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原　著

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要　　旨：日本産赤米・黒米・白玄米のぬか成分の炎症反応に対する影響を調べるためのモデル実験としてリポポリサッカライド（LPS）で刺激したマウスマクロファージ培養細胞（RAW264.7）の一酸化窒素（NO）および炎症性サイトカインのインターロイキン6（IL-6）や腫瘍壊死因子α（TNF-α）の産生に及ぼす米ぬかメタノール抽出液の影響を検討した。実験の結果、すべての米ぬか抽出液はマクロファージからのNOおよび炎症性サイトカインの産生に対して有意な抑制作用を示したが、特に白玄米のぬか抽出液においてもっとも強い抑制作用を示した。また、これらの米ぬか抽出液中の抗炎症成分の候補としてそれぞれの抽出液中のポリフェノール含量を測定したところ、その含量と抗炎症作用の強さの間で一定の相関関係が認められた。以上の結果から日本産赤米・黒米・白玄米のぬか成分中に炎症性マクロファージのNOおよび炎症性サイトカインの産生に対する抑制成分が存在することが示唆された。

Abstract：To estimate the potent immunomodulating activity of Japanese rice bran, the effect of its extracts of red, black and brown rice was investigated on the production of nitric oxide (NO) and inflammatory cytokines in mouse macrophage cell line (RAW 264.7), induced by lipopolysaccharide (LPS). Their methanol extracts showed modulating effects on the production of NO and inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in LPS-stimulated macrophages. Among these extracts, the brown one showed the relatively strong suppressive activities against their production compared with them of red and black ones. These immunosuppressive activities of rice bran were roughly proportional to the content of phenolic compounds in their extracts. From these results, Japanese rice bran was shown to have potent immunomodulating activities against the production of NO and inflammatory cytokines in the activated macrophages.

キーワード：米ぬか、NO、TNF-α、IL-6、マクロファージ

Keywords：rice bran,, NO, TNF-α, IL-6, macrophages

Introduction

Rice bran is a byproduct during the milling process of raw rice seeds to separate the major food source, endosperm and contains various beneficial substances for human health. Among them, antioxidant or radical-scavenging substances in fat-soluble fraction such as tocopherols, tocotorienols, oryzanols are known to have potent hypocholesterolemic and antitumor activities[1-4]. Furthermore, the water-soluble polysaccharides from rice bran caused an antitumor effect in animal model experiment[5].

However, there are only a few studies units detailed immunological analysis as follows, I. Enhancement of attachment and phagocytosis activity of human and mouse macrophages by a modified arabinoxylan from rice bran and stimulated production of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and nitric oxide (NO) in the same macrophages[6], II. Enhancement of mitogen-induced proliferation and interferon-γ (IFN-γ) production of rat spleen cells by the oral supplementation of its oxtract modified by mushroom [7], III. Suppression of the release of histamine and β-hexosaminidase from the stimulated basophilic or mast cells and of the release of TNF-α, IL-1α and IL-6 production in the same inflammatory cells by black rice bran[8], IV. Suppressive effects on lectin-induced cell proliferation and the production of IL-6 and IFN-γ in mouse spleen cells by brown and black rice bran[9].

In present study, the immunomodulating activity of bran extracts of red, black and brown rice on the production of NO and inflammatory cytokines in the activated mouse macrophages (RAW264.7 cells) was confirmed and the contents of phenolic compounds in the rice bran, were shown to be associated with their immunosuppressive activities in the same cells as active substances for these immunomodulating activities.

Materials and Methods

Chemicals and reagents

Ferulic acid was kindly donated from Tsuno Life Fine Chemicals Inc. (Wakayama, Japan). Folin-Ciocalteu reagent, E coli lipopolysaccharide, Dulbecco's minimum essential medium (DMEM) and penicillin-streptomycin solution were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Hana-Nesco Bio Co. (Tokyo, Japan). Other chemical reagents used in the experiments were purchased from Wako Pure Chemicals Co. (Osaka, Japan) except for several specific reagents or assay kits which are explained in the text.

Preparation of bran extracts of Japanese rice

Raw grains of red rice (*Oryza sativa japonica*, Benizome-mochi) and black rice (*Oryza sativa japonica*, Asa-murasaki) were kindly donated from Katsuya Co. (Hiroshima, Japan). Brown rice grains (*Oryza sativa japonica*, Hino-hikari) were kindly donated from a farmer in Kikuchi City (Kumamoto, Japan). The Bran extract was prepared by modification of the previous report[10]. After 10 g of the rice grains were milled by an electric milling apparatus (IEM-100, Iwatani Sangyo Co, Tokyo, Japan) for 20 sec, the bran powder was recovered and mixed with 50 ml of methanol and kept for one overnight at room temperature. Each extract of the rice bran was separated from the precipitable residue by a centrifugation at 1,000×g for 10 min and stored in a freezer at -20° C.

Cell line and cell culture

RAW 264.7 cell, a mouse macrophage cell line, was obtained from American Type Culture Collection (ATCC, MD, USA) and cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37° C under 5% CO_2 and humidified at 95% air condition.

Measurement of NO content

RAW 264.7 cells (8×10^5 cells) were seeded in Falcon 12-well culture plate (No.353043, Becton-Dickinson Co, Lincohn Park, NJ, USA) using DMEM medium supplemented with 10% FBS at a final volume of 1.5 ml and cultured for 24 hr. The cells were treated with rice bran extract or test compound for 1 hr and then incubated for 24 hr in DMEM medium/10% FBS with or without LPS (1 μg/ml). Nitrite (a stable oxidative end product of NO) accumulation in the culture medium was determined by the Griess reaction[11]. Briefly, the cell culture supernatant was treated with Griess reagent solution (1:1 (v/v) mixture of 0.1% N-(1-naphtyl) ethylenediamine in H_2O and 1% sulfanilamide in 5% phosphoric acid) and kept for 30 min at room temperature. The absorbance of the reaction mixture was measured at 540 nm using a Shimadzu UV-265 spectrophotometer.

Assay for cytokine production

Assay for IL-6 or TNF-α production in RAW 264.7 cells was carried out as follows. RAW 264.7 cells (8×10^5 cells) were cultured with or without rice bran extract or test compound in DMEM medium containing 10% FBS in a 12 well culture plate (No.353043, Becton-Dickinson Co, Lincohn Park, NJ, USA) at 37 ℃ for 1 hr and further cultured for 24 hr in the presence of LPS (1 μg/ml). The cell culture medium was then harvested, diluted with PBS and the amount of IL-6 or TNF-α was measured by a mouse IL-6 or TNF-α ELISA kit (eBioscience Inc, San Diego, CA, USA) according to the maker manual of assay kit.

Assay for cell viability

Cell viability was determined by a dye exclusion method as described in the previous report[12]. RAW 264.7 cells (3×10^5 cells) were cultured in Falcon 24-well culture plate (No. 353047, Beckton-Dickinson Co.) for 24 hr with PBS, vehicle (0.1 or 0.5% methanol (V/V)) or test compound under the same culture conditions as in NO or cytokine assays. After the cultured cells attached to the plate were washed with PBS, 0.05% trypsin/0.01% EDTA solution was added into the culture well, and they were incubated at 37° C for 5 min with PBS including 10% FBS. Then, 0.1% trypan blue was added and mixed with the cell suspension, and the viable cell numbers were counted by a Nikon Labophot microscopic apparatus. The cell viability was calculated as (viable cell number in the culture well with the test sample/viable cell number in the culture well with PBS) ×100%.

Measurement of phenolic content

The content of phenolic comounds including flavonoids or phenolic acids was measured by a Folin-Ciocalteu method of Zielinski and Kozlowska[13] using ferulic acid as a standard phenolic compound.

Statistical analysis of experimental results

All assays for NO and cytokine production were carried out by triplicate experiments. The statistical comparison between the control and sample-treated experimental groups was carried out using Student's t test. A P value less than 0.05 was considered to be significantly different.

Results

First, we examined the effects of bran extracts of Japanese red, black and brown rice on nitric oxide (NO) production in mouse macrophages (RAW 264.7 cells) induced by LPS. Because LPS induces the remarkable expression of iNOS (inducible nitrite oxide synthase) and subsequent NO release from RAW 264.7 cells[14]. As

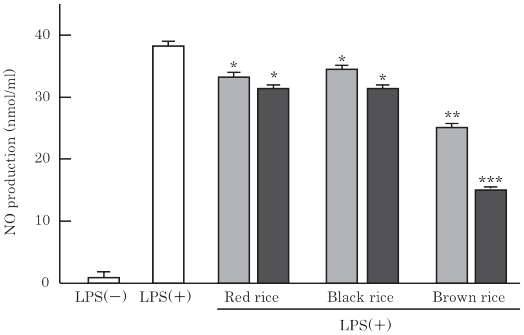

Fig.1. Effect of rice bran extracts on NO production in mouse RAW 264.7 cells induced by LPS. The column and bar represent the mean and SD of triplicate assays of NO production. Methanol extract of rice bran was dried, dissolved with the same volume of PBS (pH7.2) and applied to the cell culture system at a final concentration of 0.1 or 0.5% (v/v). As a negative control experiment, the same amount of PBS was added in place of the extract. In a positive control experiment, LPS (1 μg/ml) was added to the cell culture system without the extract. The grey and black columns show NO production by 0.1 and 0.5% extract respectively. The statistical comparison between the positive control and extract-treated experiment was carried out using Student's *t* test. *P* values are shown as follows. *: $P<0.01$, **: $P<0.001$, ***: $P<0.0001$, ■: Extract 0.1%, ▒: Extract 0.5%.

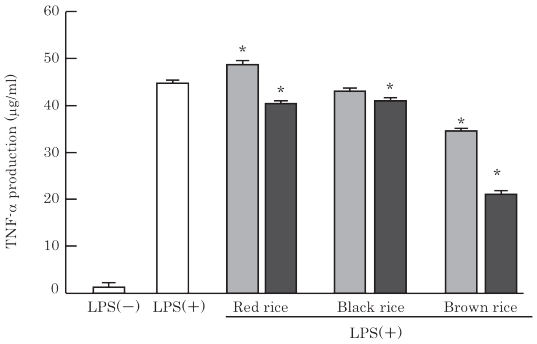

Fig.2. Effect of rice bran extracts on TNF-α production in LPS-stimulated RAW 264.7 cells. The column and bar represent the mean and SD of triplicate assays. TNF-α production in macrophages was assayed as described in Materials and Methods. Methanol extract of rice bran was dried, dissolved with the same volume of PBS (pH7.2) and applied to the cell culture system at a final concentration of 0.1 or 0.5%. As a negative control experiment, the same amount of PBS was added in place of the extract. In a positive control experiment, LPS (1 μg/ml) was added to the cell culture system without the extract. The grey and black columns show TNF-α production by 0.1 and 0.5% extract respectively. The statistical comparison between the positive control and test sample-treated experiment was carried out using Student's t test. *P* values are shown as follows. *: $P<0.01$, **: $P<0.001$, ***: $P<0.0001$, ■: Extract 0.1%, ▒: Extract 0.5%.

shown in Fig.1, when RAW 264.7 cells were exposed with LPS, remarkable NO release was observed, and all methanol extracts of rice bran suppressed significantly NO production in LPS-stimulated macrophages. Among these extracts, the extract of brown rice bran exhibited the relatively strong suppression against LPS-induced NO production (34.5-60.8% inhibition, $P<0.001$ and $P<0.0001$) with 0.1-0.5% extract (v/v) compared to red or black rice bran extract (9.9-18.3% inhibition, $P<0.01$) (Fig.1).

Second, to analyze the effects of these rice bran extracts on the production of typical inflammatory cytokines induced by LPS, we examined the effects of these extracts on TNF-α production in LPS-stimulated RAW264.7 cells. As indicated in Fig.2, although the bran extracts of red and black rice showed weak suppressive effects at a higher concentration of the extract (0.5% (v/v)) (8.5-9.6% inhibition, $P<0.01$), the bran extract of brown rice caused relatively strong suppressive effects (23.0-52.8% inhibition, $P<0.001$ and $P<0.0001$) with 0.1-0.5% extract (v/v). In addition, the red rice bran showed a weak stimulatory effect at a lower concentration of the extract (0.1% (v/v)) compared with the positive control value (9.2% stimulation, $P<0.01$) (Fig.2).

Next, we analyzed the effects of these extracts on another inflammatory cytokine (IL-6) production in LPS-stimulated macrophages. As shown in Fig.3, the extracts of black and brown rice bran caused significant suppressive effects on IL-6 production. Among them, 0.5% extract of brown rice bran exhibited the strong suppressive effect (59.4 % inhibition, $P<0.0001$). However, 0.1% extract of red rice bran exhibited a weak stimulatory effect (16.1% stimulation, $P<0.01$), although 0.5% extract of red rice bran showed a weak inhibitory effect (12.9% inhibition, $P<0.01$).

Additionally, when the cells were exposed with these rice bran extracts at 0.1-0.5% (v/v) in the absence of

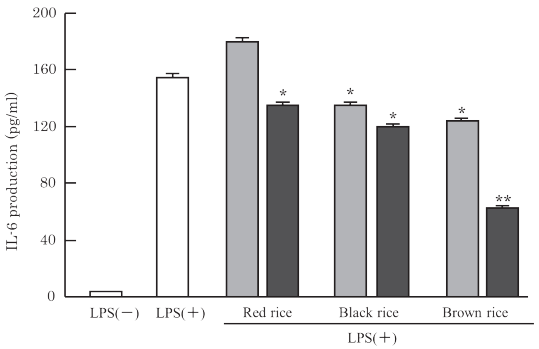

Fig.3. Effect of rice bran extracts on IL-6 production in RAW 264.7 cells induced by LPS. The column and bar represent the mean and SD of triplicate assays of IL-6 production. Methanol extract of rice bran was dried, dissolved with the same volume of PBS (pH7.2) and applied to the cell culture system at a final concentration of 0.1 or 0.5%. As a negative control experiment, the same amount of PBS was used in place of the extract. In a positive control experiment, LPS (1 μg/ml) was added to the cell culture system without the extract. The grey and black columns show IL-6 production by 0.1 and 0.5% extract respectively. The statistical comparison between the positive control and extract-treated experiment was carried out using Student's t test. *P* values are shown as follows. *: $P<0.01$, **: $P<0.0001$, ■: Extract 0.1%, ▒: Extract 0.5%.

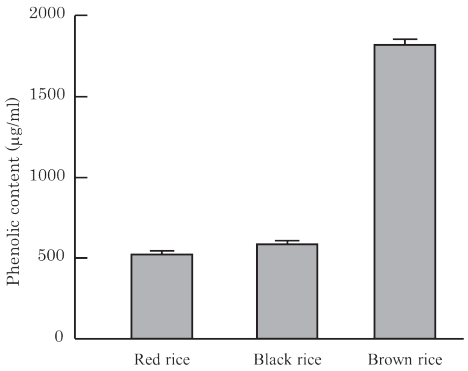

Fig.4. Measurement of phenolic content in rice bran extracts. The columns and bars represent the mean and SD of triplicate assays of phenolic content in rice bran extracts by using Folin-Ciocalteu method.

LPS, the significant effect on NO and cytokine production was not observed compared with the negative control experiment without these extracts (data not shown).

Furthermore, to evaluate whether the suppressive effects of rice bran extracts on NO and inflammatory cytokine production in mouse macrophages were affected by cytotoxic effects of these bran extracts, we analyzed the cell viability of RAW 264.7 cells exposed with the bran extracts by a dye exclusion method. However, significant cytotoxic effects were not detected by the treatment with the same amounts of rice bran extracts. Namely, the treatment of all extracts showed more than 93.8% cell viability compared with the control experiment. Thus, we concluded that the immunosuppressive effects of rice bran extracts on mouse macrophages are not derived from their cytotoxic effects.

These results suggest that the rice bran extracts have potent modulating activities against the production of NO and inflammatory cytokines such as TNF-α and IL-6 in the activated macrophages and these effects of rice bran extracts are not derived from their cytotoxic effects.

Furthermore, we assumed phenolic compounds including flavonoids or phenolic acids as possible active principles responsible for the immunosuppressive activities in the rice bran according to the results of the previous studies[15-16]. When we measured the contents of phenolic compounds in these extracts, they contained considerable amounts of phenolic compounds (Fig.4), which were roughly proportional to the immunosuppressive activities of the rice bran extracts (Fig.1-3). Especially, the extract of brown rice bran exhibited the highest concentration of phenolic compound (Fig.4), which seems to be associated with the strong suppressive activity of brown rice extract against the production of NO and inflammatory cytokines (Fig.1-3).

Discussion

In our present study, we showed that methanol extracts of Japanese rice bran caused suppressive effects on the production of NO and inflammatory cytokines such as TNF-α and IL-6 in LPS-stimulated macrophage cell line (RAW 264.7) (Fig 1-3).

Previously, we showed that methanol extracts of black and brown rice bran suppressed lectin-induced cell proliferation and IFN-γ and IL-6 production in mouse spleen cells[9]. Furthermore, the ethanol extract of black rice bran suppressed histamine-release from rat basophilic or mast cells and also inhibited TNF-α and IL-6 production in the same inflammatory cells[8].

In contrast, a previous report showed that the water soluble polysaccharides from rice bran caused antitumor effects in an animal model experiment[5]. Furthermore, the polysaccharides in water extracts of rice bran enhanced phagocytosis activity and TNF-α production of mouse macrophages[6]. Possibly, water and methanol extracts of rice bran might contain qualitatively different substances which express different immunomodulating activities.

On the other hand, as possible active principles responsible for the immunosuppressive substances in edible plants for macrophages, some phenolic compounds including flavonoids or phenolic acids were known. For example, caffeic acid derivatives suppressed the production of Inducible nitric oxide synthase (iNOS) and subsequent NO production in LPS-stimulated macrophages[15]. Furthermore, several flavonoids derived from *Waltheria indica* showed the inhibitory effects on the production of NO and TNF-α in the activated macrophages[16].

Previously, we reported that the extract of brown rice bran contains considerable amount of some phenolic acids, which were identified by HPLC analysis, and they showed radical-scavenging activities[10].

As shown in Fig.4, the considerable amount of phenolic substance was observed in the rice bran extract. Especially, the brown rice extract contained a relatively high concentration of phenolic substance compared with red and black bran extracts, which seems to be associated with the strong immunosuppressive activity of brown rice bran against NO and inflammatory cytokine production in the activated macrophages (Fig.1-3).
We analyzed another possible substance responsible for the immunosuppressive activity besides phenolic acids

in the rice bran extracts. In our separate study, the hot water extract of Japanese rice bran showed strong antioxidant and antigenotoxic activities, and the active principle in the extract responsible for these activities was identified to be an endogenous radical-scavenging protein, peroxidase[17]. Although the considerable peroxidase activity was also observed in the extract of black and brown rice brans (data not shown), the supplement of the purified peroxidase did not show significant effect on NO and inflammatory cytokine production in the activated macrophages (data not shown), suggesting that endogenous peroxidase in the rice bran is not responsible for the immunomodulating activities in our present study.

Possibly, rice bran-derived compounds might cause suppressive effects on the initiation and propagation of cardiovascular diseases such as atherosclerosis. For example, the supplementation of black rice bran significantly reduced atherosclerotic plaque formation induced by hypercholesterolemia in rabbits[18]. This pharmacological activity of rice bran might be responsible for their antioxidant and radical-scavenging activities, because the rice bran-derived phenolic compounds have potent antioxidant and radical-scavenging activities[19]. We also detected significant radical scavenging activities in red and black rice bran extracts[20] and further identified radical-scavenging phenolic acids in the extract of brown rice bran[10].

In addition, a previous report described that the treatment with NO-lowering agents such as nicotiamide or aminoguanidine prevented β cell dysfunction in pancreatic islets and hyperglycemia in obesity diabetic rats showing high NO concentration in their blood[21]. Therefore, NO-lowering activity of the rice bran extracts shown in the present study might contribute to the prevention against diabetic disease.

The activated macrophages play a key role in the development of atherosclerosis, and diet-derived flavonoids, quercetin derivatives showed a potent suppressive activity against atherosclerosis by expressing inhibitory effects on various functions of macrophages such as the incorporation of oxidized lipoprotein[22]. Furthermore, some dietary flavonoids caused suppressive effects on TNF-α induced adhesion molecule expression in human aortic endotherial cells , which is responsible for the inflammatory reactions and atherosclerosis[23].

Thus, the finding in the present study implicates a possibility of the beneficial effects of Japanese rice bran on the prevention against various chronic or allergic diseases, which are associated with their immunosuppressive effects on the activated macrophages.

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