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Inhibitory Effect of MD-Fraction on Tumor Metastasis: Involvement of NK Cell Activation and Suppression of Intercellular Adhesion Molecule (ICAM)-1 Expression in Lung Vascular Endothelial Cells

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The anti-metastatic activity of MD-Fraction extracted from the maitake mushroom (*Grifola frondosa*) was examined in an experimental murine model of lung metastasis. Intraperitoneal administration of MD-Fraction 2 d before tumor implantation significantly inhibited lung metastasis of colon-26 carcinoma and B16/BL6 melanoma cells. In this model, MD-Fraction enhanced IL-12 production from antigen presenting cells (APCs). MD-Fraction treatment activated NK cells and increased cytotoxicity against YAC-1 and colon-26 carcinoma cells. Furthermore, the depletion of NK cells with anti-asialo GM1 abolished the inhibitory effect of MD-Fraction on lung metastasis of colon-26 cells. *Ex vivo*, B16/BL6 cell adhesion to LPS-activated murine lung vascular endothelial cells was inhibited by MD-Fraction and anti-intercellular adhesion molecule (ICAM)-1 antibody. These results suggest that MD-Fraction inhibits tumor metastasis by activating NK cells and APCs, and by suppressing of ICAM-1 leading to the inhibition of tumor cell adhesion to vascular endothelial cells.

Key words *Grifola frondosa*; metastasis; natural killer; intercellular adhesion molecule-1

Recently, the number of patients with lung cancer has been increasing worldwide. Therapeutic modalities for metastatic lung cancer include surgery, radiotherapy and chemotherapy. Chemotherapy, in particular, is beneficial for preventing further metastasis as it affects not only metastatic lesions but also tumor cells in the blood stream due to the systemic effect of antitumor agents. However, chemotherapy is often associated with serious side effects that lower patients' Quality of Life significantly. Subjective side effects include nausea, anorexia, general fatigue and alopecia, while objective side effects such as anemia, thrombocytopenia and leucopenia, which are detectable by laboratory tests, cause immuno-suppression. To attenuate the severity of such events, an immuno-enhancing strategy with concomitant administration of Biological Response Modifer (BRM) agents including lentinan[1] from *Lentinus edodes* and Protein-bound Polysaccharide K (PSK)[2] from *Coriolus versicolor* is applied in the clinical setting.

We extracted (β1,3)-branched (β1,6)-glucan from the fruit body of the maitake mushroom (*Grifola frondosa*), which acts as a BRM agent and named it MD-Fraction. MD-Fraction exhibits antitumor activity against solid tumors of MM-46 carcinoma cells (breast cancer) and colon-26 carcinoma cells (colorectal cancer). The mechanism of such antitumor activity is that MD-Fraction not only activates innate immunity, including activation of IL-12 production by antigen-presenting cells (APCs), resulting in activation of NK cells, but also activates adaptive immunity, including dominant induction of Th-1 response, resulting in activation of cytotoxic T cells.[3—8] We have also reported that when Mitomycin-C is given concomitantly with MD-Fraction in a dose low enough not to cause side-effects, it can produce an antitumor effect that can only be achieved when it is given alone at a standard dose.[9]

NK cells, a major component of innate immunity, have the ability to kill tumor cells that have lost expression of MHC class I.[10] NK cells activated and its cytotoxic activity enhanced by numerous cytokines including IL-2, IL-12, IL-15, IL-18, IL-21, and IFN-α/β.[11—15] It has been reported that NK cells play important role as a primary effector cells for the control of metastasis.[16—18]

Metastasis is accomplished through a series of processes in which the tumor cells detach themselves from the primary lesion, which is followed by vascular or lymphatic invasion and their subsequent adhesion to other tissues. Each metastatic process from invasion to adhesion can be assessed by using an animal model of experimental lung metastasis. Adhesion molecules, such as ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin play a critical role in this model, and its inhibition may prevent metastasis.[19,20] It has been reported that the sugar chain exhibits an antimetastatic effect by its inhibitory effect on adhesion factor expression.[21,22]

This study therefore investigated whether the inhibitory effect of MD-Fraction on lung metastasis was due to activation of NK cells and suppression of adhesion molecule expression in lung vascular endothelial cells.

MATERIALS AND METHODS

Animals BALB/cA female mice at the age of 5 weeks (Clea Japan, Inc., Tokyo, Japan) and BDF1 female mice at the age of 10 weeks (Japan SLC, Inc., Shizuoka, Japan) were provided with drinking water and solid chow *ad libitum* until they were used for experiment.

Tumor Cell Line Cultured colon-26 carcinoma cell line provided by Kitasato University was implanted in 6-week-old BALB/cA female mice *via* the caudal vein and re-collected from nodules formed in the lungs. This procedure was repeated twice. For the experiment, we used cultured colon-26 carcinoma cell line with an affinity for lung tissue with less than 10 subculture passages. The murine lymphoma cell line YAC-1 (ATCC number: TIB-160) was purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and

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maintained in RPMI-1640 medium supplemented with 10% heat-inactivated bovine serum (FBS).

Antibodies Flow cytometry analysis was performed using the following monoclonal antibodies; anti-CD16/CD32 (Fc blocker), FITC-conjugated anti-CD49b, PE-conjugated anti-CD69, Cy-Chrome-conjugated anti-CD3 (BD Bioscience, San Jose, CA, U.S.A.), biotinylated anti-E-selectin (R & D Systems, Inc., Minneapolis, MN, U.S.A.), FITC-conjugated anti-CD54/ICAM-1, and PE-conjugated anti-CD106/VCAM-1 (Beckman Coulter, Inc., Fullerton, CA, U.S.A.). For ELISA, monoclonal anti-mouse IL-12 (clone C17.8) (Biolegend, Inc., San Diego, CA, U.S.A.), biotinylated anti-mouse IL-12 (PEPRO TECH EC, Inc., London, U.K.), and peroxidase conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.) were used.

Preparation of MD-Fraction MD-Fraction was prepared from dried powder of the fruit body of the Maitake mushroom (Yukiguni Maitake Co., Ltd., Niigata, Japan) as described previously.[7)]

Antimetastatic Activity The antimetastatic activity of MD-Fraction was studied when it was given to mice before and after the tumor cells were implanted. When MD-Fraction was initiated before the tumor cells (day 0), each group of BALB/cA female mice (6 weeks old) received 1.0, 4.0, or 8.0 mg/kg/d of MD-Fraction (as sugar weight) intraperitoneally once daily for 16 consecutive days (the pretreatment method). Mice in the control group received saline. Forty-eight hours after the first treatment of MD-Fraction, the mice had colon-26 carcinoma cells (2.5×10^4 cells/mouse) implanted *via* the caudal vein.

When the MD-Fraction was initiated after the tumor cell implantation (non-pretreatment method), each group of BALB/cA female mice (6 weeks old) was implanted with tumor cells. Intraperitoneal administration of MD-Fraction with 1.0, 4.0, or 8.0 mg/kg/d (as sugar weight) was initiated 24 h later and repeated once daily for 13 consecutive days. On day 14 post-implantation, the lung tissue was isolated and fixed in Bouin's solution for 24 h at 4 °C. The inhibitory effect on metastatic nodule formation was evaluated by grossly counting the number of nodules.

Incubation of Peritoneal Exudate Cells and Splenocytes Intraperitoneal administration of MD-Fraction (8 mg/kg/d) to each group of BALB/cA mice was initiated 48 h before implantation of tumor cells and repeated once daily. After 24 h (day 1) or 72 h (day 3) of tumor implantation, the mice were dissected. Splenocytes were collected and peritoneal exudate cells were harvested from the peritoneal cavity with PBS (−)+0.02% EDTA. After centrifugation ($3000\times\boldsymbol{g}$, 5 min, 4 °C), splenocytes (1×10^6 cells/well) and peritoneal exudate cells (1×10^5 cells/well) were seeded into a 96-well plate with RPMI-1640 medium containing 5% FBS, and incubated for 24 h at 37 °C in the presence of 5% CO_2.

Cytokine Assay After incubation of splenocytes and peritoneal exudate cells, the supernatant was collected and IL-12 assay was performed by ELISA.

Cytotoxicity Assay The splenocytes were incubated with anti-CD49b (DX-5) MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and DX-5^+ cells (NK cells) isolated using MACS Separation columns (Miltenyi Biotec GmbH). The NK cells collected were detected as CD49b^+ cells using flow cytometry and the recovery rate was confirmed to be 85% or more. The densities of effector cells (splenic NK cells) were prepared at 1×10^7, 0.2×10^7, 0.04×10^7 cells/ml with RPMI-1640 medium. The target cells (5×10^6 cells of YAC-1 cell or colon-26 cells) were suspended in 1 ml of RPMI-1640 medium, added to 15 μl of 0.1% Calcein-AM (in DMSO), then incubated for 45 min at 37 °C, in the presence of 5% CO_2. After incubation was completed, the cells were washed twice with 15 ml of PBS (−)+0.02% EDTA, then adjusted to a density of 1×10^6 cells/ml with RPMI-1640 medium. A mixture of 50 μl of effector cell preparation and the same volume of target cell preparation was prepared and 50 μl of Spontaneous medium (RPMI-1640 medium with 30% FBS) or Maximum medium (RPMI-1640 medium with 30% FBS and 6% Triton X-100) was added. Subsequently, the mixture was slowly agitated, then incubated for 2 h at 37 °C in the presence of 5% CO_2. Once incubation was completed the supernatant was collected and centrifuged at $300\times\boldsymbol{g}$ for 10 min at 4 °C to precipitate the suspended cells. Seventy-five microliters of supernatant was added to each well of a 96-well plate and its fluorescence strength was measured with an excitation wavelength of 485 nm, and a detection wavelength of 538 nm using Fluoromark (BIO-RAD Lab., Hercules, CA, U.S.A.). Cytotoxic activity was calculated using the following formula.[23,24)] Cytotoxicity (%)=(spontaneous release−test release)/(spontaneous release−maximum release)×100.

Antimetastatic Effect of MD-Fraction in NK Cell Deficient Model Mouse Intraperitoneal administration of MD-Fraction to BALB/cA mice was initiated 48 h before tumor cell implantation and followed by 2 consecutive administrations once daily. Subsequently, the mice received 0.2 mg of anti-asialo-GM1 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) *via* the caudal vein.[25)]

Inhibitory Effect on the Expression of Adhesion Factor in Lung Vascular Endothelial Cells in Mice Treated with MD-Fraction BDF1 female mice (10 weeks old) received MD-Fraction for 3 d and were sacrificed by exsanguinations. A cannula was inserted from the inferior vena cava and ligated at the right atrium. Another cannula was inserted and ligated at the left atrium. HEPES-Tyrode solution heated to 37 °C and containing 0.01% collagenase, 0.02% dyspase, and 0.005% trypsin was perfused for 20—30 min through the cannula inserted into the right atrium. The perfused solution was passed through a nylon-wool column, adjusted to a density of 5×10^4 cells/ml with RPMI-1640 medium containing 0.2% ECGS, then seeded to a 48-well plate at a volume of 0.2 ml/well (purity of endothelial cells, 89—95%). Implantation was confirmed 2 h after completing incubation and the medium was changed to a fresh one. LPS was added to the well to adjust the final concentration to 1 μg/ml, and further incubated for 2 h. After changing to fresh medium, the cells were incubated for 14 h, the medium was changed again, and 0.1 ml of fluorescent-labeled B16/BL6 melamoma cells (2×10^4 cells/ml) with CSFE was added to the well, followed by 30 min of incubation. The supernatant was removed and fluorescent strength was measured with an excitation wavelength of 448 nm, and a detection wavelength of 530 nm. ICAM-1, VCAM-1, and E-selectin expressed in vascular endothelial cells were measured with labeled antibody for

ICAM-1, VCAM-1, and E-selectin (Seikagaku Corp., Tokyo, Japan) respectively.

Statistical Analysis The measurements obtained from the experiment were expressed with mean±standard error (Mean±S.E.). The data were statistically analyzed using Student's *t*-test. The statistical significance level was set at a p value of less than 0.05. Comparison among 3 or more groups was performed using Scheffe's or Dunnett's multiple comparison procedure.

RESULTS

Difference in Antimetastatic Effect Resulting from the Timing of the Initiation of MD-Fraction Treatment We examined which administration mode of MD-Fraction, either before or after implantation of tumor cells, could inhibit lung metastasis. An inhibitory effect was observed only when MD-Fraction was administrated before implantation. As shown in Fig. 1, the number of nodules in murine lung in the MD-Fraction non-pretreatment group was not reduced, but pre-treatment group was significantly reduced to 31.8% of that of the control group. This result suggested that exerting cytotoxic activity against tumor cells by innate immune response is essential in exhibiting the inhibitory effect of MD-Fraction on lung metastatic nodule formation.

The Effect of MD-Fraction on IL-12 Production In innate immune response, NK cells which non-selectively destroy foreign bodies play an important role as well as APCs, the initiators of immune response, such as macrophages and dendritic cells. We have previously reported that MD-Fraction activates APCs and enhances IL-12 production in tumor-bearing mice.[8] Hence, in a murine model of lung metastasis, we speculated that IL-12 produced by APCs that have been activated by MD-Fraction increase the activation of NK cells, which result in the inhibition of lung metastasis. We conducted this investigation in order to elucidate such a mechanism. The effect on IL-12 production was examined in the pretreatment method. As shown in Fig. 2A, IL-12 production by peritoneal excluded cells in treatment group was twice as high on day 1 and 2.6 times on day 3 than the control group. While, IL-12 production by whole spleen cells in treatment group was higher 1.65 times on day 1 and 1.4 times on day 3 compared with control group (Fig. 2B).

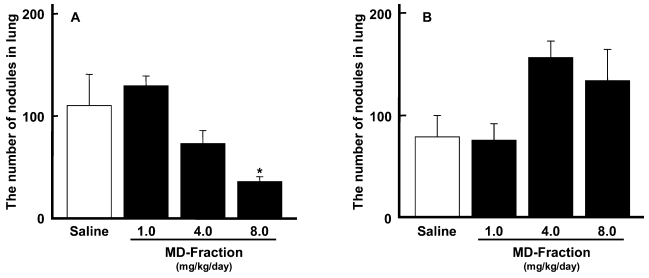

Fig. 1. Effect of MD-Fraction on Colon-26 Cells Metastasis

Colon-26 cells (2.5×10^4 cells) were implanted into the tail vein (day 0). (A) Each group of BALB/cA female mice received 1.0, 4.0, or 8.0 mg/kg/d of MD-Fraction intraperitoneally once daily for day −2 to day 13 (the pretreatment method). (B) Intraperitoneal administration of MD-Fraction with 1.0, 4.0, or 8.0 mg/kg/d was once daily for day 1 to day 13 (non-pretreatment method). On day 14 tumor implantation, the lung tissue was isolated and fixed in Bouin's solution. The metastatic nodules were counted grossly. Data are presented as means and standard error of 1 experiment (4—5 mice/experiment). Significant difference, Dunnett's test. (∗ $p<0.05$).

The Effect of MD-Fraction on Splenic NK Cells We observed that MD-Fraction enhance IL-12 production by APCs. Since IL-12 consists of 2 subunits, p40 and p35 and is an NK cell-activating cytokine,[26] we assessed NK cell activation by the increase in IL-12 production. Tumor cells were implanted after pretreatment with MD-Fraction and spleen weight was measured on day 1 and day 3 after implantation. MD-Fraction treatment increased the spleen weight by 1.46 times on day 1 and 1.78 times on day 3 (Fig. 3). The percentage of $CD69^+$ NK cells in whole spleen cells was increased in 1.25 times on day 1 and 1.48 times on day 3 (Fig. 4). NK cells were isolated from whole spleen cells and evaluated for their cytotoxic activity against NK sensitive YAC-1 cells or colon-26 cells. As shown in Figs. 5A and B, enhancement of cytotoxic activity by MD-Fraction was observed both in YAC-1 and colon-26 cells. Furthermore, depletion of NK-cells with anti-asialo GM1 antibody did not show a decrease in the number of nodules despite MD-Fraction treatment (Figs. 6A, B). These results demonstrate that the antimetastatic activity of MD-Fraction is mediated by NK cells.

Inhibitory Effect of MD-Fraction on Lung Metastasis of Tumor Cells Derived from the Inhibition of Adhesion Factor Expression B16/BL6 melanoma cells were injected into tail veins of BDF1 mice. It was resulted that the number of nodules in murine lung in the MD-Fraction treated group was significantly reduced to 20% of that of the control group (data no shown). We examined the inhibitory effect of MD-

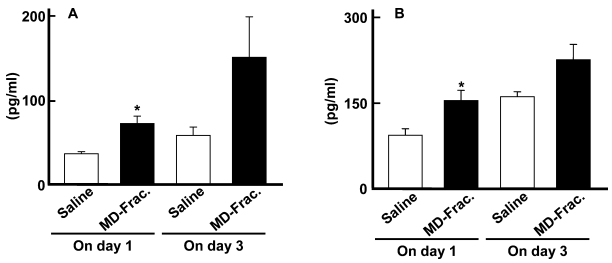

Fig. 2. Effect of MD-Fraction on IL-12 Production by Peritoneal Exudate Cells and Whole Spleen Cells of Colon-26-Bearing Mice

Continuous administration of MD-Fraction (8 mg/kg/d) was initiated 2 d before colon-26 cells (2.5×10^4 cells) implantation. After 24 h (day 1) or 72 h (day 3) of tumor implantation, peritoneal exudate cells were harvested from the peritoneal cavity and splenocytes were collected. (A) peritoneal exudate cells (1×10^5 cells/well) and (B) splenocytes (1×10^6 cells/well) were seeded into a 96-well plate with RPMI-1640 medium containing 5% FBS, and incubated for 24 h. The supernatant was collected and IL-12 assay was performed by ELISA. Data are presented as means and standard error of different 2—6 experiments. Significant difference, Student's *t*-test (∗ $p<0.05$).

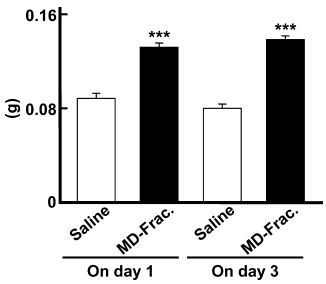

Fig. 3. Effect of MD-Fraction on Spleen Weight of Colon-26-Bearing Mice

Continuous administration of MD-Fraction (8 mg/kg/d) was initiated 2 d before colon-26 cells (2.5×10^4 cells) implantation. After 24 h (day 1) or 72 h (day 3) of tumor implantation, the spleens were weighed. Data are presented as means and standard error of different 3—6 experiments. Significant difference, Student's *t*-test (∗∗∗ $p<0.001$).

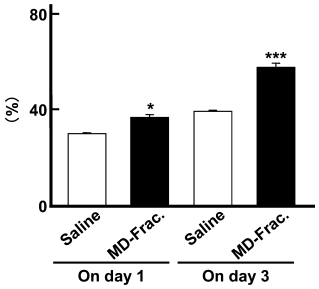

Fig. 4. Effect of MD-Fraction on Splenic NK Cell Activation of Colon-26 Bearing-Mice

Continuous administration of MD-Fraction (8 mg/kg/d) was initiated 2 d before colon-26 cells (2.5×10^4 cells) implantation. After 24 h (day 1) or 72 h (day 3) of tumor implantation, the splenocytes were collected and the percentage of activated NK cells ($CD49b^+CD3^-CD69^+$ cells) in splenic NK cells ($CD49b^+CD3^-$ cells) was analyzed by flow cytometry. Data are presented as means and standard error. Significant difference, Student's *t*-test (*** $p<0.001$, * $p<0.05$).

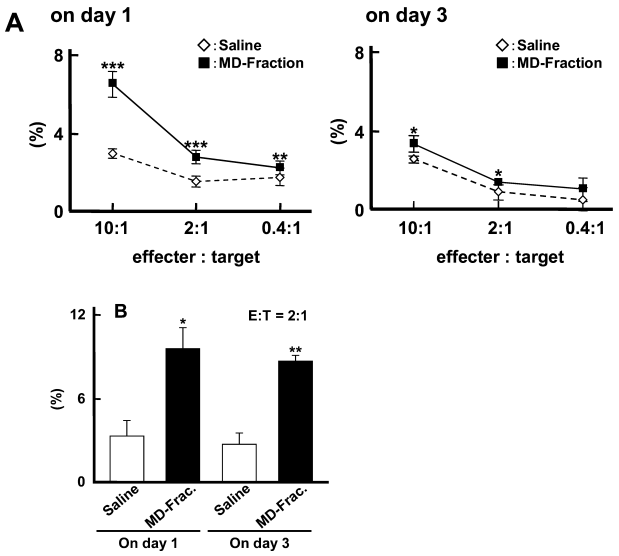

Fig. 5. Effect of MD-Fraction on Cytotoxicity of Splenic NK Cell of Colon-26-Bearing Mice against YAC-1 Cell (A) or Colon-26 Cell (B)

Continuous administration of MD-Fraction (closed square) or saline (open lozenge) was initiated 2 d before colon-26 cells (2.5×10^4 cells) implantation. After 24 h (day 1) or 72 h (day 3) of tumor implantation, the splenic NK cells isolated from whole spleen cells. The splenic NK cells and tumor cells labeled Calcein-AM were mixed and incubated for 2 h. After incubation was completed, fluorescence strength of the supernatant was measured with an excitation wavelength of 485 nm, and a detection wavelength of 538 nm. Data are presented as means and standard error of different 2 experiments. Significant difference, Student's *t*-test (*** $p<0.001$, ** $p<0.01$, * $p<0.05$).

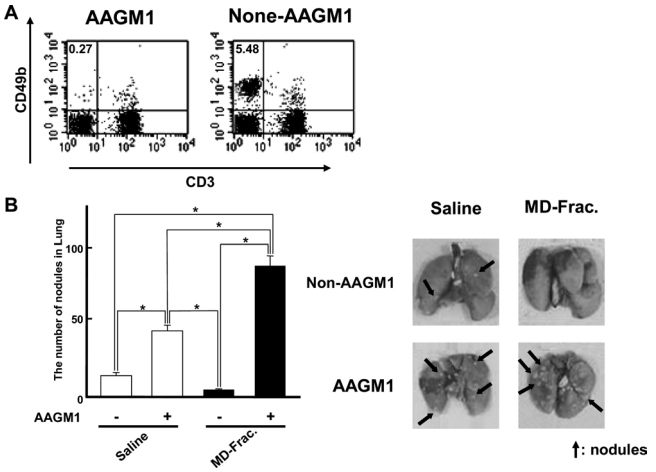

Fig. 6. Effect of MD-Fraction on NK Cell Depletion in Colon-26 Bearing-Mice

Continuous administration of MD-Fraction (8 mg/kg/d) was initiated 2 d before colon-26 cells (2.5×10^4 cells) implantation. After 2 consecutive administrations of MD-Fraction, the mice received 0.2 mg of anti-asialo-GM1 *via* the caudal vein. (A) The percentage of NK cells ($CD49b^+CD3^-$ cells) in peripheral white blood cells was analyzed by flow cytometry at 5 d after anti-asialo-GM1 administration. (B) On day 14 tumor implantation, the lung tissue was isolated and fixed in Bouin's solution. The metastatic nodules were counted grossly. Data are presented as means and standard error of different 2 experiments. Significant difference, Sheffe's test. (* $p<0.05$).

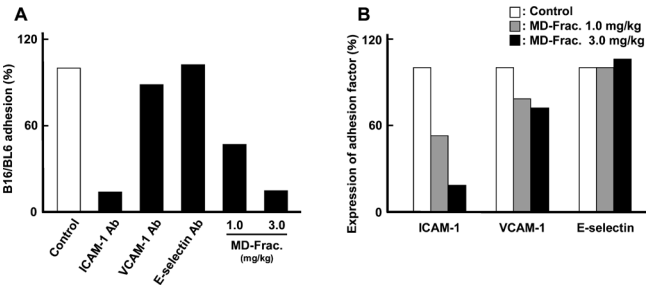

Fig. 7. Effect of MD-Fraction on Expression of Adhesion Factor

(A) LPS-treated lung vascular endothelial cells were incubated with 0.1 ml of CSFE-labeled B16/BL6 melamoma cells (2×10^4 cells/ml), followed by 30 min of incubation. The supernatant was removed and fluorescent strength was measured with an excitation wavelength of 448 nm, and a detection wavelength of 530 nm. (B) ICAM-1, VCAM-1 and E-selectin expressed in vascular endothelial cells were measured with labeled antibody for ICAM-1, VCAM-1, and E-selectin respectively. Data are presented as means and standard error of 1 experiment (4—5 mice/experiment).

Fraction on adhesion factor expression. In the normal BDF1 mice, lung vascular endothelial cells were stimulated by LPS to express ICAM-1, VCAM-1, and E-selectin, resulting in a remarkable increase in adhesion of B16/BL6 melanoma cells to these cells. The adhesion of tumor cells was not influenced by the addition of a 1 μg/well of neutralizing antibody against VCAM-1 or E-selectin. On the other hand, cell adhesion was remarkably inhibited by the addition of ICAM-1 neutralizing antibody (Fig. 7A). The lung vascular endothelial cells in mice treated with MD-Fraction also expressed VCAM-1 and E-selectin by LPS stimulation, but ICAM-1 expression showed a dose-dependent decrease with MD-Fraction treatment (Fig. 7B). Furthermore, adhesion of B16/BL6 melanoma cells to the lung vascular endothelial cells was decreased to 50—80% in MD-Fraction treated mice. Thus, MD-Fraction treatment caused selective inhibition of ICAM-1 among cell adhesion factors. This result indicates that MD-Fraction indirectly inhibits B16/BL6 melanoma cell adhesion-mediated metastasis formation.

DISCUSSION

We have found that MD-Fraction inhibits tumor cell growth by dominantly inducing Th-1 immune response and by activating cytotoxic T cells in solid-tumor-bearing mice as well as alleviating the side effects of anticancer drugs. We have also reported that MD-Fraction exhibits strong antitumor activity when given concomitantly with an anticancer drug.[9] In this study, we employed an animal model of lung metastasis to assess the inhibitory effect of MD-Fraction on lung metastasis excluded the processes of detachment and invasion found in natural metasitasis.

In the murine model of experimental lung metastasis, no T cell activation in the spleen during adaptive immune response was observed (data not shown). Therefore, we rea-

soned that MD-Fraction exhibits an antimetastatic effect by activating the innate response and by exerting cytotoxic activity against tumor cells in the blood stream during the metastatic process. We performed this investigation to confirm this hypothesis. As a result, we found that MD-Fraction can exhibit an antimetastatic effect by strongly activation the innate immune response when it is treated before tumor cell implantation. IL-12 production by peritoneal exudate cells and by splenocytes was measured to evaluate immune response. IL-12 production was increased both by peritoneal exudate cells and by splenocytes. IL-12 is a cytokine produced by activated APCs and activates NK cell. Thus, the activation and cytotoxic activity of NK cells were examined. Both were enhanced by MD-Fraction treatment. In addition, in an experiment using mice, where NK cells had been depleted with anti-asialo GM1 antibody, antimetastatic activity was clearly decreased. This demonstrates that NK cells were strongly involved in antimetastatic activity.

Since metastasis formation by tumor cells requires production of adhesion factors,[19,20] we assessed the effect of MD-Fraction on this. MD-Fraction inhibited ICAM-1 expression of vascular endothelial cells. This indicates that MD-Fraction inhibits metastasis by blocking the adhesion of tumor cells to lung tissue. However, why MD-Fraction only inhibits the ICAM-1 expression is not clear and this is currently under investigation. In this study, we revealed the inhibitory capacity of MD-Fraction in the production of adhesion factors, which indicates its effectiveness in metastitic inhibition.

As described above, MD-Fraction inhibits adhesion of tumor cells to vascular endothelial cells and exerts cytotoxic activity against tumor cells in the blood stream *via* NK cell activation during systemic circulation. With these two mechanisms, MD-Fraction exhibits antimetastatic activity against various cancers.

In this study, we demonstrated that MD-Fraction exhibits an antimetastatic effect when MD-Fraction was administrated before implantation of tumor cells. We reported previously that MD-Fraction inhibits carcinogenesis in mouse model induced by *N*-nitrosodi-*n*-butylamine.[27] *Grifola frondosa*, an edible mushroom, has been widely eaten in Asia and it's safe to take for the long term. Since MD-Fraction also exhibits an antitumor effect by oral administration,[28] MD-Fraction is effective for cancer prevention and inhibition of tumor metastasis if people eat regularly.

Acknowledgements The authors appreciate Yukiguni Maitake Co., Ltd. for supplying maitake and also thank Ms. Fumiko Yamamoto for supporting of experiment. We wish to thank Dr. Marry Bareilles for correcting this article.

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