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FULL PAPER

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Macrophage J774.1 cell is activated by MZ-Fraction (Klasma-MZ) polysaccharide in *Grifola frondosa*

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Abstract We previously reported that the (β1,3)-branched (β1,6)-glucan, MD-Fraction extracted from the fruit body of the edible maitake mushroom (*Grifola frondosa*) exhibits an antitumor effect. In this study, we separated a new polysaccharide from maitake, which we named Maitake MZ-Fraction (Klasma-MZ). MZ-Fraction is a β-glucan with a low molecular weight of about 20000, which has a β1,3- and a β1,6-bond. MZ-Fraction enhanced TNF-α and IL-12 productivity and the antigen presentation of murine macrophage cell line J774.1 in vitro. Furthermore, MZ-Fraction showed antitumor activity similar to MD-Fraction in vivo.

Key words Antitumor activity · Polysaccharide · TNF-α

Introduction

Macrophages are mononuclear cells that have a phagocytic capacity and are derived from precursors in the bone marrow via the monocytes of peripheral blood. Macrophages are known to be typical effector cells of innate immunity. The immune response of macrophages can be largely classified into two roles; the first is to exclude foreign bodies by phagocytosis or the effect of cytotoxicity, and the second is to fragment foreign bodies and to present antigen information to the T cell with major histocompatibility complex (MHC).

Polysaccharides, particularly glucans, are used in a wide variety of clinical applications as macromolecules that activate the immune system. They have been extracted from mushrooms, fungi, yeast, algae, and plants. In particular, β-glucans such as lentinan (Chihara et al. 1970), schizophyllan (Mitani et al. 1982), and krestin (Tsukagoshi et al. 1984) isolated from mushrooms display an antitumor effect as a biological response modifier (BRM).

We previously reported that the (β1,3)-branched (β1,6)-glucan, MD-Fraction extracted from the fruit body of the maitake mushroom (*Grifola frondosa*), acts as a BRM similar to lentinan. MD-Fraction activates immunocompetent cells such as the macrophages, helper T cells, and cytotoxic T cells that attack tumor cells (Adachi et al. 1987; Hishida et al. 1987; Nanba et al. 1987). MD-Fraction administered orally and by i.p. injection to MM-46 carcinoma-bearing C3H/HeN mice induced a Th-1-dominant response when MD-Fraction expressed its antitumor effect (Inoue et al. 2002).

We separated a new polysaccharide other than MD-Fraction from *Grifola frondosa* and named it Maitake MZ-Fraction. The molecular weight of MZ-Fraction is almost 20000 whereas the molecular weight of MD-Fraction is 1 to 2 million.

In the present study, to characterize the MZ-Fraction, we investigated its influence on murine macrophage cell line J774.1 in vitro and its antitumor activity in vivo.

Materials and methods

Animals

Male C3H/HeJ mice (4 weeks old) were supplied by Japan Clea (Osaka, Japan) and were raised for 1 week before being used for experiments.

Antibodies

The following monoclonal antibodies were purchased from BD Bioscience Pharmingen (San Diego, CA, USA): CD16/CD32, FITC-conjugated I-A/I-E, R-PE-conjugated CD86, R-PE-conjugated TNF-α. Purified antimouse IL-12 (p40/70) was purchased from Biolegend, Inc. (San Diego, CA,

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USA). Recombinant murine IL-12, biotinylated antimurine IL-12, recombinant murine TNF-α were purchased from PeproTech EC Ltd. (London, UK). Purified antimouse TNF-α was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Biotinylated antimouse TNF-α was purchased from R&D Systems, Inc (Minneapolis, MN, USA).

Preparation of MZ-Fraction (Klasma-MZ)

A dried powder prepared from the maitake mushroom was obtained from Yukiguni Maitake (Niigata, Japan); 100 g of the powder was mixed with 1000 ml milli Q water and heated for 20 min at 120°C. After centrifugation (7000 *g*, 10 min), ethanol of equiponderance was added to the supernatant, and a floating substance was obtained that was dissolved in sterilized water. The solution was applied to DEAE Cellulofine (φ12.5 × 10 cm; Seikagaku Industry, Tokyo, Japan) and eluted with 12.5 mM Tris-HCl (pH 8.0). The solution obtained was treated with an equal volume of EtOH, and the ethanol layer was evaporated. The supernatant was again treated with 4 volumes of EtOH. The resulting pellet was dissolved in sterilized water and then lyophilized. The sugar content of the final product was 84%, as determined by the Anthrone method, and contained about 16% protein as determined by Lowry's method. The level of contaminated lipopolysaccharide (LPS) contained in MZ-Fraction was determined by using an Endospecy ES-20S Set (Seikagaku Industry), and when the contaminated ratio (%) of LPS in MZ-Fraction was less than 4×10^{-10}%, we used the purified MZ-Fraction for the experiment.

MD-Fraction and MX-Fraction were prepared from the powder according to the method described previously (Nanba et al. 1987; Kubo et al. 1994).

Enzymatic digestion

β1,6-Glucanase (final concentration, 0.1182 U/ml) and β1,3-glucanase (final concentration, 0.15 U/ml), which were purified from filtered culture medium of *Trichoderma harzianum* (ATCC number: MYA-1175), were each reacted with MZ-Fraction (final concentration, 6.0 mg/ml), and incubated for 4 h at 45°C. The polysaccharide degraded by the enzyme was separated by high pressure liquid chromatography (HPLC).

Cell culture

Murine macrophage cell line J774.1 was obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Miyagi, Japan) and grown in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS), potassium penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) at 37°C in an atmosphere of 95% air – 5% CO_2. J774.1 cells are a monocyte-derived macrophage cell line from BALB/c mice.

Induction of tumor necrosis factor (TNF)-α or interleukin (IL)-12 from macrophage-like J774.1 cells

J774.1 cells were harvested and suspended in fresh medium. The cells (1×10^6 cells/ml in each well of 96-well dishes) were allowed to adhere to the plastic for 2 h at 37°C, washed twice with medium, and incubated 18–24 h with 30 μg/ml polymyxin B and MZ-, MD-, or MX-Fraction.

Determination of released TNF-α or IL-12 by enzyme-linked immunosorbent assay

J774.1 cells were cultured with MZ-, MD-, or MX-Fraction for 4–24 h. The concentration of TNF-α or IL-12 in the supernatant was determined by enzyme-linked immunosorbent assay (ELISA).

Flow cytometry

J774.1 cells were analyzed for expression of major histocompatibility complex (MHC) class II and CD86 by flow cytometry. The cells were mixed with anti-CD16/CD32 monoclonal antibody in a tube and reacted at 4°C for 5 min. The cells were then stained with fluorescein isothiocyanate (FITC)-conjugated antimouse I-A/I-E monoclonal antibody and R-PE-conjugated antimouse CD86 monoclonal antibody at 4°C for 30 min. Flow cytometric analyses were performed with a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA).

For intracellular cytokine detection, J774.1 cells were applied to a 24-well plate, and ionomycin (750 ng/ml) and phorbol-12-myristate-13-acetate (50 ng/ml) were added to each well. The plate was then incubated with 1.4 μl Goldi stop at 37°C in 5% CO_2 for 4 h. After stimulation, anti-CD16/CD32 monoclonal antibody was added and reacted at 4°C for 5 min. The cells were washed with staining buffer [0.09% NaN_2 and 3% FBS in phosphate-buffered saline (PBS)], incubated with 100 μl Cytofix/Cytoperm (BD Bioscience Pharmingen) at 4°C for 30 min, and washed again with Perm/Wash (BD Bioscience Pharmingen). The stained cells were reacted with R-PE-conjugated antimouse TNF-α monoclonal antibody at 4°C for 30 min, then washed with Perm/Wash, suspended in 0.4 ml PBS containing 1% paraformaldehyde, and counted by FACScan.

RT-PCR detection of TNF-α or IL-12 p40 mRNA expression by MZ-Fraction

J774.1 cells were cultured with MZ-Fraction for 24 h. The cells were harvested, and the total RNA was extracted by Cepasol RNA II Super (Nakarai Tesque, Kyoto, Japan); then, 1 μg total RNA was reverse transcribed to cDNA using oligo (dT) primers and then amplified with a reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Toyobo, Osaka, Japan). The synthetic oligonucleotides were 5′-GGCAGGTCTACTTTGGAGTCATTGC-3′ and 5′-ACATTCGAGGCTCCAGTGAATTCGG-3′ for TNF-

α, and 5′-AACTGGCGTTGGAAGCACGG-3′ and 5′-GAACACATGCCCACTTGCTG-3′ for IL-12 p40. The G3PDH gene with 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ primers served as a control.

Dosage of MZ- and MD-Fraction

MM-46 carcinoma cells (1×10^6) were implanted in mice of the C3H/HeJ strain (6 weeks old) in the right axillary region. After 24h, MZ- or MD-Fraction (4mg/kg/day) was injected into the MM-46 carcinoma-bearing mice intraperitoneally (i.p.) for 10 consecutive days, and tumor weight was measured 27 days after tumor cell implantation. As a control, saline was also injected for 10 consecutive days. Tumor Inhibition ratio (TIR, %) was calculated as follows: [1 – (weight of tumor mass from animals treated with drug/weight of tumor mass from animals receiving no treatment)] × 100.

Statistical analysis

Values are expressed as means ± standard error, and significant differences across the groups were analyzed with Student's *t* test.

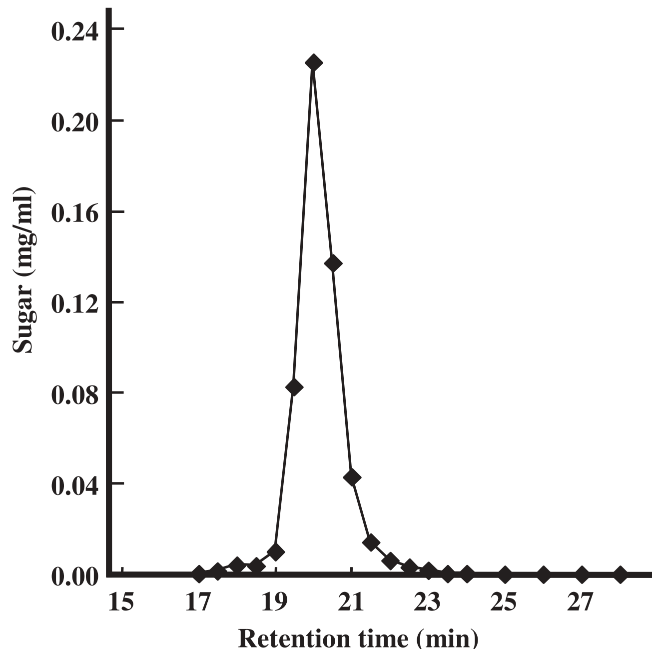

Fig. 1. High pressure liquid chromatography (HPLC) analytical profile of MZ-Fraction. Column, Shodex OHpak SB-2006M (20 × 300mm); buffer, 5mM phosphate (pH 7.2); flow rate, 3.0ml/min; fractions, 1.5ml; injection, 1.0ml containing 1.0mg/ml

Results

Possible structure of MZ-Fraction

As shown in Fig. 1, MZ-Fraction was indicated as a single peak corresponding to authentic dextran (MW, 20000) by HPLC analysis. However, it was not analyzed by mass spectrum analysis because it had a high molecular size. To examine its structure, MZ-Fraction was digested with 1N HCl or β1,3- or β1,6-glucanase.

HCl (final concentration, 1N) was added to MZ-Fraction (final concentration, 9.8mg/ml) and hydrolyzed for 4h sat 75°C. When the sample by which MZ-Fraction was digested was analyzed by thin-layer chromatography (TLC), the digested MZ-Fraction presented in a green color produced by the anthrone sulfuric acid. As a result of the *Rf* value, MZ-Fraction consisted of glucose only (data not shown). MZ-Fraction was hydrolyzed by β1,3- or β1,6-glucanase into oligosaccharide and glucose, indicating it to be a β-glucan that has a β1,3- and a β1,6-bond (data not shown).

Effect of MZ-, MD-, or MX-Fraction on the production of TNF-α by J774.1 mouse macrophages

MZ-Fraction induced significant increases in TNF-α production for 18h by J774.1 cells in vitro (Fig. 2A), which depended on the concentration of MZ-Fraction. On the other hand, MD- and MX-Fraction did not influence TNF-α production in vitro.

To exclude the possible contamination of LPS in MZ-Fraction, polymyxin B was added to the incubation medium. When the same amount (500μg/ml) of LPS contamination in MZ-Fraction was added, TNF-α production showed almost the same value as the control (data not shown). This result suggests, therefore, that there is no influence on the production of TNF-α by contamination of LPS in MZ-Fraction.

Effect of MZ-, MD-, or MX-Fraction on intracellular TNF-α and TNF-α mRNA expression by J774.1 mouse macrophages

Because MZ-Fraction induced significant increases in TNF-α production by J774.1 cells, we investigated intracellular TNF-α and TNF-α mRNA expression in J774.1 cells. MZ-Fraction induced increases intracellular TNF-α production by J774.1 cells significantly (Fig. 2B). Moreover, MZ-Fraction enhanced the expression of TNF-α mRNA in J774.1 cells (Fig. 2C). These results correspond to the results of the extracellular production of TNF-α by J774.1 cells.

Effect of MZ-, MD-, or MX-Fraction on the production of IL-12 by J774.1 mouse macrophages

IL-12 production of J774.1 cells was increased dose-dependently by MZ-Fraction but not MD- or MX-Fraction (Fig. 3A). The IL-12 mRNA expression in J774.1 cells was also increased by MZ-Fraction (Fig. 3B).

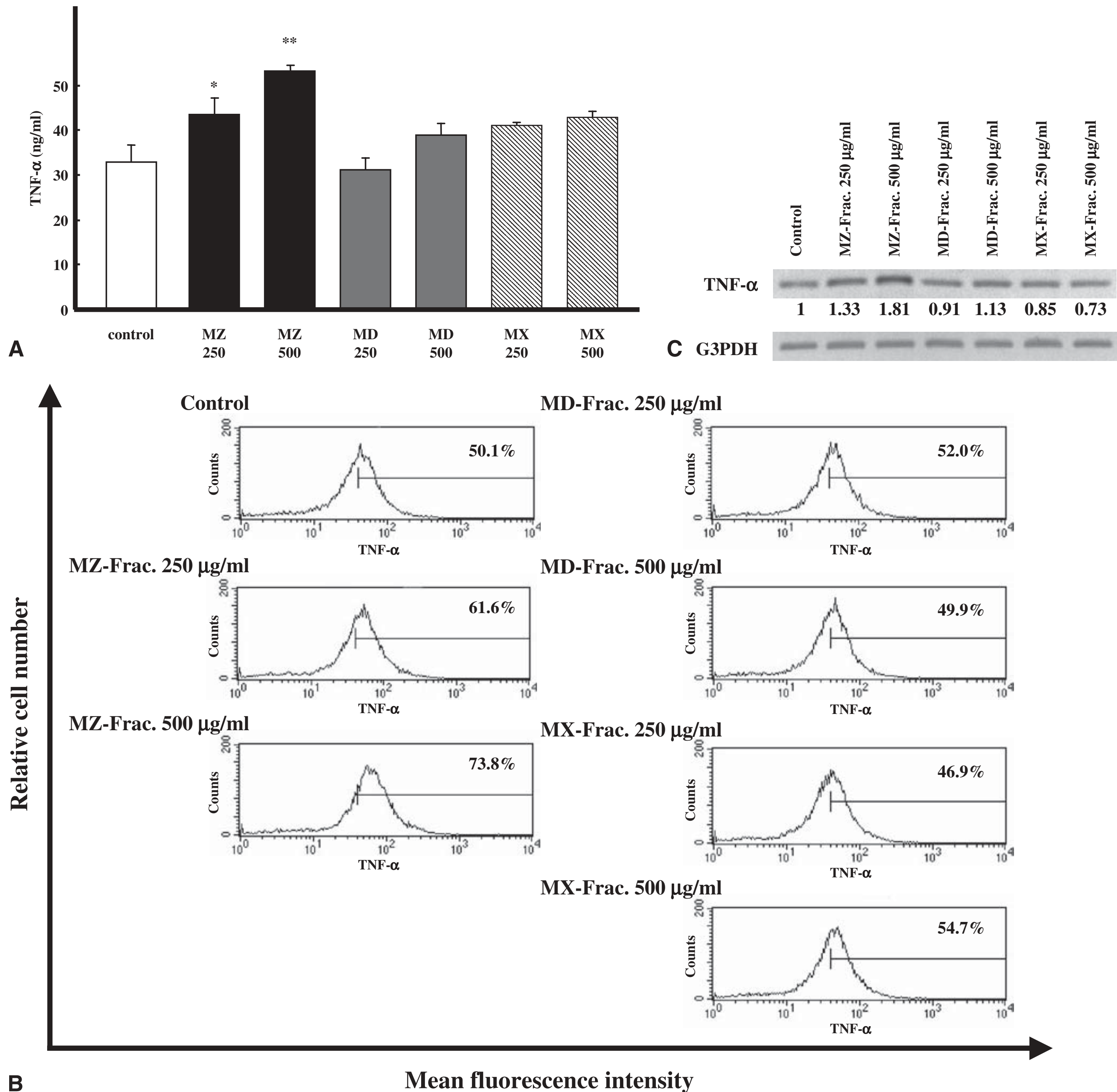

Fig. 2. Effect of MZ-, MD-, or MX-Fraction (250, 500 μg/ml) on the production of tumor necrosis factor (TNF)-α by J774.1 mouse macrophage. J774.1 cells were stimulated with various concentrations of MZ-, MD-, or MX-Fraction (250, 500 μg/ml) for 18 h (**A**) or 24 h (**B**, **C**). **A** TNF-α production. Culture supernatants were collected for detecting the level of TNF-α by enzyme-linked immunosorbent assay (ELISA). **B** Expression of intracellular TNF-α was determined by flow cytometry. **C** The expression of TNF-α mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR). Values are expressed as the mean ± SE. Significant difference from the control group was determined using Student's *t* test. *$P < 0.05$, **$P < 0.01$

Effect of MZ-Fraction on the expression of MHC class II and CD86 by J774.1 mouse macrophages

Next, we examined the influence that MZ-Fraction exerted on the expression of MHC class II and CD86 of the cell-surface antigen. MZ-Fraction enhanced the expression of MHC class II and CD86 antigen of the cell surface (Fig. 4). As a result, we assume that MZ-Fraction enhances the antigen presentation of macrophage cell line J774.1.

Antitumor activity of MZ-Fraction

Because the proposed structure of MZ-Fraction is very similar to MD-Fraction, which possesses antitumor activity, and MZ-Fraction activates macrophages in vitro more strongly than MD-Fraction, we investigated the antitumor activity of MZ-Fraction. When mice were treated with MZ-Fraction (4 mg/kg/day), TIR was 70.3%. When treated with MD-Fraction (4 mg/kg/day), TIR was 72.9%. These results

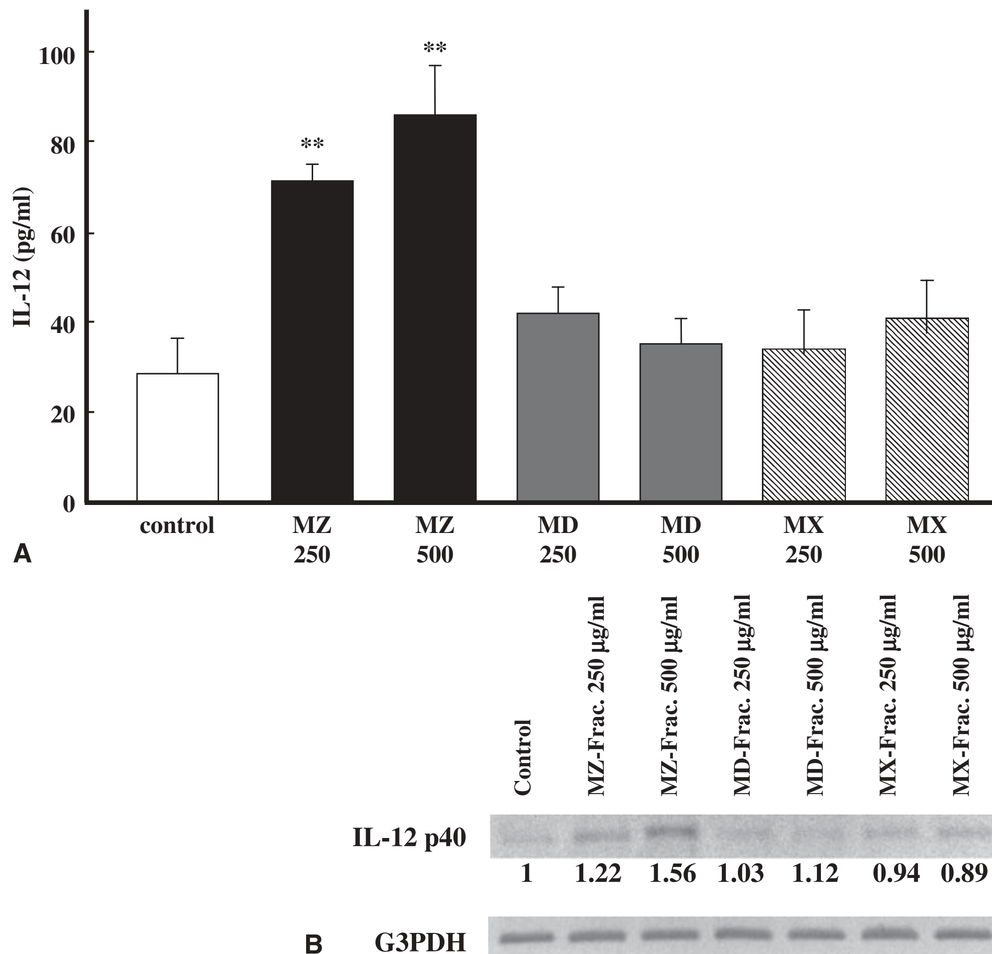

Fig. 3. Effect of MZ-, MD-, or MX-Fraction (250, 500 µg/ml) on the production of interleukin (IL)-12 by J774.1 mouse macrophage. J774.1 cells were stimulated with various concentrations of MZ-, MD-, or MX-Fraction (250, 500 µg/ml) for 24 h. **A** IL-12 production. Culture supernatants were collected for detecting the level of TNF-α by ELISA. **B** The expression of IL-12 mRNA by RT-PCR. Values are expressed as the mean ± SE. Significant difference from the control group was determined using Student's *t* test. $^{**}P < 0.01$

suggest that the antitumor activity of MZ-Fraction is as great as that of MD-Fraction.

Discussion

MZ-Fraction is a β-glucan polysaccharide with a low molecular weight of about 20 000 that has a β1,3- and a β1,6-bond. When MZ-Fraction was digested by β1,6- or β1,3-glucanase, the oligosaccharide was obtained when it was digested by β1,6-glucanase, whereas most of the MZ-Fraction digested by β1,3-glucanase was a glucose. These results suggest MZ-Fraction is a polysaccharide composed of a β1,6 main chain and a β1,3 side chain. This structure is very similar to MD-Fraction, which shows antitumor activity, but MZ-Fraction has a low molecular weight whereas the molecular weight of MD-Faction is 1 000 000–2 000 000.

MZ-Fraction enhanced TNF-α, IL-12 productivity, and the antigen presentation of macrophage cell line J774.1 in vitro. On the other hand, MD-Fraction does not show effectiveness as MZ-Fraction in vitro (Figs. 2, 3). After cutting of the β1,3 side chain in MD-Fraction, we could not detect the antitumor activity (data not shown). This fact suggests that there is a relationship of the antitumor action and the molecular structure of the polysaccharide. It is believed that this is because of differences not only in molecular weight but also in the stereochemistry of MZ-Fraction and MD-Fraction. In addition, to activate macrophages, transmission of an activation signal is necessary, and low molecular weight material causes stimulation remarkably more strongly via the receptor than the macromolecule. However, we are still not clear about this aspect and need to examine the problem further.

Moreover, as shown in Fig. 4, MZ-Fraction upregulated MHC class II and CD86 antigen expression in macrophages. These results suggest that the upregulation of these molecules may assist the interaction between antigen-presenting cells and helper T cells and also the expression of antitumor activity in vivo.

Because a strong activation of macrophages was observed in vitro, we investigated the antitumor activity of MZ-Fraction. As a result, it was shown that MZ-Fraction had antitumor activity similar to MD-Fraction. MZ-Fraction directly activated macrophages in vitro. It is believed that MZ-Fraction shows antitumor activity by activated macrophages.

The activated macrophage by MZ-Fraction produced TNF-α and IL-12. The tumoricidal activity of macrophages is performed mainly through production of NO and TNF-α

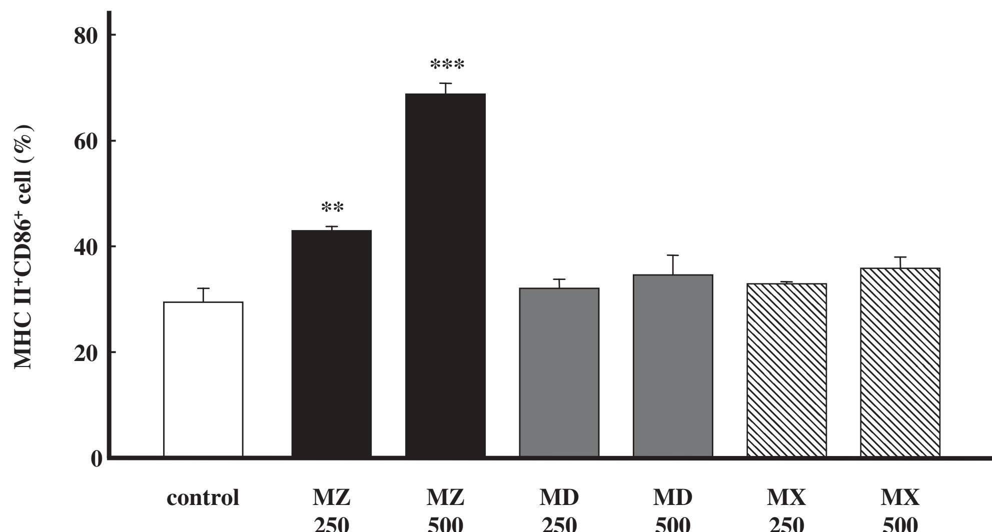

Fig. 4. Effect of MZ-, MD-, and MX-Fraction on the expression of MHC class II and CD86. J774.1 cells were stimulated with various concentrations of MZ-, MD-, or MX-Fraction (250, 500 μg/ml) for 18 h. Expression of MHC class II and CD86 were determined by flow cytometry. Values are expressed as the mean ± SE. **$P < 0.01$, ***$P < 0.001$ (Student's *t* test)

(Kim et al. 2004; Zheng et al. 2005). TNF-α is known as an important host protection cytokine that destroys the tumor cells. The production and gene expression of TNF-α by activated macrophages may be connected with the cytotoxic effect on the malignant cells (Urban et al. 1986; Decker et al. 1987). The production of TNF-α was elevated by MZ-Fraction, which can effectively destroy tumor cells. IL-12 was identified as a disulfide-linked heterodimeric cytokine that activated the natural killer cells and induced the production of IFN-γ (Kobayashi et al. 1989). IL-12 is a 70-kDa (p70) glycoprotein that is composed of two subunits of 40 kDa (p40) and 35 kDa (p35). p35 is produced constantly. On the other hand, p40 is produced by activated macrophages and B cells. IL-12 not only activates the natural killer cell and the T cell but also plays an essential role in the induction of an effective Thl type of cell-mediated immune response against tumors (Chehimi et al. 1994; Tsung et al. 1997). It is thought that IL-12, which is produced by MZ-Fraction-activated macrophages, was related to antitumor response by enhanced cell-mediated immune responses, dependent on Th1 cell development.

It is already clear that MD-Fraction, in vivo, shows an antitumor effect by working as a biological response modifier (BRM), activating the immune system by inducing cell-mediated immunity, but it is not effective in vitro. On the other hand, MZ-Fraction has immunological activity both in vivo and in vitro. It might be easier for low molecular weight material with a simplified structure to make the connection to the receptor of the signal transmission than material with a complex helix structure, or for the low molecular weight to be easily phagocytosed and cause the intracellular signal transmission and thus activate macrophages (Thornton et al. 1996; Brown and Gordon 2001; Brown et al. 2002; Kataoka et al. 2002; Shao et al. 2004). The receptor of MZ-Fraction requires further examination.

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