CD4+ T cell activity from a type 2-dominant state to type 1-dominant, which was effective in inhibiting hematogenous metastasis in a mouse melanoma model.15,17) However, it has not been confirmed whether Z-100 is effective on lymphatic metastasis and the contribution of CD8+ T cells, Natural Killer (NK) cells and Natural Killer T (NKT) cells by Z-100 is also unclear.

The B16 melanoma lymphatic metastasis model is widely used to verify the effect of drugs on lymphatic metastasis.18–20) In particular, the B16-BL6 melanoma subline was selected based on its invasive ability through the mouse bladder membrane, and transplantation into the footpad results in a high incidence of spontaneous metastases.21,22) Here, to evaluate the effect of Z-100 on lymphatic metastasis, we investigated the effect of Z-100 on lymph node metastasis using a mouse model of B16-BL6 melanoma metastasis. Moreover, in order to clarify the inhibition of lymph node metastasis by Z-100, we investigated the involvement of immune cells in inguinal lymph node. In addition, we investigated the combined effect of Z-100 and irradiation on lymphatic metastasis in this model.

MATERIALS AND METHODS

Cell Line and Animals B16-BL6 melanoma cells obtained from Tohoku University (Sendai, Japan) were cultured in RPMI Medium 1640 (Life Technologies Japan, Tokyo, Japan).
Japan) supplemented with 10% fetal bovine serum (HyClone Laboratories, UT, U.S.A.), 100 U/mL of penicillin (Meiji Seika Pharma, Tokyo, Japan), and 100 µg/mL streptomycin (Meiji Seika Pharma), and kept at 37°C in 5% CO₂/95% air. Male C57BL/6N strain mice purchased from Charles River Laboratories Japan (Yokohama, Japan) were housed in pathogen-free conditions and treated in accordance with the guidelines of the Animal Ethics Committee of Zeria Pharmaceutical Co., Ltd. And this study was performed according to Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006).

**Z-100** Z-100 (2.2 mg/mL), developed by Zeria Pharmaceutical Co., Ltd., Japan, was diluted to 0.002, 0.02, 0.2 or 2 mg/mL in physiological saline for single daily subcutaneous administration in the right inguinal region. Physiological saline was injected subcutaneously into control mice under the same schedule. The volume of Z-100 and physiological saline was 5 mL/kg.

**Tumor Model** B16-BL6 melanoma cells (1.5×10⁶/mL) were suspended in Dulbecco's phosphate-buffered saline (DPBS, Life Technologies Japan), and 20 µL of the cell suspension was injected subcutaneously into the right hind footpad of 8-week-old male C57BL/6N strain mice using a Hamilton syringe with a 27 gauge needle (3×10² cells/mouse). On day twenty-one after the injection, the right inguinal lymph nodes were excised, and the presence of metastases was assessed *via* stereomicroscopy under blinded conditions.

**Cell Viability Assay** Cell viability was determined using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) that uses WST-8 for colorimetric detection of viable cells. Briefly, B16-BL6 melanoma cells (7.5×10⁵ cells/well) were seeded into 96-well culture plates and cultured for 24 h. Physiological saline, Z-100 (final concentration 100 µg/mL), or cisplatin (final concentration 1 µg/mL in physiological saline; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was then added to each well. After 48 h of incubation, the cells were reected with 10 µL of Cell Counting Kit-8 reagent for 3 h, and absorbance was measured at 450 nm with reference wavelength of 600 nm using a microplate reader (Sunrise Remote, Tecan Austria GmbH, Grödig, Austria). Each value is the mean of triplicate wells, and results were obtained by subtracting the absorbance of the blank. The data represent the mean of six experiments. Cell viability (%) was calculated by dividing each absorbance of the treated group by the mean absorbance of the saline group.

**Flow Cytometry** Cell suspensions were prepared from four right inguinal lymph nodes per mice. Cells were stained with fluorochrome-labeled mouse antibodies and analyzed using a MACSQuant Analyzer (Miltenyi Biotec, Cologne, Germany). The following monoclonal antibodies were used: anti-CD4-FITC (Miltenyi Biotec), anti-mouse CD8a PE-Cy7 (eBioscience, San Diego, CA, U.S.A.), CD49b-PE (Miltenyi Biotec), and APC/Cy7-conjugated, anti-mouse TCRβ chain (BioLegend, San Diego, U.S.A.). CD4-positive, TCRβ-positive cells were identified as CD4+ T cells, while CD8-positive, TCRβ-positive cells were identified as CD8+ T cells. CD49b-positive, TCRβ-negative cells were identified as natural killer (NK) cells, while CD49b-positive, TCRβ-positive cells were identified as NKT cells. A total of 20,000 events per sample were recorded, and dead cells identified by propidium iodide (Miltenyi Biotec) staining were excluded from analysis. Enumeration of immune cells was expressed as the absolute cell number per lymph node. The number of each cell type was calculated by multiplying the percentage of positive cells by the total number of cells in the lymph node.

**Enzyme-Linked Immunosorbent Assay (ELISA)** The amount of granzyme B protein in the excised lymph nodes was assessed by ELISA (Usen Life Science, Wuhan, China) in accordance with the manufacturer’s instructions, with samples tested in duplicate. The microtiter plate provided in this kit was pre-coated with a biotin-conjugated antibody specific for granzyme B. Briefly, tissue lysates prepared from three right inguinal lymph nodes were diluted and added to the microtiter plate wells. Avidin-conjugated horseradish peroxidase was then added to each well, and the microplate was incubated. 3,3′,5,5′-Tetramethylbenzidine substrate solution was then added and the microplate was incubated for a further 15 min. Lastly, sulphuric acid solution was added to each well, and absorbance was measured using a microplate reader at 450 nm. The concentration of granzyme B (pg/mL) in lysates was determined by comparing the absorbance of the standard curve. The amount of granzyme B was calculated using the following formula:

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\text{granzyme B (pg/lymph node)} = \frac{\text{concentration of granzyme B (pg/mL)}}{\text{lysat volume (0.3 mL)}} \times \text{pooled numbers of lymph nodes (} n = 3 \text{)}
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**Radiation Therapy** Non-anesthetized, tumor-bearing mice were irradiated using a soft X-ray unit (M-150WE; Softex, Tokyo, Japan) with a 10-mm acrylic filter. Mice were placed in an acrylic holder in the prone position, and only the primary tumor was exposed to radiation, with the holder shielded with a lead sheet. Animals received a single daily dose of 5 Gy at 4, 7 and 11 d after tumor injection. Twenty-one days after tumor injection, the size of the primary tumor was assessed by measuring the thickness of the right hind paw using a thickness gauge (Peacock, Tokyo, Japan).

**Statistical Analysis** All data values are expressed as mean±standard error (S.E.), except for the assessment of lymph node metastases. Bartlett’s test or the F-test was used to analyze data homogeneity. Homogenous data were analyzed using Student’s *t*-test while non-homogeneous data were analyzed using the Aspin–Welch *t*-test. Dunnett’s test was used to perform multiple comparisons of parametric data. Fisher’s exact test was used to assess categorical data. The *p* values less than 0.05 were considered statistically significant.

**RESULTS**

**Z-100 Inhibited Lymph Node Metastases in Mice Injected with B16-BL6 Cells** We first examined the effect of Z-100 on spontaneous lymph node metastasis of B16-BL6 melanoma cells in mice. The presence of inguinal lymph node metastases was confirmed after injection of metastasis-prone B16-BL6 cells into the footpad of C57BL/6N mice (Fig. 1). Z-100 was given to mice injected with 3×10⁵ B16-BL6 cells in the right hind footpad, and the right inguinal lymph nodes were excised and examined using stereomicroscopy 21 d after the injection. As shown in Fig. 2, Z-100 significantly reduced
the number of animals with metastasis in a dose-dependent manner, with respective metastasis rates of 85.7%, 42.9%, 7.1%, and 0.0% in the saline, 0.1 mg/kg Z-100, 1 mg/kg Z-100, and 10 mg/kg Z-100 groups. To evaluate the direct cytotoxic effect of Z-100, we examined the influence on viability of cultured B16-BL6 melanoma cells by the exposure of Z-100. Results showed that Z-100 had no effect on the cell viability (92.7%) of B16-BL6 melanoma cells, in contrast to cisplatin (1 µg/mL), which was associated with a viability of 70.1% (Fig. 3). These results suggest that Z-100 inhibits lymph node metastasis without exerting a direct cytotoxic effect.

**Z-100 Increased the Number of Immune Cells in the Lymph Nodes**

The right inguinal lymph nodes were examined by flow cytometry to investigate the role of immune cells in the inhibition of lymph node metastasis by Z-100. Lymph node cell suspensions were prepared from four excised lymph nodes as described above. Results showed an approximate five-fold increase in the number of CD4+ T, CD8+ T, NK and NKT cells in the Z-100 group compared with the saline group (Figs. 4A–D). Thus, Z-100 may inhibit lymph node metastasis by causing a non-specific increase in the number of immune cells in the lymph nodes.

**Z-100 Increased the Amount of Granzyme B in the Lymph Nodes**

Granzyme B induces cell apoptosis and is expressed by cytotoxic T lymphocytes, NK and NKT cells. To examine the potential role of granzyme B in the inhibition of lymph node metastasis by Z-100, we examined the levels of granzyme B in the right inguinal lymph nodes from Z-100-treated mice using ELISA. As shown in Fig. 5, the level of granzyme B was significantly higher in the Z-100 group (215.6 ± 42.8 pg/lymph node) than the saline group (23.1 ± 3.4 pg/lymph node), suggesting that Z-100 increases the cytotoxic activity of immune cells in the lymph nodes.

**Combined Low-Dose Z-100 and Irradiation Inhibited Lymph Node Metastasis**

We examined the effect of Z-100 combined with irradiation on spontaneous lymph node metastasis in mice injected with B16-BL6 melanoma cells. The right hind paw of mice was irradiated 4, 7 and 11 d after injection. On the first day of irradiation, either physiological saline or Z-100 (0.01, 0.1, 1 mg/kg) was administered once daily for 17 d, and the thickness of the right hind paw was measured 21 d after injection. In addition, the right inguinal lymph nodes were excised and examined using stereomicroscopy. The metastasis rates of the saline group, 0.01 mg/kg Z-100 group, irradiation with saline group, irradiation with 0.01 mg/kg Z-100 group, and irradiation with 1 mg/kg Z-100 group were 83.3%, 83.3%, 72.2%, 44.4%, 27.8%, and 5.6%, respectively. The metastasis rates of the irradiation with Z-100 groups (0.01, 0.1, 1 mg/kg) were significantly lower than that of the saline group (Fig. 6A). Measured right hind paw thickness of the saline group,

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Fig. 1. Lymphatic Metastasis of B16-BL6 Cells in Mice after Injection of B16-BL6 Cells into the Footpad

Photographs of inguinal lymph node with metastases (A), and a normal inguinal lymph node (B).

Fig. 2. Z-100 Inhibited Lymph Node Metastases in Mice Injected with B16-BL6 Cells

B16-BL6 cells (3×10⁵) were injected into the right hind footpad of C57BL/6N mice (n=14) and were given either physiological saline or Z-100 (0.1, 1, 10 mg/kg) subcutaneously in the right inguinal region once daily for 21 d. Afterwards, the right inguinal lymph nodes were excised, and the presence of metastases was assessed via stereomicroscopy under blinded conditions. Significant differences between saline and Z-100-treated groups: *p<0.05, ***p<0.001 (Fisher’s exact test).

Fig. 3. Effect of Z-100 on the Viability of Cultured B16-BL6 Cells

B16-BL6 cells (7.5×10² cells/100 µL/well) were seeded into 96-well culture plates and cultured for 24 h, followed by the addition of physiological saline, Z-100 (final concentration 100 µg/mL) or cisplatin (final concentration 1 µg/mL) to each well. After 48 h of incubation, the cells were reacted with Cell Counting Kit-8 reagent for 3 h and absorbance was measured at 450 nm (reference wavelength 600 nm) using a microplate reader. Values were obtained by subtracting the absorbance of the blank. Cell viability (%) was calculated by dividing each absorbance of the treated group by the mean absorbance of the saline group. The data shown represent the mean±S.E. of experiments using six culture flasks. Significant difference between saline and cisplatin-treated groups: ***p<0.001 (Student’s t-test).

Fig. 4. Effect of Z-100 on the Number of Immune Cells in the Lymph Nodes

Photographs of inguinal lymph node with metastases (A), and a normal inguinal lymph node (B).

Fig. 5. Effect of Z-100 on the Amount of Granzyme B in the Lymph Nodes

Photographs of inguinal lymph node with metastases (A), and a normal inguinal lymph node (B).

Fig. 6. Combined Low-Dose Z-100 and Irradiation Inhibited Lymph Node Metastasis

Photographs of inguinal lymph node with metastases (A), and a normal inguinal lymph node (B).
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0.01 mg/kg Z-100 group, irradiation with saline group, irradiation with 0.01 mg/kg Z-100 group, and irradiation with 1 mg/kg Z-100 group were 2.6±0.2 mm, 2.7±0.2 mm, 1.8±0.0 mm, 1.8±0.0 mm, 1.7±0.0 mm, and 1.8±0.0 mm, respectively (Fig. 6B). Therefore, irradiation combined with Z-100 at 0.01 mg/kg significantly reduced the occurrence of lymph node metastasis but Z-100 at 0.01 mg/kg or irradiation alone did not significantly inhibit on the metastasis. However, irradiation was successful in inhibiting the growth of the primary tumor, and the addition of Z-100 did not interfere with its inhibitory effect on primary tumor growth.

DISCUSSION

Lymphatic metastasis is common in advanced-stage carcinomas, and although it indicates a poor prognosis, few effective means of inhibiting this metastasis are available. In this study, we showed that Z-100 prevented lymph node metastasis via a nonspecific increase in the number of immune cells and granzyme B in lymph nodes. Notably, administration of Z-100 resulted in an increase in the number of CD4+ T, CD8+ T, NK, and NKT cells, which are believed to contribute to the anti-tumor effect in the lymph nodes. Furthermore, Z-100 did not show the cytotoxic effect against B16 melanoma cells in vitro, suggesting that inhibition of lymph node metastasis by Z-100 mediate immune response. In addition, Z-100 in combination with irradiation was successful in reducing lymph node metastasis. These findings suggest that Z-100 may represent a novel therapy for the treatment of lymph node metastasis.

Effector cells, such as CD4+ T, CD8+ T, NK and NKT cells, are considered to exert anti-cancer effects. Selective depletion of NK cells by monoclonal antibody treatment exacerbated lymph node metastasis of B16 melanoma cells in...
A clinicopathological study reported that oral cavity squamous cell carcinoma samples without lymph node metastasis had a significantly higher number of peritumoral granzyme B-positive cells than those with lymph node metastasis. Moreover, patients with a high density of peritumoral granzyme B-positive cells had a longer survival time than those with a lower density of these cells. Granzyme B is produced by CD8+ T, NK and NKT cells, and induces apoptosis in tumor cells. The increase in granzyme B in the lymph nodes by Z-100 in the present study suggests that Z-100 may induce apoptosis of tumor cells in lymph nodes by enhancing granzyme B production.

In the present study, Z-100 inhibited lymph node metastasis without affecting anti-tumor effect of irradiation to primary tumor. In addition, low-dose Z-100 combined with irradiation was superior to either Z-100 or irradiation alone. Although the immunological effects of radiation are not fully understood, recent studies have reported the benefits of combined radiation and immunotherapy. We believe that radiation and Z-100 may be effective in inhibiting the primary tumor and metastatic lesions, respectively.

A previous study has been reported that the main polysaccharide of Z-100 was mannan, glucan, arabino-mannan, and lipomannan. In general, these polysaccharides are known to induce various immune responses. For example, a few kinds of lipomannan and lipoarabinomannan are known as ligands for Toll-like receptor 2, and induce activation of the innate immune system including IL-12 production. In addition, the polysaccharides including the core structure of mannan have been shown to bind mannose receptors and to regulate macrophages and dendritic cells. Furthermore, it has been reported that mannose receptors on lymphatic endothelial cells involve in leukocyte trafficking and contribute to the metastasis of cancer. These immune responses of polysaccharides may cooperatively contribute to the inhibition of lymph node metastasis by Z-100.

In conclusion, our findings suggested that Z-100 prevents lymph node metastasis by immune cell responses, expecting as the novel therapeutics for lymphatic metastasis.

REFERENCES


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