Antimetastatic Effect of Yeast Mannan–Bleomycin Conjugate against Mouse Lewis Lung Carcinoma

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A conjugate of bleomycin (BLM) and the mannan of bakers' yeast (*Saccharomyces cerevisiae* wild type strain) (WNM) was synthesized. The assay of its antimetastatic effect on Lewis lung carcinoma (3LL) implanted in C57BL/6 mice showed that this conjugate exhibited a higher antimetastatic effect and longer life-span elongation than those of free bleomycin and mannan with corresponding doses. This conjugate was also found to kill the 3LL cells *in vitro*. ¹⁴C-Labeled mannan–bleomycin conjugate was much more bound than ¹⁴C-labeled dextran–bleomycin conjugate to the 3LL. It was concluded that the anti-cancer mechanism of this conjugate, WNM–BLM possessed a specific binding effect to the tumor cells and exhibited a cytotoxic effect on the 3LL target cells.

Keywords metastasis; mannan; bleomycin; pro-drug; Lewis lung carcinoma; *Saccharomyces cerevisiae*

Much of the recent work in clinical cancer chemotherapy has been aimed at developing methods to enhance the effects and to decrease the side effects of antitumor agents. These side effects of antitumor agents are due to the low selectivity of their cytotoxic effect between tumor and normal cells. Attempts to bind anticancer agents to monoclonal antibodies of tumor cells have been tested by many researchers.¹⁾ Because of the heterogeneity of tumor cells, however, these conjugates of antitumor agents and antibodies were scarcely able to contribute to the suppression of tumors against expectation. Theoretically, the selective cytotoxicity of anticancer agents should be enhanced by conjugation to substances which possess the ability to bind to tumor cells, tissues and organs. Reports dealing with the synthesis of a dextran-mitomycin C (MMC) conjugate published by Sezaki *et al.* are of interest because this conjugate shows an appropriate autodegradation rate with low side effects.^{2,3)} The fact that reports dealing with the presence of a mannose/*N*-acetylglucosamine receptor on the liver cells^{4,5)} indicates the adequacy of the use of these sugars as ligand molecules.

In a previous study, we synthesized the bakers' yeast mannan–mitomycin C conjugate (WNM–MMC). The WNM–MMC conjugate was found to affect antitumor efficacy on mouse transplantable tumors.⁶⁾ This conjugate was able to kill the tumor cells in a high ratio, and it is likely that the introduction of the mannose residue to anticancer agents might lead to development of highly specific anti-hepatoma agents. Previously, we reported the distribution of WNM in mice by intravenous administration,⁷⁾ and found a high affinity of WNM to lung, liver, kidney and ileum.

In the present study, in order to obtain an anti-lung cancer agent, we tried to synthesize mannan–bleomycin (WNM–BLM) conjugate, and have described the antimetastatic activity of this conjugate to mouse Lewis lung carcinoma (3LL) *in vivo* and *in vitro*.

MATERIALS AND METHODS

Materials Bleomycin (BLM) was kindly provided by Nippon Kayaku Co., Ltd., Tokyo, Japan. The bakers' yeast mannan used in this study was the same specimen as that of the neutral fraction designated as WNM (neutral fraction of a *Saccharomyces cerevisiae* wild type strain) in a previous study.^{8,9)} Dextran T40 (Dex) was purchased from Pharmacia Fine Chemicals, Upsala, Sweden.

Preparation of WNM–BLM and Dex–BLM Preparation of WNM–BLM and Dex–BLM involved the same method used in our previous report⁶⁾ in accordance with the description by Kojima *et al.*²⁾ The content of BLM in WNM–BLM and Dex–BLM is 6.0 and 5.5%, respectively.

Preparation of ¹⁴C-Labeled WNM–BLM and Dex–BLM This was the same method used for the preparation of mannan–mitomycin C conjugate, reported previously.⁶⁾ For the synthesis of ¹⁴C-labeled WNM–BLM and Dex–BLM, ¹⁴C-aminobutyric acid was introduced to WNM as a spacer.²⁾ The radioactivity of ¹⁴C-labeled WNM–BLM and Dex–BLM was 1.0 and 1.3 kBq/mg, respectively.

Animals Male C57BL/6 SPF mice, 5 weeks old, bred by Nihon SLC Co., Ltd., (Shizuoka, Japan) were used throughout this study. These mice were housed six/cage in air-conditioned quarters, and were provided food and water *ad libitum*.

Tumor Cells 3LL cells used in this study were supplied by the Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan, and were continuously maintained in our laboratory in ascites form in C57BL/6 mice.

Epithelial Cells The lines of EBTr cells from fetal calf and NCTC2544 cells from human used in this study were obtained from Dainihon Pharmaceutical Co., Ltd., Osaka, Japan.

***In Vivo* Antimetastatic Assay of WNM–BLM against 3LL Cells in Lung** This assay was done using the method

of Wexler.¹⁰⁾ For the antimetastatic assay test, 3LL cells (5×10^5 cells) were inoculated into mice by subcutaneous (s.c.) implantation in the right groin or intravenous (i.v.) implantation through the tail vein, and the animals were randomly divided into experimental groups of 10 to 12 mice each. The sample was given on 3, 6, 9, 12, 15, and 18 d into s.c. tumor implanted mice and 0, 3, 6, 9, 12, and 15 d into i.v. tumor implanted mice at a dose of 1 or 10 mg/kg, intravenously. The effect on its metastatic spread in the lungs was evaluated on day 21,¹¹⁾ and the assays were repeated three times. Body weights of mice were measured on days 0, 3, 6, 9, 12, 15, and 18.

In Vivo Life-Span Elongation of WNM-BLM against 3LL Implanted C57BL/6 Mice The tumor cells (5×10^5 cells) were inoculated in mice intravenously. Samples were given on 0, 3, 6, 9, 12, and 15 d in a dose of 1 or 10 mg/kg, intravenously. The mice were observed for 60 d during their life-span elongation. These assays were repeated two times.

Assay of Direct Cytotoxicity of WNM-BLM against 3LL Cells and Two Normal Cell Lines in Vitro In the culture plate insert (Millicell-HA, No. PIA01200 Millipore, Japan) set in the 24-well microplate (3047, Becton Dickinson, Oxnard, CA, U.S.A.), 350 μ l of a suspension of 3LL cells or two normal cell lines (5×10^5 cells/well) was mixed with 350 μ l of WNM-BLM in RPMI 1640 medium containing 10% fetal bovine serum, and the mixtures were preincubated for 60 min at 37°C in an atmosphere of humidified 5% CO₂-containing air. The cells were then washed twice with Ca²⁺, Mg²⁺-free phosphate-buffered saline (PBS(-)), and then added to 700 μ l of fresh RPMI 1640 medium, and incubated for 24 h. The number of viable tumor cells was counted by the trypan blue dye exclusion method, and the value was compared with that of the non-treated group.⁶⁾ These assays were repeated three times.

In Vitro Binding Assay of ¹⁴C-WNM-BLM and ¹⁴C-Dex-BLM to 3LL Cells Into the 24-well microplate, 1×10^7 3LL cells were mixed with either the ¹⁴C-WNM-BLM or ¹⁴C-Dex-BLM solution of RPMI 1640 medium. After cultures were incubated for 60 min at 37°C in a humidified atmosphere of 5% CO₂ in air, the tumor cells were centrifuged and washed with PBS(-) three times. The pellet was lysed with 0.5 ml of 1 N NaOH. The radioactivity of the lysate was measured using a liquid scintillation counter. This assay was repeated two times.

Statistical Analysis The data were represented as the mean $\pm$ standard error (S.E.) of the combined experiments, and differences were analyzed for significance according to the Student's *t*-test and Mann-Whitney *U* test.

RESULTS

Body Weight Changes of Mice Administered WNM-BLM The body weight of mice administered WNM-BLM (1 and 10 mg/kg), BLM alone (0.6 mg/kg), and WNM plus a BLM mixture (10 mg + 0.6 mg) were increased gradually similarly to the tumor-bearing non-

Fig. 1. Body Weight Changes of Mice Injected with WNM-BLM or BLM

For this test, 3LL cells were implanted into C57BL/6 mice intravenously. WNM-BLM was given in a dose of 1 or 10 mg/kg/d and BLM was given in a dose of 0.6 mg/kg/d on 0, 3, 6, 9, 12 and 15 d, respectively. The body weight of the mice was measured on day 0, 3, 6, 9, 12, 15, and 18, respectively. —○—, control; —●—, WNM-BLM 1 mg/kg $\times$ 6; —△—, WNM-BLM 10 mg/kg $\times$ 6; —▲—, WNM + BLM 10 + 0.6 mg/kg $\times$ 6; —□—, BLM 0.6 mg/kg $\times$ 6.

TABLE I. Effect of WNM-BLM on the Pulmonary Metastasis of 3LL Implanted C57BL/6 Mice

Sample	Dose ^{a)} (mg/kg/d)	Number of nodules (mean $\pm$ S.E.)	Inhibition ratio (%)	<i>p</i> ^{b)} vs. control
Experiment 1 ^{c)}				
Control		29.3 $\pm$ 2.80		
WNM-BLM	10	16.4 $\pm$ 0.88	44	<0.01 (<0.01)
	1	23.9 $\pm$ 2.13	18	ND ^{d)}
WNM + BLM	10 + 0.6	24.1 $\pm$ 2.26	18	ND ^{d)}
BLM	0.6	25.1 $\pm$ 2.25	14	ND ^{d)}
Experiment 2 ^{e)}				
Control		41.6 $\pm$ 4.88		
WNM-BLM	10	20.5 $\pm$ 2.46	51	0.01 (<0.01)
	1	27.0 $\pm$ 1.46	35	<0.05
WNM + BLM	10 + 0.6	33.6 $\pm$ 2.58	19	(0.01 < <i>p</i> < 0.05)
BLM	0.6	34.2 $\pm$ 3.68	18	ND ^{d)}

a) mg/kg/d $\times$ 6. b) Student's *t* test (Mann-Whitney *U* test). c) In experiment 1, mice were inoculated with 3LL cells subcutaneously on day 0. d) Not significant. e) In experiment 2, mice were inoculated with 3LL cells intravenously on day 0.

treated mice (control) (Fig. 1).

Anti-metastatic Assay of WNM-BLM on Mouse 3LL From the results in Table I, the body weight of the tumor-bearing mice administered with 0.6 mg/kg BLM, 6 times, was not significantly difference from the nontreated tumor-bearing mice. Therefore, we designated the dose of BLM, 0.6 mg/kg, 6 times, as harmless but having no effect on the tumor in the mouse in this experiment. Experiments 1 and 2 in Table I show the results of the antimetastatic activities of WNM-BLM on mouse implanted 3LL. WNM-BLM inhibited the lung metastasis of 3LL cells when this tumor was inoculated in C57BL/6 mice subcutaneously or intravenously, and the inhibition ratio was 44 and 51% in a dose of 10 mg/kg/d for 6 d. On the other hand, the mice treated with 0.6 mg of BLM or

Fig. 2. Life-Span Elongation of WNM-BLM against 3LL Implanted Mice

For this test, 3LL cells were implanted into C57BL/6 mice intravenously. WNM-BLM was given in a dose of 1 or 10 mg/kg/d and BLM was given in a dose of 0.6 mg/kg/d on days 0, 3, 6, 9, 12 and 15, respectively. The mice were observed for 60 d for life-span elongation. —○—, control; —●—, WNM-BLM 1 mg/kg $\times$ 6; —△—, WNM-BLM 10 mg/kg $\times$ 6; —▲—, WNM + BLM 10 + 0.6 mg/kg $\times$ 6; —□—, BLM 0.6 mg/kg $\times$ 6.

Fig. 3. Direct Cytotoxicity of WNM-BLM against 3LL Cells *in Vitro*

In the culture plate insert set in the microplate, a suspension of 3LL cells was mixed with samples in RPMI 1640 medium, and the mixtures were pre-incubated for 60 min at 37°C. The cells were washed, added to fresh RPMI 1640 medium, and incubated for 24 h. The number of viable tumor cells was counted by the trypan blue dye exclusion method.

Fig. 4. Direct Cytotoxicity of WNM-BLM against 3LL, EBTr and NCTC2544 Cells *in Vitro*

A suspension of the cells was mixed with samples in RPMI 1640 medium, and the mixtures were pre-incubated for 60 min at 37°C. The cells were washed, added to fresh RPMI 1640 medium, and incubated for 24 h. The number of viable tumor cells was counted by the trypan blue dye exclusion method. □, 3LL; ▨, EBTr; ■, NCTC 2544.

Fig. 5. Binding Activity of ^{14}C -WNM-BLM and ^{14}C -Dex-BLM to 3LL Cells *in Vitro*

Into the 24-well microplate, the 3LL cells were mixed with 1, 2, 5, and 10 mg/well of ^{14}C -WNM-BLM or ^{14}C -Dex-BLM solution in RPMI 1640 medium. After the cultures were incubated for 1 h at 37°C , tumor cells were centrifuged and washed with PBS (—). The pellet was lysed and the radioactivity of the lysate was measured in a liquid scintillation counter. —○—, ^{14}C -WNM-BLM; —●—, ^{14}C -Dex-BLM.

a combination of 10 mg/kg WNM plus 0.6 mg/kg BLM did not exhibit inhibition of the tumor.

Life-Span Elongation of WNM-BLM against 3LL Implanted C57BL/6 Mice Figure 2 indicates the life span elongation of WNM-BLM on 3LL implanted mice. In the mice administered with WNM-BLM in a dose of 1 and 10 mg/kg/d for 6 d, life span elongation was observed, and 50 to 70% of mice survived for 60 d. The mice administered WNM or BLM alone, or WNM plus BLM died on days 20 to 26, respectively.

Direct Cytotoxicity Assay of WNM-BLM against 3LL and Several Normal Cells *in Vitro* To examine the direct cytotoxic effect of WNM-BLM, 3LL cells and NCTC 2544 and EBTr normal cells were tested using the trypan blue dye exclusion method. WNM-BLM manifested a strong direct cytotoxic effect on 3LL cells *in vitro* (Fig. 3). On the other hand, the mixture of WNM and BLM, BLM alone and Dex-BLM did not show any cytotoxicity on the same tumor cells. WNM-BLM also did not show any activity against normal cells, NCTC 2544 and EBTr (Fig. 4).

Binding Assay of ^{14}C -WNM-BLM on 3LL Cells *in Vitro* The result of this binding assay indicates that significant incorporation was observed between ^{14}C -labeled WNM-BLM and the 3LL cells. Reactivity of ^{14}C -Dex-BLM under the same condition did not show any binding effect to the same tumor cells (Fig. 5).

DISCUSSION

In this article, we showed that the WNM-BLM conjugate exhibited an antimetastatic effect (Table I) and life-span elongation on 3LL implanted mice (Fig. 2). No decrease in the body weights of the mice was observed (Fig. 1) and no toxic effect was observed in WNM-BLM administered mice. Imanishi, *et al.* reported that the effective dose of BLM alone to mouse transplantable 3LL cells was 1 to 3 mg/kg/d for 11 d.¹²⁾ In the present report, we showed that WNM-BLM exhibited antitumor activity on 3LL in a dose of 1 to 10 mg/kg/d (dose of BLM:

0.06 to 0.6 mg/kg/d) for 6 d (Table I and Fig. 2). These results indicate that the antitumor activity of BLM in WNM-BLM conjugate was elevated about 9 to 30 times compared with that of BLM alone.

In order to analyze the action mechanism of WNM-BLM, direct cytotoxic assay to the 3LL cells and two cell lines was estimated. WNM-BLM exhibited tumoricidal activity on 3LL cells, but exhibited lower activity against EBTr and NCTC2544 epithelial cells (Figs. 3, 4). ^{14}C -Labeled WNM-BLM also showed cytotoxic activity and binding activity to 3LL cells, while ^{14}C -Dex-BLM did not show this activity *in vitro* (Fig. 5). The above results suggest that one of the antitumor mechanisms of WNM-BLM depends on the specific targeting effect of WNM against 3LL cells and depository to the lung.⁷⁾ Therefore, the introduction of mannose residue to anticancer agents might lead to the development of highly specific anti-lung cancer agents. The specific binding effect of WNM-BLM to the 3LL cells might also contribute to the exclusion of the side effects of WNM-BLM.

WNM possesses antitumor activity and antimicrobial effects against several opportunistic infections, according to its enhancement of immunity of host animals.¹³⁻¹⁵⁾ These papers suggested that released WNM from WNM-BLM conjugate might cause the enhancement of a host defense mechanism in tumor-bearing mice, and may inhibit the metastasis and relapse of a tumor.

The conjugates of a monoclonal antibody and anti-cancer agent showed only a slight antitumor effect because of the heterogeneity of tumor antigens on the surface of tumor cells and tissues.¹⁾ However, the ligand of WNM-BLM fits to a common mannan binding site on tumor cells. The present results thus suggest that mannan serves as a useful drug carrier for an anti-lung cancer pro-drug.

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