# Effects of D-Fraction, a Polysaccharide from *Grifola frondosa* on Tumor Growth Involve Activation of NK Cells

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**Natural killer (NK) cells are directly cytotoxic for tumor cells and play a primary role in regulating immune responses. We monitored levels of NK cell cytotoxic activity in cancer patients receiving D-Fraction extracted from maitake mushrooms (*Grifola frondosa*). Elevated levels of cytotoxic activity were maintained for one year. To elucidate the mechanisms underlying long-term activation of NK cells during treatment with D-Fraction, we examined tumor volume and levels of IFN-$\gamma$ and TNF-$\alpha$ in MM46-bearing C3H/HeN mice to which D-Fraction was administered for 19 d. D-Fraction markedly suppressed tumor growth, corresponding with increases in TNF-$\alpha$ and IFN-$\gamma$ released from spleen cells and a significant increase in TNF-$\alpha$ expressed in NK cells. This suggests that the D-Fraction activates NK cells even on the 20th day after treatment. Furthermore, D-Fraction increased macrophage-derived interleukin (IL)-12, which serves to activate NK cells. These results suggest that NK cells are not only responsible for the early effects of D-Fraction on tumor growth, but also for the long-term tumor-suppressive effects of D-Fraction through increased IL-12 released from macrophages.**

**Key words** IL-12 production; macrophage; NK cell

We previously reported that the (1→3)-branched (1→6)-$\beta$-glucan termed D-Fraction, extracted from the fruit body of the maitake mushroom (*Grifola frondosa*) (Fig. 1), is a biological response modifier (BRM) like lentinan, which is found in *Letinus edodes*.[1—3] These polysaccharides enhance the activity of immunocompetent cells such as macrophages, helper T cells, and cytotoxic T cells, which attack tumor cells. These effects of BRMs led us to hypothesize that oral administration of D-Fraction would be an effective treatment for cancer patients. Orally administered D-Fraction was shown to reduce tumor size in mice, without causing unwanted side effects.[2] Therefore, D-Fraction has been commercialized as a health material, the safety of which has been confirmed by the Consumer Product Testing Co. (New Jersey, U.S.A.) We have already tried a non-randomized clinical trial of D-Fraction therapy for patients with lung and breast cancer in stages II—IV.[4] In this trial, we found that IL-2 production was enhanced in D-Fraction-treated patients, suggesting that macrophages and T cells were activated by D-Fraction. In addition, D-Fraction is revealed to be effective for human immunodeficiency virus (HIV) infection[5] and listeriosis,[6] by reinforcing the immune system. These results indicate that one network of the immuno-response pathway by which D-Fraction enhances anti-tumor effects is dependent on an adaptive immunity associated with cytotoxic T cell activation.

Natural killer (NK) cells rapidly recognize and lyse a large variety of tumor or virus-infected cells, without the need for either prior sensitization or MHC-dependent recognition, which is different from cytotoxic T cell.[7] Pham-Nguyen *et al.* have reported that the early NK cell response alone is not sufficient for tumor clearance, and that both T cells and NK cells are required in the development of the long-term survival in the hepatic tumor model by IL-12-mediated gene therapy.[8] In our clinical study, we also found that D-Fraction activated NK cells in lung, breast and liver carcinoma patients for long periods of time, exceeding one year (Table 1). To investigate maintenance of NK cell activation by D-Fraction, we examined NK cell activation in MM-46 carcinoma-bearing mice treated with D-Fraction for 19 d. Our data in this paper may support a critical role for NK cell activation in immunotherapy with D-Fraction for cancer patients.

## MATERIALS AND METHODS

**Materials** For the detection of human NK activity, chromium-51 (Daiichi Kagaku Yakuhin Co., Tokyo) and lymphopacel (d=1.077) (IBL Co., U.S.A.) were prepared.

**Animals** Male C3H/HeN mice (4-weeks-old) were obtained (Japan Crea Co., Osaka) and were raised for one week before being used for experiments. Food and water were given freely to these mice until used for experiments.

**Cells** MM-46 carcinoma cells were kindly donated by Dr. Kanki Komiyama. $^{51}$Cr labeled K-562 cells were used as target cells of human NK.

**Preparation of D-Fraction** D-Fraction was prepared from the dried powder of the fruit body of maitake mushrooms (*Grifola frondosa*) (Yukiguni Maitake Co., Niigata), according to the method described in our previous paper.[6] The level of LPS contained in D-Fraction was determined by using Endospecy ES-20S Set (Seikagaku Industry Co., Tokyo), and the ratio (%) of LPS in D-Fraction was less than

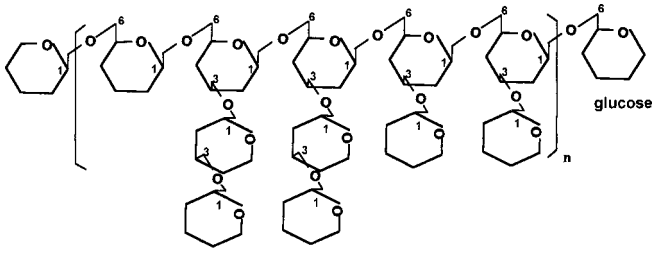


Fig. 1. Chemical structure of D-Fraction

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0.000007%. In the experiment using macrophage cell line RAW 264.7 cells, D-Fraction was pretreated with polymixin B (30 $\mu$g/ml) for 2 h.

**Administration of D-Fraction to Cancer Patients** Dr. Kiyoshi Komuta of the Osaka Police Hospital gave informed consent to 8 cancer patients. Eight stage II—IV cancer patients between 43- and 74-years-old were orally administered 100 mg of D-Fraction on consecutive days for 34 months and their NK activity was examined.

**Administration of D-Fraction to MM46 Carcinoma-Bearing C3H/HeN Mice** MM-46 carcinoma cells ($2\times10^6$) were implanted in the right axillary region of 5-week-old male C3H/HeN mice. After 24 h, D-Fraction (5 mg/kg/d) was administered to MM-46 carcinoma-bearing mice intraperitoneally (i.p.) for 19 consecutive days. Tumor inhibition ratio (T.I.R.) was calculated as follows: [1-(weight of tumor mass from mice treated with D-Fraction)/(weight of tumor mass from mice treated with PBS)]×100.

**Detection of Human NK Cell Activity** NK cells ($1\times10^6$) obtained from blood using the Conray–Ficoll method[9] were mixed with $2\times10^7$ of $^{51}$Cr-labelled K-562 (target cell of NK cell) cells in a tube. After incubation for 1 h, release of $^{51}$Cr into the culture supernatant was detected using a $\gamma$-counter. NK activity was calculated as: NK activity (%)= [(experimental release−spontaneous release) cpm/(max-release−spontaneous release) cpm (by 1 N HCl treatment)]× 100.

## RESULTS AND DISCUSSION

**Effects of D-Fraction on MM46-Carcinoma Cell Growth** On the 20th day of D-Fraction administration to MM-46 tumor-bearing mice, D-Fraction significantly decreased tumor growth as compared with mice administered phosphate bufferent saline (PBS), when the T.I.R. was 82% (Fig. 2). The effect of D-Fraction was also investigated in C3H/HeJ mice that are non-response to LPS; the T.I.R. was 78%. These results indicated that D-Fraction inhibited the growth of tumor.

**Effects of D-Fraction on NK Cell Activation** D-Fraction has already been reported to enhance cellular immunity by stimulating cells such as macrophages, helper T cells and cytotoxic T cells.[2] To detect NK cell activation, we examined IFN-$\gamma$ and TNF-$\alpha$ release from whole spleen cells on the 20th day of D-Fraction administration by mouse IFN-$\gamma$ and TNF-$\alpha$ ELISA kits (Genzyme Co., Minneapolis, U.S.A.). As shown in Figs. 3A and 3B, levels of IFN-$\gamma$ and TNF-$\alpha$ were significantly increased by 1.5- and 2.5-fold, respectively, compared to control mice. Intracellular expression of TNF-$\alpha$ in splenic NK cells under D-Fraction administration was also investigated using flow cytometric analysis. To detect NK cells, $2\times10^6$ cells were stained with R-PE-conjugated PanNK and Cy-Chrom$^{TM}$-conjugated CD3$\varepsilon$ antibodies (PharMingen Co., San Diego, CA, U.S.A.) and CD3$\varepsilon$ negative and PanNK positive was determined to represent the NK cell. Fluorescein isothiocyanate (FITC)-conjugated TNF-$\alpha$ antibody (PharMingen Co.) was used for detecting TNF-$\alpha$ expression in NK cells. After staining, NK cells expressing TNF-$\alpha$ were analyzed using a FACSCalibur analyzer (Beckton Dickinson Co., Grenoble, France) and histograms were calculated with CellQuest software (Becton Dickinson, Mountain View, CA, U.S.A.). As shown in Fig. 3C, D-Fraction administration resulted in a 1.3-fold increase in intracellular TNF-$\alpha$ expression in splenic NK cells compared to control mice. TNF-$\alpha$ is a cytokine released from activated NK cells in the same way as IFN-$\gamma$,[10,11] and has various functions relating to inflammatory and cytotoxic reactions, in addition to cytotoxicity and the ability to directly cause hemor-

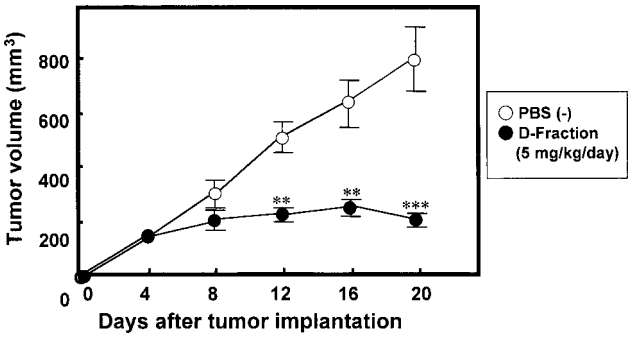
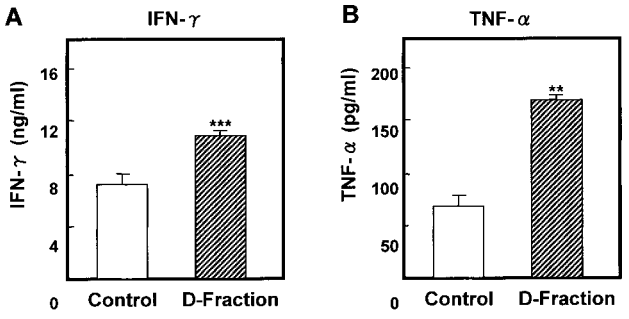
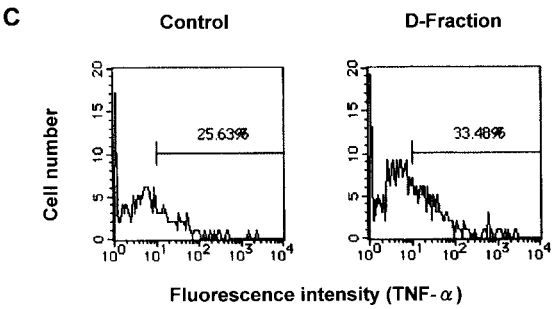



Fig. 2. Effect of D-Fraction on MM-46 Carcinoma Cell Growth

D-Fraction (5 mg/kg/d) was administered to MM-46 carcinoma-bearing mice i.p. for 19 consecutive days, and then tumor volume (mm$^3$=longest diameter×shortest diameter$^2$/2) was measured on day 20.[16] Data are expressed as mean±S.E.M. of 8 experiments. **, $p<0.01$; ***, $p<0.001$ compared to control mice (Student's $t$-test).




Figs. 3A and 3B. Effects of D-Fraction on Release of IFN-$\gamma$ or TNF-$\alpha$ from Whole Spleen Cells in MM-46 Carcinoma-Bearing Mice

On day 20 in mice administered D-Fraction (5 mg/kg/d) or PBS (−) i.p., splenocytes were collected according to previously described methods.[6] Splenocytes were plated at $1\times10^6$ cells/well in a 96-well plate and cultured with Con A (10 $\mu$g/ml) for 24 h in 5% $CO_2$ at 37 °C. After activation, levels of INF-$\gamma$ and TNF-$\alpha$ in culture supernatant were assessed using ELISA. Data are expressed as mean±S.E.M. of 4—8 experiments. **, $p<0.01$; ***, $p<0.001$ compared to control mice (Student's $t$-test).

Fig. 3C. Effect of D-Fraction on Intracellular TNF-$\alpha$ Expression in NK Cells of MM-46 Carcinoma-Bearing Mice

On day 20 in mice administered D-Fraction (5 mg/kg/d) or PBS (−), intracellular TNF-$\alpha$ expression in splenic NK cells was investigated using flow cytometric analysis. Shown are representative histograms of triple-color flow cytometric analysis of splenocytes stained with R-PE-conjugated PanNK, Cy-Chrom$^{TM}$-conjugated CD3$\varepsilon$, and FITC-conjugated TNF-$\alpha$ antibodies.

Table 1. Activities of NK Cells in Cancer Patients

| Patient | Treatments (100 mg of D-Fraction) | |
|---|---|---|
| | Before[a] (date) | After[a] (date) |
| SCLC (small cell lung carcinoma) (74-year-old female, stage III) | 23% ('99.6) | 44% ('99.9), 51% ('00.2) |
| SCLC (small cell lung carcinoma) (54-year-old male, stage III) | 21% ('98.5) | 42% ('99.7), 47% ('00.9) |
| Alveolar cell carcinoma (54-year-old female, stage III-B) | 24% ('98.4) | 48% ('98.9), 44% ('99.4), 42% ('00.5), 44% ('01.2) |
| Pulmonary adenomatosis (54-year-old male, stage-IV) | 21% ('99.7) | 42% ('00.9), 47% ('01.2) |
| Gallbladder carcinoma (48-year-old female, stage II) | 28% ('98.7) | 40% ('98.8), 39% ('00.1), 48% ('01.5) |
| Bronchogenic cancer (58-year-old male, stage III-B) | 26% ('99.5) | 33% ('00.10), 39% ('01.1), 31% ('01.6) |
| Masto carcinoma (breast cancer) (43-year-old female, stage III) | 30% ('98.10) | 47% ('99.9), 53% ('00.12) |
| Parathyroid carcinoma (69-year-old male, stage III-B) | 23% ('99.6) | 38% ('99.9), 44% ('00.4), 62% ('01.6) |

*a*) NK activity (%) examined. Standard level of NK activity on human, 18—40%.

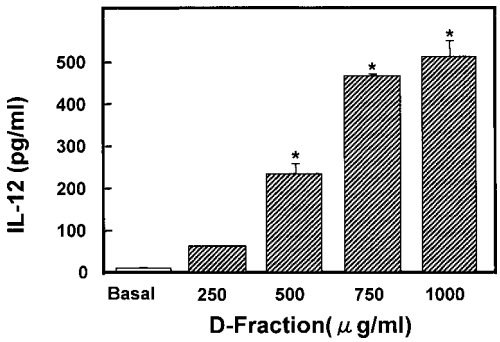



Fig. 4. IL-12 Release from RAW 264.7 Cells Stimulated with D-Fraction

RAW 264.7 cells ($1\times10^6$ cells/well in a 24-well plate) were stimulated for 18 h with D-Fraction. To remove any possible LPS contamination, D-Fraction was treated with polymyxin B (30 $\mu$g/ml) for 2 h before use. At the indicated time of incubation, IL-12 in the culture supernatants was measured using ELISA. Data are expressed as mean±S.E.M. of 3 experiments. *, $p<0.05$ as compared with the basal (0 $\mu$g/ml of D-Fraction) (Scheffe's *F*-test).

rhagic tumor necrosis.[12] Consequently, significant increases in release of IFN-$\gamma$ and TNF-$\alpha$ suggest activation of NK cells by D-Fraction.

**Effects of D-Fraction on IL-12 Release from Macrophages** IL-12 is a cytokine released by monocytes and macrophages, and is critical to the functions of NK and T cells. It induces IFN-$\gamma$ release from both NK and T cells.[12—14] NK cells can lyse a variety of different tumor cells by exocytosis of perforin-containing granules, and subsequent formation of lytic pores by perforin on the target cell membrane. Activation of NK cells with IL-2 and IL-12 has recently been reported to increase the binding of perforin to the target cell membrane and subsequent lysis of tumor cells.[15] IL-12 is released from monocytes and macrophages. To investigate the effect of D-Fraction on IL-12 release, macrophage cell line RAW 264.7 cells were incubated with various concentrations of D-Fraction and secretion of IL-12 was assessed. When cells were treated with D-Fraction (500—1000 $\mu$g/ml) for 24 h, the amount of IL-12 released significantly exceeded basal levels (0 $\mu$g/ml D-Fraction) (Fig. 4). Also, the effect of D-Fraction on IL-12 production was investigated *in vitro* using peritoneal macrophages from normal C3H/HeJ mice. When the normal peritoneal macrophages were stimulated with D-Fraction (500 $\mu$g/ml) for 24 h, IL-12 production showed an increase compared with that of the control (data not shown). These results suggested that the long-term anti-tumor effects of D-Fraction are attributable to activation of NK cells via macrophage-derived IL-12, and to T cell activation. NK cells may be further activated by INF-$\gamma$ released from not only themselves but also activated T cells.

**Effect of D-Fraction on the Activation of NK Cells in Cancer Patients** We concluded that D-Fraction was safe with no toxicity according to data confirmed by the Consumer Product Testing Co. Then, a non-randomized clinical trial of D-Fraction was conducted to check the effect in NK cells of 8 cancer patients, who agreed to the trial. As shown in Table 1, the NK cell activities of these patients were enhanced 1.2—2.7 times by treatment with D-Fraction. Even though these tests were limited and in a non-controlled trial, these results indicated that D-Fraction was effective for activation of NK in cancer patients.

In conclusion, D-Fraction represents an important BRM for NK cells by enhancing IL-12 release from macrophages. In immunotherapy using D-Faction for cancer patients, NK cells are responsible for early anti-tumor responses, while both NK and T cells are responsible for long-term anti-tumor responses. Although the dependence of the immune system on NK cells requires further study, our results also indicate the possibility of immunotherapy by various agents, which enhance the activity of NK cells for cancer patients.

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