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Short Communication

Antimetastatic and immunostimulatory properties of fermented brown rice

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The antimetastatic effect of fermented brown rice by *Aspergillus oryzae* (FBRA), a processed food, was examined in an animal model of metastasis to the liver using mouse colon cancer cells. Mice fed a diet containing 10% FBRA were inoculated with colon 26-L5 cells via the portal vein on day 14. Liver metastasis on day 28 was significantly inhibited by the FBRA-containing diet without an increase in body weight. To investigate the immunostimulatory activity, the cellular functions of macrophages were examined. Intracellular glutathione levels were increased in peritoneal macrophages (PEMs) prepared from mice fed FBRA for 18 days. FBRA did not induce the production of interferon-γ (IFN-γ) by itself, though enhanced the ability of PEMs to produce IFN-γ, but not interleukin-12 or tumor necrosis factor-α, in response to lipopolysaccharide (LPS). These results indicated that oral administration of FBRA inhibited the metastasis of colon 26-L5 cells to the liver through a mechanism leading to a Th1-dominant immune state and activation of macrophages via anti-oxidative properties.

Key words fermented brown rice, tumor metastasis, macrophage, interferon-γ, glutathione.

Introduction

Metastasis is the major cause of mortality in cancer patients. The liver is the most common target of the metastasis of gastrointestinal tract cancer, especially colon cancer and the prognosis for patients with hepatic metastasis is extremely bad.[1,2] If occult metastases, which is already established at the time of surgery, could be inhibited, then the prognosis of patients with colon carcinoma would improve.[3] To evaluate the efficacy of treatment against metastasis to the liver, we have used a liver-metastatic variant (colon 26-L5) of the colon 26 carcinoma cells obtained by an *in vivo* selection method.[4-6] Colon 26-L5 cells predominantly metastasize to the liver after their injection via the portal vein in BALB/c mice.[5] This model has provided some information about the efficacy of treatments for hepatic metastasis, especially for occult micrometastasis.[7] We have previously reported that juzentaihoto, a Japanese traditional Kampo medicine, potently suppressed metastasis of colon 26-L5 cells via the activation of macrophages and T cells.[8,9]

Toll-like receptors (TLRs) have recently been characterized as receptors of innate immunity.[10,11] TLRs are mainly expressed on macrophages and dendritic cells, and their recognition of pathogens provokes a rapid activation of innate immunity by inducing production of pro-inflammatory cytokines and upregulation of co-stimulatory molecules. Activated innate immunity subsequently leads to effective acquired immunity including anti-tumor immunity. Lipopolysaccharide (LPS) is the best-characterized TLR4-stimulating pathogen and is a potent activator of macrophages.

Fermented brown rice by *Aspergillus oryzae* (FBRA) is a processed food prepared by fermenting brown rice and rice bran with *A. oryzae*. FBRA has been shown to have potent inhibitory effects on chemical-induced carcinogenesis of the colon, liver, bladder, and esophagus in rodents.[12-15] Anti-oxidative effects are suggested to be involved in the chemopreventive potential of FBRA. However, the effects of FBRA on tumor metastasis and immunostimulatory effect on cytokine production have yet to be examined.

In the present study, we focused on the antimetastatic activity of FBRA in an animal model of metastasis to the liver using colon 26-L5 cells. In addition, the effects on LPS-induced production of cytokines and glutathione levels in peritoneal exudate macrophages were examined.

Materials and Methods

Preparation of FBRA. FBRA was provided by Genmai Koso Co., Ltd., Sapporo and was prepared as follows. Briefly, brawn rice with bran was crushed to make a powder, immersed in water for 1-2 h, and steamed at 85-100°C for 45-60 min. The material (1 kg) thus obtained was fermented with *A. orysae* (5 mg) for 48 h and then dried to produce FBRA powder (lot. No. 0106-TF118). For experiments *in vivo*, this preparation was mixed with a normal diet (CE2, Oriental Yeast). 10% FBRA-containing diet was used in animal models and the dose per weight is approximately 30-times as compared to the standard dose of human.

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HPLC analysis of FBRA. A HPLC-based analysis, the so-called 'fingerprint' method, was performed to assess the homogeneity of the formulation and to prepare batches of constant formulation and efficacy, as described previously.[16] FBRA (500 mg) was refluxed with 10% ethanol (25 ml) for 1h. The extracted solution was filtered and analyzed by HPLC (HP-1090, Hewlett-Packard) under the following conditions: column, TSK gel 80 Ts octadecyl silica (ODS) (4.6 x 250 mm); mobile phase, 10 mM phosphoric acid: CH3CN (linear gradient, 95:5→40:60, for 40 min); flow rate, 0.8 ml/min; oven temperature, 40℃; injection volume, 5 μl.

Animals. Specific pathogen-free female BALB/c mice, 7 weeks old, were purchased from Japan SLC Inc. (Hamamatsu, Japan), and maintained under laminar air-flow conditions. This study was conducted in accordance with the standards outlined in the Guidelines for the Care and Use of Laboratory Animals of University of Toyama.

Liver metastasis. A highly metastatic line of colon 26-L5 carcinoma cells was obtained by selection *in vivo*.[5] Colon 26-L5 cells were maintained as monolayer cultures in RPMI-1640 medium supplemented with 10% FCS at 37 ℃ in a humidified atmosphere of 5% CO2/95% air. Log-phase cultures of the cells were harvested with 1 mM EDTA in phosphate-buffered saline (PBS), washed three times with PBS, and resuspended at appropriate concentrations in PBS. BALB/c mice under ether anesthesia underwent a laparotomy using an upper median incision, and the duodenal loop was exposed. An injection of colon 26-L5 cells (1-2 x 10^4 cells/200 μl) was given into the portal vein through a 29-gauge needle attached to a 1-ml syringe. A sterile absorbable cotton swab was placed over the injection site as the needle was withdrawn to prevent bleeding and peritoneal dissemination of the tumor cells. The mice were sacrificed 14 days after the inoculation and the liver was weighed to evaluate the hepatic metastasis as previously described.[17]

Preparation of peritoneal exudate macrophages. Mice were divided into two groups. One group was fed a 10% FBRA-containing diet and the other, a normal diet. After 14 days, the mice were injected intraperitoneally with 2 ml of 3% thioglycollate medium (Sigma). The administration of FBRA was continued for 4 days after the injection. Peritoneal exudate cells (PECs) were collected by lavage with cold phosphate-buffered saline (PBS). PECs were seeded in RPMI1640 medium (GIBCO) supplemented with 10% fetal calf serum, 2 mM of L-glutamine, 100 units/ml of penicillin and 100 g/ml of streptomycin. After 2 h at 37℃ in 5% CO_2, cells were washed two times with PBS to remove nonadherent cells. Adherent cells were regarded as peritoneal exudative macrophages (PEMs), and were incubated for an adequate time in complete medium at 37℃ in 5% CO_2 before each experiment after incubation.

ELISA. PEMs were purified as described above. After a 4-h incubation, LPS (0.1 μg/ml) was added to the medium, and then incubation continued for 24 h. The supernatant was collected and IFN-γ, TNF-α, and IL-12 p40 concentrations were measured by ELISA (BD Pharmingen) according to the manufacturer's instruction manual.

Statistical analysis. All values are shown as mean ± S.E. Statistical significance was determined by use of Student's two tailed *t*-test. Values of $p < 0.05$ were considered significant.

Results and Discussion

HPLC analysis of FBRA. To assess the homogeneity of the constituents and to prepare batches of constant formulation and efficacy, the influence of the fermentation of brown rice and rice bran by *A. Orysa* was investigated by comparing the constituents before and after the fermentation. Fig. 1A shows the HPLC profiles of FBRA in terms of single monitoring (220 nm), and demonstrated that contents of some peaks were changed between *Oryza sativa* and Fermented *Orysa sativa* (*i.e.* FBRA). One peak was identified as ferulic acid, one of the antioxidants in brown rice, which was increased slightly by the reaction (Fig. 1A). Three-dimensional HPLC using a photodiode array system as a detector identified a peak for ferulic acid through comparison with the retention time and the UV spectra of standard compound.

Inhibition of experimental liver metastasis by FBRA. We first examined the effect of FBRA on metastasis to the

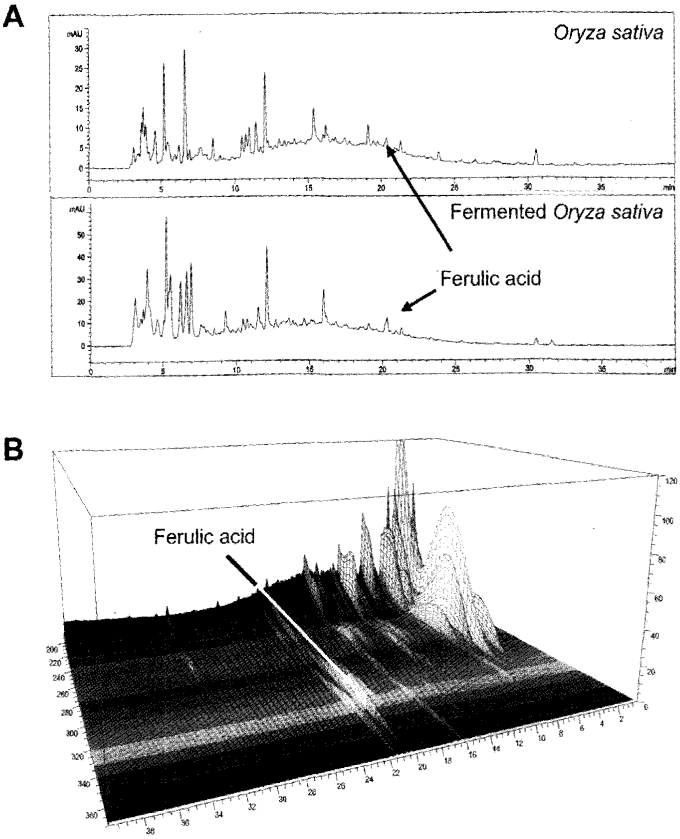

Fig. 1. HPLC profile and UV spectra of 10% ethanol soluble portion of FBRA.
FBRA (500 mg) was refluxed with 10% ethanol (25 ml) for 1h. The extracted solution was filtered and analyzed by HPLC. A) HPLC pattern obtained by measuring absorbance at 220 nm. UV spectra of main peaks, Peak 12 was identified as ferulic acid. B) Three-dimensional HPLC pattern.

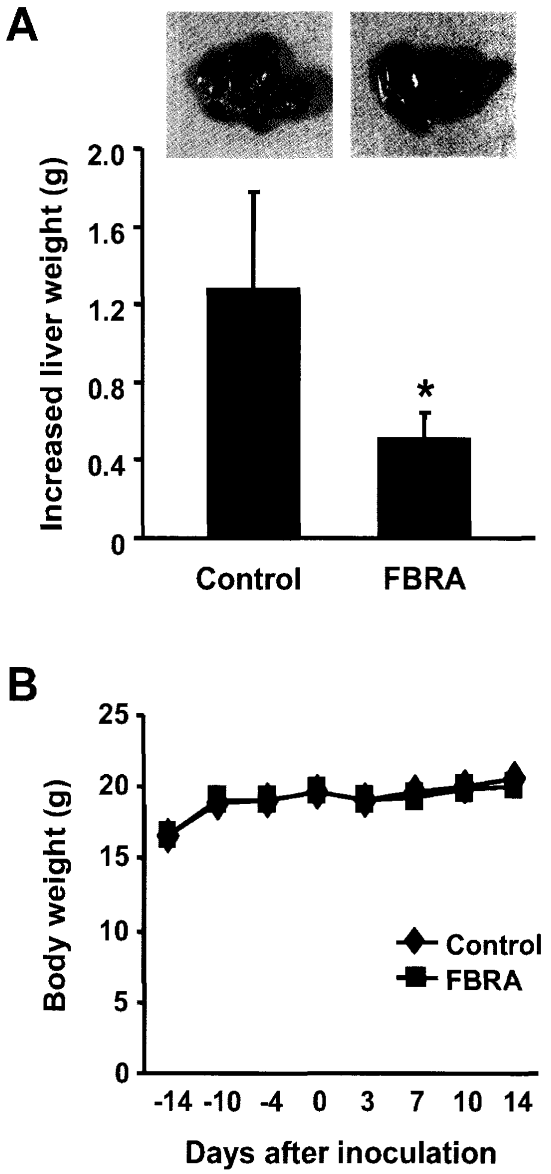

Fig. 2. Effect of FBRA on liver metastasis of colon 26 L5 cells.
Effect of the FBRA-containing diet on experimental metastasis to the liver following an intraportal injection of colon 26-L5 carcinoma cells. Female BALB/c mice (n = 6) were inoculated intraportally with colon 26-L5 cells ($1x10^4$). FBRA was administered orally for 14 days before the inoculation. A) Mice were maintained with continuous administration of FBRA and the liver weight was measured on day 14 after the inoculation. The results represent the mean ± S.D. *, $p < 0.05$ as compared to the untreated control using Student's two tailed *t*-test. Macroscopic views of the livers of mice injected with colon 26-L5 cells are presented. B) Body weight during the experiment.

liver caused by the injection of colon 26-L5 cells into the portal vein. Mice were divided into two groups; one given the diet containing 10% FBRA and the other, fed a normal diet for 14 days before the inoculation of tumor cells. Mice were maintained with continuous administration of FBRA and the liver weight was measured on day 14 after the inoculation. Fig. 2A shows that the oral administration of FBRA significantly attenuated the increase in liver weight. No apparent side effects such as a decrease in body weight were not observed (Fig. 2B). These results clearly indicate that FBRA is effective at preventing experimental liver metastasis.

This is consistent with observations that a diet containing 10% FBRA prevented chemical-induced carcinogenesis in several tissues in rodents.[12-15] In addition, ferulic acid may have a chemopreventive effect in these animal models.[17] Metastasis is a complex process involving multiple steps, in which oxidative stress plays a critical role.[18] These results suggested that FBRA suppressed metastasis by reducing oxidants in the process of metastasis as well as carcinogenesis. Several reports have clearly demonstrated that natural products containing antioxidants are effective at inhibiting metastasis. We have previously shown that the metastasis of colon 26-L5 cells to the liver was suppressed by juzentaihoto, a traditional Kampo medicine.[8-9] In addition, juzentaihoto contains many anti-oxidative compounds such as ginsenosides which are critical to the pharmacological activities of the formulation.[19] Collectively, these observations suggest that the anti-metastatic activity of FBRA is partly dependent on antioxidative activities. It is also important to reveal whether the anti-metastatic activities of these natural products reflect to the increase in survival rate of tumor-baring mice.

Effect of FBRA on the intracellular glutathione level. Evidence has been accumulating that redox state influences cellular functions.[20] Glutathione, one of the most abundant reducing peptides in the cells, has multiple cellular functions including effects on gene expression.[21] We tried to examine the effect of FBRA on glutathione levels in macrophages, because FBRA contains several antioxidants such as ferulic acid. Mice were maintained on the FBRA-containing diet for 14 days, and then injected thioglycollate medium intraperitoneally. PEMs were isolated four days after the injection with continuous administration of FBRA. Fig. 3 shows that the total intracellular concentration of glutathione was increased in PEMs prepared from mice fed 10% FBRA. A major proportion of glutathione existed as a reduced form (GSH) in the cell; therefore, total amounts of glutathione reflected the reduced form. In fact, we could not

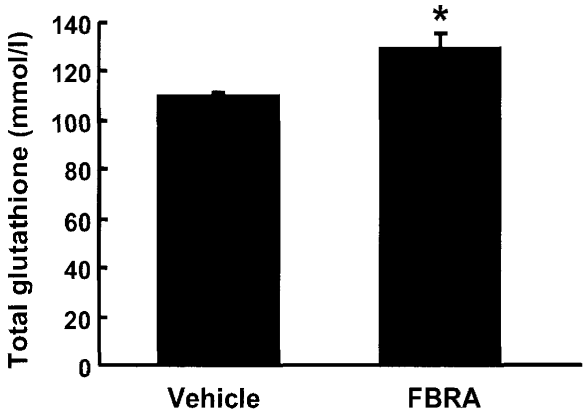

Fig. 3. Effect of FBRA on intracellular glutathione levels in macrophages.
Mice were fed a diet containing 10% FBRA for 14 days, and then injected with thioglycollate medium intraperitoneally. Four days after the injection, PEMs were harvested and the total glutathione level was measured. The values represent the mean ± S.E. from triplicate determinations, *$p < 0.05$.

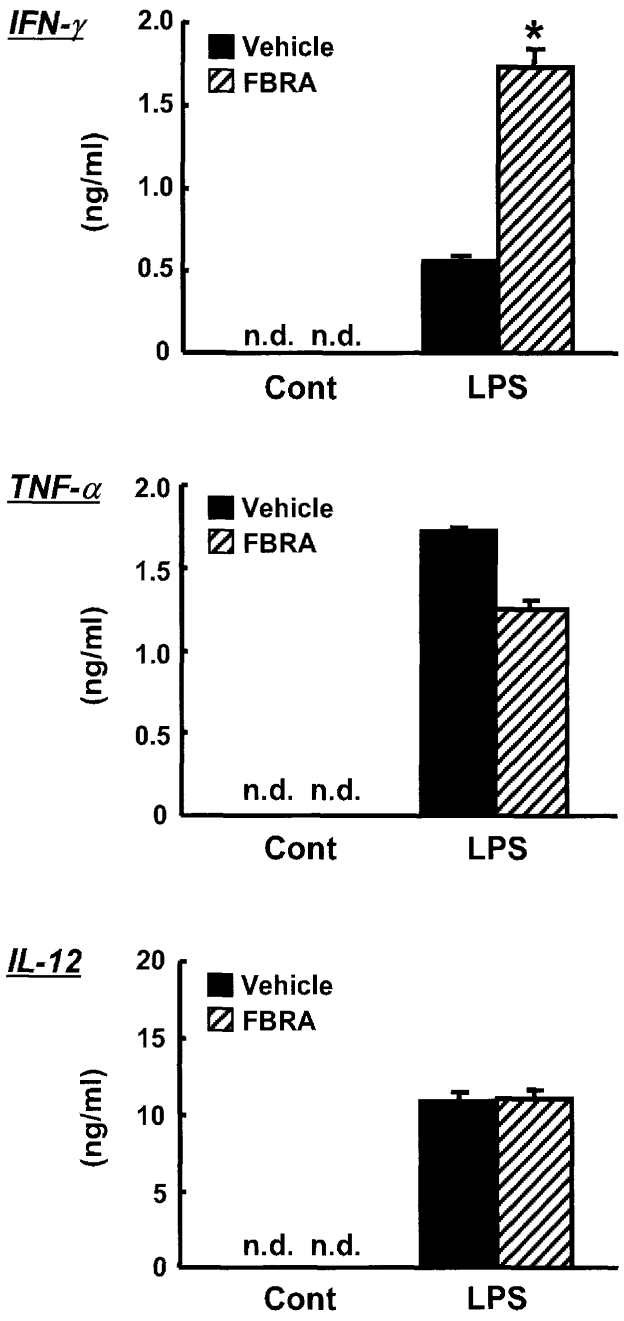

Fig. 4. Effect of FBRA on cytokine production in macrophages.
Mice were fed a diet containing 10% FBRA diet for 14 days, and then injected with thioglycollate medium intraperitoneally. Four days after the injection, PEMs were collected and stimulated with LPS (0.1 μg/ml) for 24 h. Concentration of INF-γ, TNF-α, and IL-12 p40 in the supernatant were measured by ELISA. The values represent the mean ± S.E. from triplicate determinations, n.d. means not detected. *$p < 0.01$.

detect the oxidative form of glutathione in PEMs (data not shown). These results indicated that the FBRA-containing diet enhanced the reducing activity of glutathione in macrophages.

Our previous study demonstrated that the anti-metastatic effect of juzentaihoto was associated with the activation of macrophages,[8,9] and also enhanced the intracellular glutathione level in PEMs (our unpublished data). These results indicate that the anti-oxidative activity of FBRA in macrophages is involved in the anti-metastatic potential of these natural products.

Effect of FBRA on cytokine production. To investigate the effects of FBRA on the cellular functions of macrophages, we next focused on the LPS-induced production of IFN-γ in PEMs. Mice received a daily oral administration of FBRA for 14 days, and then were injected with thioglycollate medium intraperitoneally. Four days after the injection, PEMs were isolated and stimulated with LPS *in vitro* for 24 h. LPS potentially induced production of IFN-γ, a cytokine generating type I helper T cell (Th1), in both groups, however, the level of IFN-γ production was significantly augmented in the FBRA-administered group compared with the vehicle-treated group (Fig. 2). In contrast, production of IL-12 p40 and TNF-α was not enhanced. These results demonstrated that FBRA had the potential to selectively promote TLR4-mediated IFN-γ production in macrophages.

Viewing this phenomenon in the Th1/2 balance,[22] FBRA induces a Th1-type response, which means an enhancement of cellular immunity. Innate immunity plays an important role not only in recognizing various pathogens but also in inducing acquired immunity. Activation of TLRs provokes the production of pro-inflammatory cytokines and upregulation of co-stimulatory molecules by antigen-presenting cells such as macrophages and dendritic cells.[10-11] Although T cells and NK cells are believed to be a major source of IFN-γ, it has recently been suggested that macrophages are another important source of this cytokine.[23] IFN-γ is a promising Th1 inducer and plays a critical role in tumor immunity; therefore, the secretion of IFN-γ by macrophages may partly explain the mechanism of anti-metastatic activity resulting from the oral administration of FBRA.

In conclusion, this is the first report evaluating the anti-metastatic activity of FBRA. This food supplement significantly suppresses the metastasis of colon cancer cells to the liver. We also demonstrated that FBRA affected the cellular functions of macrophages; however, the mechanisms underlying these effects are still unclear. Elucidation of these mechanisms will further reveal the merits of this supplement in suppressing the incidence of cancer and metastatic spread.

Acknowledgments

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Japanese abstract

マウス結腸がん細胞の実験的肝転移モデルにおいて，玄米を *Asperigillus oryzae* で発酵させた健康食品 FBRA の抗転移効果を検討した。FBRA を粉末飼料に10％で混ぜ14日間飼育し，その後 colon 26-L5 細胞を門脈内に移植した。さらに14日間10% FBRA 飼料で飼育し肝転移を評価した結果，FBRA 投与により肝転移が有意に抑制された。一方，実験期間中の体重増加には影響しなかった。次に，免疫増強作用を検討するため，マクロファージの細胞機能に対する FBRA の効果を検討した。10％FBRA 飼料で18日間飼育したマウスから調製した腹腔滲出マクロファージにおいて，細胞内グルタチオン濃度が増加していた。また，FBRA 投与だけではサイトカイン産生は誘導しないが，LPS 刺激による IFN-γ 産生を増強した。一方，TNF-α および IL-12産生には影響を及ぼさなかった。これらの結果から，FBRA の経口投与により colon 26-L5 細胞の肝転移が阻害されたが，それには抗酸化効果を介したマクロファージの活性化に基づく Th1 有意な免疫増強が関与していることが示唆された。

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