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SHORT COMMUNICATION

Effect of a hot water-soluble extraction from *Grifola frondosa* on the viability of a human monocyte cell line exposed to mitomycin C

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Abstract The effects of a hot water-soluble extract of *Grifola frondosa* on the viability of multiple immune cell lines were examined in vitro. The extract and the 1000-cut extract, a low molecular weight fraction, improved the viability of a human monocyte cell line, THP-1, treated by the anticancer drug mitomycin C. DNA microarray and immunoassay results revealed increases in interleukin (IL)-8 expression by THP-1 cells in response to the 1000-cut extract. These data suggest that the extract would enhance the immune system through induction of IL-8 production by human blood monocytes, which leads to activated neutrophils.

Keywords Maitake mushroom · IL-8 · Extract · Viability

Chemotherapeutic agents such as alkylating agents, antimetabolites, antitumor antibiotics, and mitotic inhibitors not only damage cancer cells, but also adversely affect the cancer patient's immune system, and can cause serious side effects including hair loss, pain, nausea, and appetite loss. Immunostimulatory agents such as monoclonal antibodies, growth factors, vaccines, cytokines, and polysaccharides are known to activate immune cells through enhancing their cytokine production and proliferative activity, and it has been suggested that combining immunostimulatory agents with anticancer therapies may improve the quality of life (QOL) for patients (Urushizaki 1989; Saji et al. 1999; Shibata et al. 2002; Hurwitz 2004). However, some agents including interferon and interleukin also cause adverse symptoms such as depression, anxiety, and appetite loss (Illman et al. 2005; Asnis and De La Garza 2006). In contrast to other immunostimulatory agents, polysaccharides from medicinal mushrooms have been reported to eliminate cancer cells indirectly by enhancing the activity of cytotoxic T cells and natural killer (NK) cells, and they do so without any adverse reactions (Borchers et al. 1999; Kodama et al. 2002a,b; Li et al. 2007). In 1987, we identified a polysaccharide from the edible maitake mushroom *Grifola frondosa* (Dicks) Gray, which we named maitake D-fraction (MD-fraction) (Adachi et al. 1987; Nanba et al. 1987). In animal experiments, MD-fraction enhanced activation of macrophages, dendritic cells (DCs), NK cells, and T cells and exhibited antitumor effects (Inoue et al. 2002; Kodama et al. 2002a,b; Harada et al. 2003). When immunostimulatory agents are clinically administered to patients, it is essential for them to be pure and well characterized and for their mechanisms of action to be elucidated at the molecular and cellular levels. Although most polysaccharides extracted from edible mushrooms exhibit antitumor effects in animal models, their approval for use as anticancer therapies or as immunomodulatory drugs for cancer patients has not yet been achieved. Although the

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whole mushrooms are edible, the dried fruiting bodies and hot water-soluble extracts are commercially available and can be purchased as an easy-to-take health food supplement. For people, especially elderly people, young children, and those suffering from illnesses who need increased immune defense against bacterial and viral infections, it may be preferable to take a daily supplement containing water-soluble nutrients including amino acids, proteins, sugars, peptides, and vitamins. Therefore, our present study specifically targets water-soluble extracts from edible mushrooms rather than polysaccharides themselves to improve the host immune system that has been damaged by exposure to toxic chemicals such as anticancer drugs as well as to boost weak immune systems of elderly people and young children.

Recently, the effect of a hot water-soluble extract of *Agaricus blazei* Murrill on gene expression was investigated in the human monocyte cell line, THP-1 (Ellertsen et al. 2006). Microarray analysis found selective upregulation of genes related to immune function in response to the extract, particularly increases in expression of proinflammatory genes such as interleukin (IL)-8. Interleukin-8, also known as neutrophil chemotactic factor (NCF), serves as a chemical signal that attracts neutrophils to the site of inflammation (Mukaida et al. 1995). The primary function of IL-8 is the induction of chemotaxis in its target cells, such as neutrophils and granulocytes, thereby increasing defenses against bacterial, viral, and fungal infections. Based on this evidence, we expected that a hot water-soluble extract from the maitake mushroom might also enhance the host immune system by increasing the proliferative activity and cytokine production of immune cells. However, our only examination thus far has been of a polysaccharide purified from the hot water-soluble extract, MD-fraction. Although this fraction possesses antitumor activity, it has no effect on the production of cytokines by macrophages, monocytes, T cells in vitro (data not shown). Thus, we speculated that other immunostimulating materials exist in the maitake hot water-soluble extract.

The extract from the maitake mushroom may restore the viability of a weakened immune system from exposure to toxic chemicals. Mitomycin C (MMC), a toxic antitumor antibiotic agent, induces apoptosis of both cancer and normal cells, blocking the cell cycle at the G_2 phase and inhibiting DNA synthesis by cross-linking DNA at guanine and adenine residues, thereby disrupting base pairing. Using an animal model, we previously found that despite the decrease in splenocyte counts, weight loss, and survival rate, tumor size clearly reduced in tumor-bearing mice during the MMC treatment (Kodama et al. 2005). Therefore, we investigated the effect of the maitake hot water-soluble extract on the proliferation and viability of a variety of leukocyte cell lines (THP-1, Jurkat, NK-92MI, HL60, KG-1, KG-1a, RAW 264.7, and J774A.1) in vitro, in the presence or absence of the toxic antitumor antibiotic MMC. To investigate the effect of the extract on activation and cytokine production by THP-1 cells in detail, a microarray and immunoassay were performed.

In this article, we describe the possible use of a maitake mushroom extract to relieve the adverse side effects of cancer drugs by preventing damage to healthy leukocytes and enhancing neutrophil activation through induction of IL-8 production by blood monocytes. Furthermore, because the extract is derived from an edible mushroom, it could also be used extensively as a nonprescription remedy against cancer, infections, and immunocompromising diseases.

Dried powder from the fruiting body of the edible maitake mushroom (*G. frondosa*) was kindly provided by Yukiguni Maitake Co. (Niigata, Japan). The dried powder was suspended in deionized water and autoclaved for 15 min at 110°C to obtain a hot extract (hot extract). Aliquots of hot extract were also filtered individually, by centrifugation, to obtain substances of less than 5000, 3000, or 1000 MW, named 5000-cut, 3000-cut, and 1000-cut, respectively.

THP-1 (human monocyte cell line), Jurkat (human T-cell line), NK-98MI (human NK cell line), HL60 (human acute promyelogenous leukemia cell line), KG-1 (the human acute myelogenous leukemic cell line), and KG-1a, a variant subline, composed of undifferentiated promyeloblasts of KG-1, RAW 264.7 (murine macrophage-like cell line), and J774A.1 (murine macrophage-like cell line) were provided by the Cell Resource Center for Biomedical Research at Tohoku University. Except for the NK98-MI cells, cells were cultured in RPMI-1640 medium (Nissui Seiyaku, Tokyo, Japan) containing P/S (penicillin/streptomycin) and 10% inactivated fetal bovine serum (FBS) and kept at 37°C in a 5% CO_2 incubator. The NK-98MI cells were cultured in alpha minimum essential medium containing 12.5% inactivated horse serum, 12.5% inactivated FBS, 0.1 mM mercaptoethanol, 0.2 mM inositol, and 0.02 mM folic acid.

To determine cell viability in response to various doses of extract, extract was added to the cells (4.8×10^4/0.1 ml in a 96-well plate) and then incubated with and without 10 μM MMC for 15 h at 37°C in a 5% CO_2 incubator. After incubation, 10 μl WST-8 reagent (Nacalai Tesque, Kyoto, Japan) was added to the cultured cells and the 96-well plate was incubated for an additional 2 h. Changes in absorbance measured at 450 nm (reference, 650 nm) created by a reaction catalyzed by mitochondrial dehydrogenase were used to determine cell viability. Sterilized milliQ water was used as a control. Cell viability (%) was calculated according to the following formula.

Viability (%)
= (Absorbance of each fraction sample with MMC)/(absorbance of control without MMC) × 100.

For gene expression experiment, THP-1 cells (2.6×10^6/1 ml in a 6-well plate) were added to 15 µl 1000-cut extract and then incubated with 10 µM MMC for 18 h at 37°C in a 5% CO_2 incubator. After stimulation, the total RNA was extracted with the RNeasy Plus Mini kit (Qiagen, Tokyo, Japan). DNA microarray analysis (Human Genome U133 Plus2.0) results were provided by the Gene Chip Analysis Service with GeneChip operating Software ver 1.4 (Affymetrix 690036) (Bio Kurabo, Osaka, Japan).

To determine the level of IL-8 production, THP-1 cells (4.8×10^4/0.1 ml in a 96-well plate) were added to 1000-cut extract, and cultures were then incubated with 10 µM MMC for 18 h at 37°C in a 5% CO_2 incubator. The culture supernatant was collected by centrifugation, and IL-8 levels were determined by enzyme-linked immunosorbent assay (ELISA) (Thermo Fisher Scientific, Rockford, IL, USA).

Analysis of variance was used to determine differences among mean values of each extract-treated group and the control in the presence or absence of MMC. The least significant difference test was used for multiple comparisons (Scheffe's test, $^{*}P < 0.05$). A t test was used to determine the difference between mean values of the 1000-cut-treated group and the control in the presence or absence of MMC (Student's t test, $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$). Values are represented as mean ± SD of three independent experiments.

An aqueous extract from the edible mushroom *A. blazei* upregulates proinflammatory cytokine genes, in particular IL-1 and IL-8, in a monocyte cell line, THP-1, after direct in vitro stimulation of the cells with the extract (Ellertsen et al. 2006). Therefore, we investigated the effects of hot water-soluble extracts derived from dried maitake mushroom powder on the growth of THP-1 cells. Figure 1 shows the effects of hot extract, 5000-cut, 3000-cut, and 1000-cut on the viability of THP-1 cells. Without MMC treatment, each extract increased the viability of THP-1 to about 1.2 times that of the control, indicating that the active material was likely a low molecular weight product, present at equal concentration in each of the extracts. When THP-1 cells were treated with MMC, the control viability level decreased to 38% of that without MMC. In contrast, addition of 5000-cut, 3000-cut, and 1000-cut extracts prevented MMC-induced decreases in THP-1 cell viability, restoring viability to 66% of the control without MMC. In addition, we examined the cytotoxicity of the hot extract and the other extracts on THP-1 cells with trypan blue

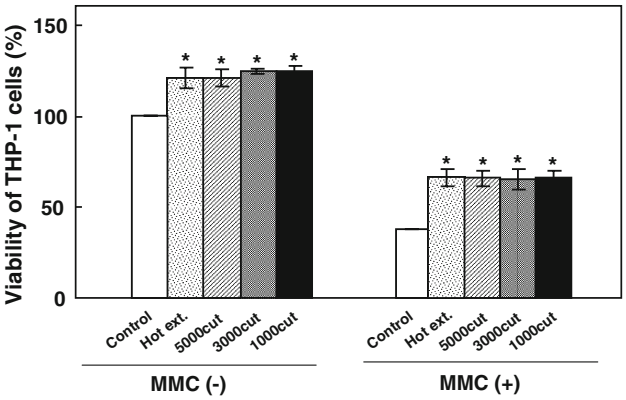

Fig. 1 Effects of hot extract, 5000-cut, 3000-cut, and 1000-cut extracts on the viability of mitomycin C (MMC)-treated THP-1 cells. The effects of hot extract, 5000-cut, 3000-cut, and 1000-cut extracts on THP-1 cells (1 µl in a 96-well plate) on viability were investigated in the presence or absence of 10 µM MMC for 15 h. Values are represented as mean ± SD of three independent experiments

reagent and found no toxicity (data not shown). These results suggest the existence of a promising low molecular weight product in hot extract, 5000-cut, 3000-cut, and 1000-cut that enhances viability of THP-1 cells. This effect is independent of the polysaccharide, MD-fraction, which has no effect on viability of THP-1 cells.

To avoid the influence of contaminating higher MW substances including MD-fraction and immunostimulating factors such as lipopolysaccharide (LPS), we investigated the effects of the 1000-cut extract on the viability of the human and murine blood immune cell lines (Fig. 2). We used the following cell lines: human THP-1 as a monocyte; murine RAW 264.7 and J774A.1 as a macrophage; human NK-98MI as a NK cell; human Jurkat as a T cell; human HL60 as a promyelogenous leukemic cell; human KG-1 as a myelogenous leukemic cell; and human KG-1a as a variant subclone of KG-1, composed of undifferentiated promyeloblasts. The 1000-cut extract significantly increased the viability of the Jurkat and THP-1 cells in the absence of MMC. In contrast, in the presence of MMC treatment with 1000-cut promoted the viability of RAW 264.7 cells and THP-1 cells but not Jurkat cells. These results show that the extract affected the THP-1 cells rather than the Jurkat cells and more than other cells of the immune system. Regarding promyelocytic cells (HL60) and myelocytic cell lines (KG-1 and KG-1a), the viability of HL60 cells was improved by the extract but KG-1 cells and KG-1a cells were not influenced. Because the HL60 cell line was originated from peripheral blood and HL60 cells can differentiate to granulocytes and monocytes spontaneously, the 1000-cut extract may have acted on differentiated cells, a part of HL60 cells. In contrast, KG-1 and KG-1a cell lines were originated from bone marrow and KG-1 cells can differentiate to granulocyte- and

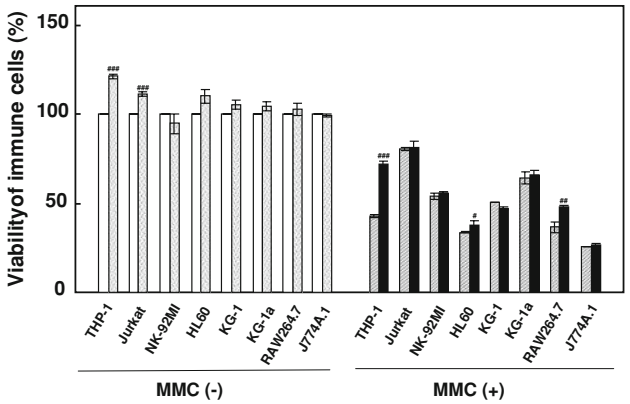

Fig. 2 Effects of 1000-cut extract on the viability of human and mouse immune cell lines treated with MMC. The effects of 1000-cut (1 μl in a 96-well plate) on THP-1 cells, Jurkat cells, NK-98MI cells, HL60 cells, KG-1 cells, KG-1a cells, RAW 264.7 cells, and J774A.1 cells on viability were investigated in the presence or absence of 10 μM MMC for 15 h. Values are represented as mean ± SD of three independent experiments. *Open bars*, nontreated; *dotted bars*, 1000-cut-treated; *slashed bars*, MMC-treated; *filled bars*, 1000-cut + MMC-treated

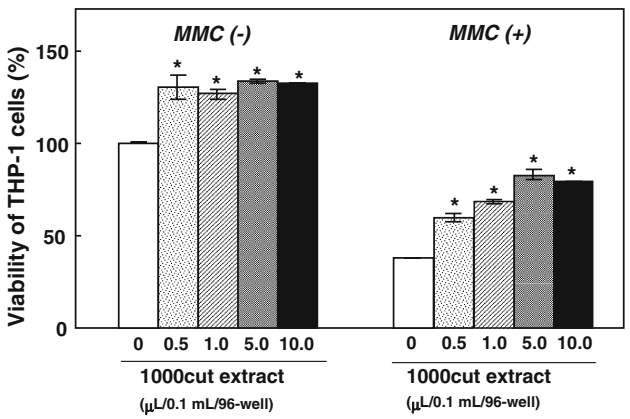

Fig. 3 Effects of various concentrations of 1000-cut extract on the viability of MMC-treated THP-1 cells. The effects of 1000-cut (0, 0.1, 1.0, 5.0, or 10.0 μl in a 96-well plate) on viability were investigated in the presence or absence of 10 μM MMC for 15 h. Values are represented as mean ± SD of three independent experiments

macrophage-like cells, but KG-1a cannot. KG-1a cells are morphologically, cytochemically, and functionally less mature than the parent KG-1. From these results, we suggest that the extract might interact with a receptor expressed on differentiated immune cells such as monocytes, macrophages, and T cells.

We next investigated the optimum concentration of 1000-cut extract, resulting in increased viability of cells treated with MMC, and found an increase from 38% to 83% viability with addition of 5.0 μl 1000-cut extract to 100 μl culture volume (Fig. 3). The same profile was not seen in the absence of MMC treatment because of the medium limitations of the cell culture.

Table 1 Effects of 1000-cut extract on gene expression of cytokines by mitomycin C (MMC)-treated THP-1 cells

Gene	Signal		Change	Change *P* value
	Control	1000-cut extract		
IL-1α	198.2	169.1	NC	0.50000
IL-1β	664.4	612.8	NC	0.42914
IL-6	90.7	14.3	NC	0.99590
IL-8	8068.9	11004.0	I	0.00005*
IL-10	19.1	11.3	NC	0.50000
IL-12 p35	12.4	16.9	NC	0.24259
TNF-α	437.1	578.3	I	0.00013*

NC no change; *I* increase; *$P < 0.001$, in comparison to the control

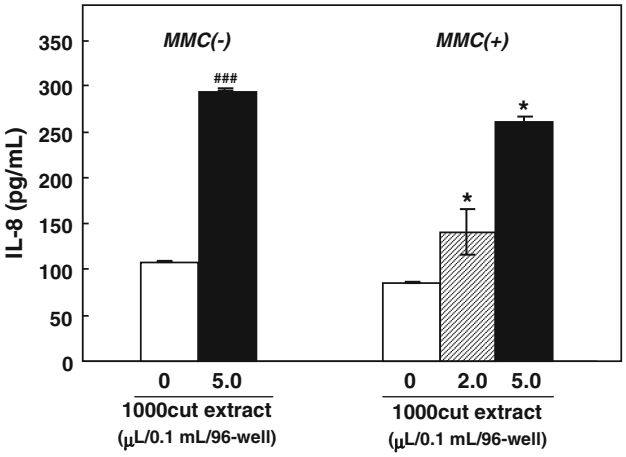

Fig. 4 Effects of various concentrations of 1000-cut extract on interleukin (IL)-8 production by MMC-treated THP-1 cells. The effects of 1000-cut (0, 2.0, or 5.0 μl in a 96-well plate) on IL-8 production were investigated in the presence or absence of 10 μM MMC for 15 h. Values are represented as mean ± SD of three independent experiments

To investigate the effect of the 1000-cut extract on the production of cytokines by monocytes, we performed a DNA microarray for MMC- and 1000-cut-treated THP-1 cells. The results indicated that the expression of IL-8 and tumor necrosis factor (TNF)-α were slightly but specifically increased in response to 1000-cut extract, whereas other cytokines produced by monocytes and macrophages such as IL-1, IL-6, IL-10, and IL-12 were unchanged (Table 1). To confirm the microarray result, we assayed by ELISA the production of TNF-α and IL-8 by THP-1 cells with MMC and 1000-cut extract. The extract significantly increased IL-8 production in treated THP-1 cells (Fig. 4), whereas production of TNF-α was not observed in the culture medium although intracellular TNF-α production was increased. In cells treated with 1000-cut extract alone, the maximal dose of 1000-cut extract increased IL-8 production by 2.7 times. Although MMC decreased baseline of IL-8 production to 0.8 times that of the control, addition of

1000-cut extract increased IL-8 production to 2.4 times that of the control.

In a previous study, the extract significantly increased the viability of normal mouse splenic cells from 100% to 188.9% in vitro. In addition, in an animal experiment, we had found that the viability of normal mouse splenic cells had been increased after administration of the extract for 7 consecutive days, in which macrophages were activated (unpublished data). In contrast, there were no data about human immune cells. In our study, the hot water-soluble extract from the edible maitake mushroom (*G. frondosa*) was found to possess immunostimulatory activity for the human monocyte cell line, THP-1, by increasing its viability and increasing production of IL-8, which activates neutrophils (Figs. 1, 2, 3, and 4; Table 1). These results suggest that the extract may enhance the immune system not only in healthy people but also in people exposed to toxic agents. We also found identical activities of the extract and the filtrated extracts without any dilution, concentration, and addition of organic solvent (see Fig. 1). Small amounts of each extract were enough to promote the viability and IL-8 production by THP-1 cells. After addition of ethanol at a final concentration of 50% to the hot water-soluble extract, the precipitates no longer had an effect on THP-1 cells similar to what was observed with MD-fraction, whereas the supernatant retained the ability to increase viability (data not shown). Therefore, we thought that the activity of the extract must be dependent on water-soluble materials with low molecular weight. The active portion of the extract thus is thought to be absorbed through the bloodstream, intestine, and skin, where phagocytic cells such as neutrophils, monocytes, macrophages, and dendritic cells are present. About 60% of peripheral blood mononuclear cells are neutrophils, which are activated and induced to undergo chemotaxis by IL-8. The intake of the extract or the filtrated extracts daily could contribute to preventing cancer and infectious diseases such as those caused by bacteria, fungi, and viruses.

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