Immunostimulatory effect of dried bonito extract on mouse macrophage cell lines and mouse primary peritoneal macrophages

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Abstract Dried bonito is a preserved food used in Japan, which contains abundant flavor ingredients and functional substances. We focused on the immunostimulatory effect of dried bonito extract (DBE) on mouse macrophage-like J774.1 cells, RAW264.7 cells, and mouse primary peritoneal macrophages. DBE significantly stimulated the production of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) by

both J774.1 cells and peritoneal macrophages by enhancing the cytokine gene expression levels. In addition, DBE stimulated nitric oxide production by enhancing the expression of inducible nitric oxide synthase in RAW264.7 cells. DBE also increased the phagocytosis activity of J774.1 cells. Immunoblot analysis revealed that DBE has an immunostimulatory effect on macrophages through activation of mitogen-activated protein kinase and nuclear factor- κ B cascades. TNF- α production enhanced by DBE was partially inhibited by treatment with TLR4 inhibitor TAK-242, whereas IL-6 production enhanced by DBE was almost inhibited. These results suggested that DBE is thought to strongly stimulate the TLR4 signaling pathway for macrophage activation, and its activation is also involved in other signaling. Finally, the phagocytosis activity of peritoneal macrophages from DBE-administered BALB/c mice increased significantly, suggesting that DBE has the potential to stimulate macrophage activity in vivo. In conclusion, these data indicated that DBE contributes to

Practical Application: Although dried bonito contains abundant flavor ingredients and functional substances, it was unclear whether dried bonito affects macrophage activation leading to enhancement of the immune system. In this study, the extract from dried bonito showed immunostimulatory effects on experimental animals as well as on cell lines. Our findings suggest that DBE would be a novel functional food for immunostimulation with academic significance and that daily intake of DBE contributes to the maintenance of human health with enhancement of the immunity due to macrophage activation.

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activating host defense against pathogens by activating innate immunity.

Keywords Immunostimulatory effect · Dried bonito · J774.1 cells · Peritoneal macrophages · Phagocytosis activity

Introduction

Inflammation is a protective response by the body to ensure removal of detrimental stimuli such as microbial infection and tissue injury (Medzhitov 2008). In an acute inflammatory response, the innate immune system is the major contributor and the response is mainly activated by neutrophils and macrophages. Macrophages recognize the molecular structures called pathogen-associated molecular patterns (PAMPs) of microbial pathogens or damage-associated molecular patterns (DAMPs) of unnecessary cells such as damaged, dead, or tumor cells through pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) (Aderem and Ulevitch 2000; Takeuchi and Akira 2010). Recognition of them by macrophages causes the activation of mitogen-activated protein kinase (MAPK) and nuclear factor- κ B (NF- κ B) cascades, and the activated macrophages secrete proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) (Aderem and Ulevitch 2000; Takeuchi and Akira 2010). TNF- α is involved in protection against infection and the antitumor effect by regulating expression of cell adhesion molecules, enhancing antibody production by plasma cells, and inducing apoptosis. IL-6 is another important cytokine in acute inflammatory response inducing the synthesis of a variety of other inflammatory mediators, stimulating neutrophil production in the bone marrow, and differentiating IL-17-producing helper T cells. Furthermore, macrophages produce nitric oxide (NO) synthesized from L-arginine by inducible nitric oxide synthase (iNOS) in response to cytokines and microbial pathogens (Moncada et al. 1991). NO is a biological effector with a wide range of activities such as antitumor and antibacterial activities in the immune system (Nathan 1992). Concurrently, macrophages phagocytize and present fragmented microbial pathogens to T cells as an antigen. A fragmented peptide from microbial pathogens is in the major histocompatibility complex (MHC) class II-containing peptide-loading compartment bound

to MHC class II molecules (Ma et al. 2003). The MHC class II-antigen peptide complex is presented to the T cell receptor on CD4⁺ T lymphocytes, resulting in the activation of the adaptive immune response (Pu et al. 2002). Thus, innate immunity is of great importance in activating adaptive immunity, and macrophage activation leads to enhancing the immune system.

Dried bonito (also called katsuobushi) is made in Japan and Taiwan from the flesh of skipjack tuna (*Katsuwonus pelamis*) by boiling, smoking, and drying to wood-like hardness to allow it to be preserved for a long period (Lin and Hwang 2008). Dried bonito is commonly shaved into flakes with a plane and used to make soup stock in Japanese cuisine, because it is rich in inosinic acid, an amino acid that produces a strong umami taste. Dried bonito is classified into two types: “arabushi”, which is produced by smoking after boiling, and “karebushi”, which is produced from arabushi by molding with *Aspergillus* species (Miyake et al. 2009; Aoki et al. 2013). A hydrolysate of dried bonito digested with thermolysin has been reported to produce strong inhibitory activity against angiotensin-converting enzyme and to be the basis of a dietary supplement with antihypertensive activity (Yokoyama et al. 1992; Curtis et al. 2002; Kouno et al. 2005). In addition, a microbial protease-resistant fraction of dried bonito has been reported to alleviate atopic dermatitis-like skin lesions in NC/Nga mice (Matsumoto et al. 2007). The dried bonito samples used in these reports were “karebushi”, and their activities are often derived as a product of microbial fermentation. The powder of arabushi is discarded during the shaving process, even though the powder might contain many beneficial substances that could contribute to good health. So far, we have found that the extract from arabushi powder stimulates immunoglobulin (Ig) production by human hybridoma HB4C5 cells and mouse spleen lymphocytes (unpublished data). These results suggest that arabushi has the potential to stimulate the immune system. However, it was unclear whether arabushi affects macrophage activation leading to enhancing the immune system. We investigated the immunostimulatory effect of dried bonito extract (DBE) from arabushi on mouse macrophage-like J774.1 cells, RAW264.7 cells, and mouse primary peritoneal macrophages. In addition, we investigated the ex vivo effect of DBE on peritoneal macrophages derived from DBE-orally administered mice.

Materials and methods

Reagents

Roswell Park Memorial Institute-1640 (RPMI-1640) medium, Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, bovine serum albumin (BSA), fetal bovine serum (FBS), lipopolysaccharides (LPS) from *Escherichia coli* 026/B6, and polymyxin B sulfate salt (polymyxin B) were products of Sigma-Aldrich (St. Louis, MO, USA). Goat anti-actin antibody and anti-goat IgG antibody labelled with horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-labelled anti-rabbit IgG antibody, HRP-labelled anti-mouse IgG antibody, mouse anti-I κ B α antibody, and rabbit antibodies against histone H3, NF- κ B p65, extracellular signal-regulated protein kinases (ERK)1/2, phosphorylated ERK1/2, c-Jun N-terminal kinase (JNK), phosphorylated JNK, p38 MAPK, and phosphorylated p38 MAPK were purchased from Cell Signaling Technology (Danvers, MA, USA). Ethyl (6*R*)-6-[*N*-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242) was purchased from Chemscene (Monmouth Junction, NJ, USA). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless otherwise noted.

Sample preparation

Dried bonito powder was provided by Senmi Ekisu Co., Ltd (Ehime, Japan). The shaving powder of dried bonito before fermentation, "arabushi", was suspended in distilled water and heated at 100 °C for 2 h. The suspension was then centrifuged at 10,000 $\times g$ at 4 °C for 30 min, and the supernatant was collected and dialyzed using a dialysis membrane with molecular weight cut off (MWCO) of 14,000 (Wako Pure Chemical Industries) against 10 mM sodium phosphate buffer (pH 7.4) at 4 °C for 24 h to reduce the salt concentration. The dialyzed supernatant was then filtrated through a 0.22 μ m membrane and used as DBE. The protein concentration of DBE was determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with BSA as a standard. To evaluate the effect of proteolytic enzyme, DBE (8.0 mg protein/mL) was treated with 500 μ g/mL of proteinase K (Wako Pure Chemical Industries)

at 37 °C for 15 h. The treated DBE was subsequently heated at 100 °C for 10 min to inactivate the enzyme.

Mice

BALB/c mice were purchased from Japan SLC (Shizuoka, Japan) and kept in an animal room under a 12 h light/dark cycle at a temperature of 24 ± 1 °C. Animals received a pelleted basal diet and water ad libitum. All animal experiments described in this study were carried out in accordance with the protocol approved by the Laboratory Animal Care Committee of Ehime University. Mice were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals of Ehime University.

Cells and cell culture

Mouse macrophage-like cell lines J774.1 cells and RAW264.7 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). J774.1 cells were cultured in RPMI-1640 medium supplemented with 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 10% FBS at 37 °C under humidified 5% CO₂. RAW264.7 cells were cultured in DMEM supplemented with 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 10% FBS at 37 °C under humidified 5% CO₂. In the subsequent experiments, cells were detached using phosphate buffered saline (PBS) containing 0.02% ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA; Dojindo Laboratories, Kumamoto, Japan).

Mouse peritoneal macrophages were prepared as previously described (Putra et al. 2012) with some modifications. In brief, 8-week-old female BALB/c mice were injected with 3.0% thioglycollate medium (2 mL/mouse) into the peritoneum following a 1-week acclimation. Four days after injection, mice were sacrificed and injected with 3 mL of 0.05% EDTA-PBS into the peritoneum to harvest thioglycollate-elicited peritoneal macrophages. Collected cells were centrifuged at 160 $\times g$ for 5 min at 4 °C, and the cell pellet was washed with PBS and centrifuged again. The cell pellet was then suspended in RPMI-1640 medium supplemented with 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 10% FBS and cultured in a culture dish (BD Falcon, Franklin Lakes, NJ, USA). After incubation at 37 °C for 1 h, the cells were washed with PBS three times to remove

unattached cells such as neutrophils. The purity of peritoneal macrophages prepared using this method was estimated to be higher than 85% according to previous reports (Ding et al. 1988; Vodovotz et al. 1993; Schindler et al. 2001). In the subsequent experiments, peritoneal macrophages were detached by pipetting in cold PBS.

Cytokine production assay

J774.1 cells or peritoneal macrophages suspended in 10% FBS-RPMI-1640 medium were seeded into a 48-well culture plate (BD Falcon) at 1.5×10^5 cells/well and cultured at 37 °C overnight under humidified 5% CO₂. After washing with PBS, the cells were treated with 0.5 mL of 10% FBS-RPMI-1640 medium containing various concentrations of DBE or 10 mM sodium phosphate buffer as control. After incubation at 37 °C for 6 h, the concentrations of TNF- α and IL-6 in culture media were measured by enzyme-linked immunosorbent assay (ELISA) using Mouse TNF- α DuoSet ELISA (R&D Systems, Minneapolis, MN, USA) and Mouse IL-6 ELISA MAX Standard (BioLegend, San Diego, CA, USA), respectively, according to the manufacturer's instructions.

To examine the contamination of endotoxin, 8.0 mg protein/mL of DBE, 10 mM sodium phosphate buffer as control, or 10 ng/mL of LPS as positive control were treated with 10 μ g/mL of polymyxin B at room temperature for 30 min. J774.1 cells suspended in 10% FBS-RPMI-1640 medium were seeded into a 96-well culture plate (BD Falcon) at 6.0×10^4 cells/well and cultured at 37 °C overnight under humidified 5% CO₂. After washing with PBS, the cells were treated with 200 μ L of 10% FBS-RPMI-1640 medium containing polymyxin B-treated sample and incubated at 37 °C for 6 h. After incubation, the concentration of cytokines in culture medium was measured by ELISA as described above.

NO production assay

RAW264.7 cells suspended in 10% FBS-DMEM were seeded into a 48-well culture plate at 1.5×10^5 cells/well and cultured at 37 °C overnight under humidified 5% CO₂. After washing with PBS, the cells were treated with 0.5 mL of 10% FBS-DMEM containing various concentrations of DBE or 10 mM sodium phosphate buffer as control. After incubation at 37 °C for 24 h, NO concentration in the culture medium was

measured using Griess Reagent System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Real-time RT-PCR

J774.1 cells or peritoneal macrophages suspended in 10% FBS-RPMI-1640 medium were seeded into a 48-well cell culture plate at 1.5×10^5 cells/well and cultured at 37 °C overnight under humidified 5% CO₂. After washing with PBS, the cells were treated with 0.5 mL of 10% FBS-RPMI-1640 medium containing various concentrations of DBE or 10 mM sodium phosphate buffer as control and incubated at 37 °C for 6 h. Total RNA was then isolated from the cells using Sepasol-RNA I Super G (Nacalai Tesque) according to the manufacturer's instructions and used as a template for cDNA synthesis with MMLV-reverse transcriptase (Promega, Tokyo, Japan) and an oligo-(dT)₂₀ primer (Toyobo, Osaka, Japan). The real-time PCR mixture, with a final volume of 20 μ L, consisted of Thunderbird SYBR qPCR Mix (Toyobo), 10 pmol of a forward primer, 10 pmol of a reverse primer, and 0.1 μ g of a cDNA sample. Thermal cycling conditions were 20 s at 95 °C, and 40 cycles of 3 s at 95 °C and 30 s at 60 °C. PCR products were measured on a StepOnePlus Real-time PCR System (Applied Biosystems, Foster City, CA, USA), and relative gene expression was calculated based on the comparative CT method using StepOne Software v2.1 (Applied Biosystems). Expression of the β -actin gene was used as an endogenous control. Specific oligonucleotide sequences for each gene are as follows. Mouse β -actin: sense, 5'-CA TCCGTAAGACCTCTATGCCAAC-3' and antisense, 5'-ATGGAGCCACCGATCCACA-3'; mouse TNF- α : sense, 5'-CTACTCCCAGGTTCTCTTCAA-3' and antisense, 5'-GCAGAGAGGAGGTTGACTTTC-3'; mouse IL-6: sense, 5'-AAGCCAGAGTCCTTCAGAGA GAT-3' and antisense, 5'-TTGGATGGTCTTGCTCCT-TAGC-3'; mouse iNOS: sense, 5'-CCAAGCCCTCACC-TACTTCC-3' and antisense, 5'-CTCTGAGGGCTGACACAAGG-3'.

Phagocytosis activity

J774.1 cells suspended in 10% FBS-RPMI-1640 medium were seeded into a 48-well cell culture plate at 1.5×10^5 cells/well and cultured at 37 °C overnight under humidified 5% CO₂. After washing with

PBS, the cells were treated with 0.5 mL of 10% FBS-RPMI-1640 medium containing 8.0 mg protein/mL of DBE or 10 mM sodium phosphate buffer as control and incubated at 37 °C for 6 h. After washing with PBS, 0.5 mL of 10% FBS-RPMI-1640 medium containing 20 µg of Texas Red-conjugated Zymosan A (*Saccharomyces cerevisiae*) BioParticles (Molecular Probes, Eugene, OR, USA) was added to each well and incubated for 1 h under a dark condition. After removing the culture medium, the cells were collected in PBS and centrifuged at $160\times g$ for 5 min at 4 °C. The cell pellet was suspended in 1 mL of 2% FBS-PBS, and phagocytosis activity was measured on a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Immunoblot analysis

J774.1 cells suspended in 10% FBS-RPMI-1640 medium were seeded into a 35 mm culture dish (BD Falcon) at 1.0×10^6 cells/dish and cultured at 37 °C overnight under humidified 5% CO₂. After washing with PBS, the cells were treated with 2 mL of 10% FBS-RPMI-1640 medium containing 8.0 mg protein/mL of DBE, 10 mM sodium phosphate buffer as control, or 100 ng/mL of LPS as positive control and incubated at 37 °C for 15 min. Cytosolic and nuclear proteins were prepared using a CellLytic NuCLEAR Extraction Kit (Sigma-Aldrich) according to the manufacturer's instructions. Denatured proteins were then separated using SDS-PAGE and transferred onto a PVDF membrane (Hybond-P; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Immunoblotting with various antibodies was performed as previously described (Ishida et al. 2013).

Treatment with TLR4 inhibitor

Treatment with TLR4 inhibitor TAK-242 was performed as previously described (Ishida et al. 2016) with some modifications. In brief, J774.1 cells suspended in 10% FBS-RPMI-1640 medium were seeded into a 96-well cell culture plate at 6.0×10^4 cells/well and cultured at 37 °C overnight under humidified 5% CO₂. After washing with PBS, the cells were treated with 0.5 mL of 10% FBS-RPMI-1640 medium containing 0.2 µM TAK-242 for 1 h. After washing with PBS, the cells were treated with 0.5 mL of 10% FBS-RPMI-1640 medium containing

8.0 mg protein/mL of DBE, 10 mM sodium phosphate buffer as control, or 10 ng/mL of LPS as positive control and incubated at 37 °C for 6 h. The concentrations of TNF- α and IL-6 in culture media were measured by ELISA as described above.

Immunostimulatory effect of DBE on peritoneal macrophages ex vivo

An ex vivo experiment was performed as previously described (Putra et al. 2012; Kumalasari et al. 2013) with some modifications. In brief, 6-week-old female BALB/c mice were placed into three groups (5 mice per group) following a 1-week acclimation. They were orally administered with 20 µL of 10 mM sodium phosphate buffer for the control group, 20 µL of high-dose DBE (16 mg protein/kg body weight) for the high-dose group, or 20 µL of low-dose DBE (1.6 mg protein/kg body weight) for the low-dose group for 14 consecutive days from day 0 to day 13. Mice were injected with 3.0% thioglycollate medium (2 mL/mouse) into the peritoneum on day 10. On day 14, the mice were sacrificed and injected with 0.05% EDTA-PBS into the peritoneum, and peritoneal macrophages were collected and seeded into a 24-well cell culture plate at 3.0×10^5 cells/well. After culturing for 24 h, the phagocytosis activity was measured on a flow cytometer as described above.

Statistical analysis

Data obtained were expressed as mean $\pm$ standard deviation. One-way ANOVA followed by Dunnett's test or the Tukey–Kramer test was used to assess the statistical significance of the difference. Values with $*P < 0.05$ or $**P < 0.01$ were considered statistically significant.

Results

Effect of DBE on cytokine production

The effect of DBE on cytokine production by J774.1 cells and peritoneal macrophages was first examined. DBE was added to the cell culture media at various concentrations, and the cytokine concentration in the medium was measured by ELISA after incubation for 6 h. As shown in Fig. 1, DBE significantly enhanced

the production of TNF- α and IL-6 by both J774.1 cells and peritoneal macrophages in dose-dependent manners. When J774.1 cells were treated with 8.0 mg protein/mL of DBE, the production of TNF- α and IL-6 increased 8-fold and 1100-fold, respectively, compared with control (Fig. 1a). The production of TNF- α and IL-6 by peritoneal macrophages increased 16-fold and 24-fold, respectively, compared with control, by treating with 8.0 mg protein/mL of DBE (Fig. 1b). In addition, DBE showed no toxicity to either cell culture within the tested concentrations (data not shown). From these results, further experiments were performed with 8.0 mg protein/mL of DBE, which

showed the highest effect on cytokine production without cytotoxicity.

Effects of DBE on cytokine gene expression

As described above, DBE enhanced cytokine production by J774.1 cells and peritoneal macrophages. Hence, the effect of DBE on cytokine gene expression was examined. DBE was added to the cell culture media at various concentrations, and the transcription level of the cytokine genes was evaluated by real-time RT-PCR using total RNA isolated from the treated cells. As shown in Fig. 2, DBE significantly increased

Fig. 1 Effect of dried bonito extract (DBE) on cytokine production by J774.1 cells and peritoneal macrophages. J774.1 cells (a) or peritoneal macrophages (b) were treated with RPMI-1640 medium containing 10% fetal bovine serum and various concentrations of DBE or 10 mM sodium phosphate buffer (pH 7.4) as control. After incubation at 37 °C for 6 h, the concentrations of TNF- α and IL-6 in culture media were measured by enzyme-linked immunosorbent assays. Data are expressed as the mean $\pm$ SD ($n = 3$). ** $P < 0.01$ against control by Dunnett's test

the expression levels of the TNF- α and IL-6 genes in both J774.1 cells and peritoneal macrophages in dose-dependent manners. When the J774.1 cells were treated with 8.0 mg protein/mL of DBE, the transcription levels of the TNF- α and IL-6 genes increased 2-fold and 740-fold, respectively, compared with control (Fig. 2a). The transcription levels of the TNF- α and IL-6 genes in the peritoneal macrophages increased 8-fold and 27-fold, respectively, compared with control, by treating with 8.0 mg protein/mL of DBE (Fig. 2b). These results indicated that DBE stimulates cytokine production by promoting the transcription processes.

Effect of DBE on NO production and iNOS gene expression

Macrophages produce NO that exhibits antitumor activity and antibacterial activity. NO is synthesized by iNOS, which is not generally present in resting macrophages but is induced in response to cytokines and microbial pathogens. We therefore examined the effect of DBE on NO production and iNOS gene expression in RAW264.7 cells. RAW264.7 cells were treated with various concentrations of DBE for 24 h and used for the assays. As shown in Fig. 3a, DBE significantly enhanced NO production by RAW264.7

Fig. 2 Effect of dried bonito extract (DBE) on mRNA expression of cytokines by J774.1 cells and peritoneal macrophages. J774.1 cells (a) or peritoneal macrophages (b) were treated with RPMI-1640 medium containing 10% fetal bovine serum and various concentrations of DBE or 10 mM sodium phosphate buffer (pH 7.4) as control. After incubation at 37 °C for 6 h, the mRNA expression levels of TNF- α and IL-6 were evaluated using real-time RT-PCR. Data are expressed as the mean $\pm$ SD ($n = 3$). * $P < 0.05$; ** $P < 0.01$ against control by Dunnett's test

(A) J774.1 cells

(B) Peritoneal macrophages

cells in a dose-dependent manner. In addition, DBE also significantly increased the gene expression level of iNOS in the RAW264.7 cells in a dose-dependent manner (Fig. 3b), indicating that DBE stimulates NO production by enhancing iNOS gene expression.

Effect of DBE on the phagocytosis activity of J774.1 cells

Macrophages are phagocytic cells differentiated in tissues from monocytes. Phagocytosis is the first step in triggering host defense and inflammation. We thus examined the effect of DBE on the phagocytosis activity of J774.1 cells using Texas Red-labelled Zymosan A. J774.1 cells were treated with 8.0 mg protein/mL of DBE for 6 h, and Texas Red-labelled Zymosan A was added to the cell culture media after washing the cells with PBS. As shown in Fig. 4a, the Zymosan A-mediated phagocytosis activity of the J774.1 cells was strongly enhanced by DBE compared with control. As shown in Fig. 4b, the phagocytosis rate of the DBE-treated cells increased significantly to 39.8% ($P < 0.01$ vs. control), whereas that of the control cells was 12.2%.

These results suggested that DBE stimulates the phagocytosis activity of macrophages.

Effect of DBE on the signaling pathways involved in macrophage activation

Macrophages are activated by stimuli such as microbial pathogens through MAPK and NF- κ B cascades. We therefore examined how DBE affects the signaling pathways involved in macrophage activation. J774.1 cells were treated with 8.0 mg protein/mL of DBE for 15 min, and the protein levels of the signal molecules were evaluated by immunoblot analysis. LPS, a major outer membrane component of gram-negative bacteria, was used as a positive control that binds to TLR4 on the cell surface of macrophages and leads to macrophage activation through MAPK and NF- κ B cascades (Sanghera et al. 1996; Hambleton et al. 1996). As shown in Fig. 5a, the phosphorylation levels of JNK and p38 were increased in the J774.1 cells by DBE, whereas that of ERK was not affected. In addition, DBE promoted the phosphorylation and degradation of I κ B α and the translocation of NF- κ B

Fig. 3 Effect of dried bonito extract (DBE) on nitric oxide (NO) production and mRNA expression of inducible nitric oxide synthase (iNOS) in RAW264.7 cells. **a** RAW264.7 cells were treated with DMEM containing 10% fetal bovine serum (FBS) and various concentrations of DBE or 10 mM sodium phosphate buffer (pH 7.4) as control. After incubation at 37 °C for 24 h, the concentration of NO in the culture media was measured by

Griess assay. **b** RAW264.7 cells were treated with 10% FBS-DMEM containing various concentrations of DBE or 10 mM sodium phosphate buffer as control. After incubation at 37 °C for 6 h, the mRNA expression level of iNOS was evaluated using real-time RT-PCR. Data are expressed as the mean $\pm$ SD ($n = 3$). * $P < 0.05$; ** $P < 0.01$ against control by Dunnett's test

Fig. 4 Effect of dried bonito extract (DBE) on phagocytosis activity of J774.1 cells. J774.1 cells were treated with RPMI-1640 medium containing 10% fetal bovine serum and 8.0 mg protein/mL of DBE or 10 mM sodium phosphate buffer (pH 7.4) as control and incubated at 37 °C for 6 h. After washing with phosphate-buffered saline, the cells were treated with Texas

Red-labeled Zymosan A for 1 h under a dark condition, and the phagocytosis activity of the cells was measured on a flow cytometer. A representative histogram from three independent experiments is shown. Data are expressed as the mean $\pm$ SD ($n = 3$). ** $P < 0.01$ against control by the Tukey–Kramer test

into the nucleus (Fig. 5b). These results revealed that DBE stimulates macrophage activation through upregulated MAPK and NF- κ B cascades.

Effect of TLR4 inhibition on DBE-induced macrophage activation

Macrophages recognize the molecular structures of microbes through PRRs such as TLRs on the cell surface, thereby activating MAPK and NF- κ B cascades. We thus examined the effect of TLR4 inhibition on DBE-induced macrophage activation using TAK-242, an inhibitor that selectively inhibits TLR4

signaling (Li et al. 2006; Matsunaga et al. 2011). J774.1 cells were treated with 0.2 μ M TAK-242 for 1 h. The cells were subsequently treated with 8.0 mg protein/mL of DBE for 6 h after washing with PBS, and then the cytokine concentration in the culture media was measured by ELISA. As shown in Fig. 6a, the TNF- α production stimulatory activity of DBE was inhibited about 40% by treatment with TAK-242, whereas that of LPS was inhibited about 60% ($P < 0.01$ between DBE and LPS treatments in the presence of TAK-242). These results suggested that DBE enhances TNF- α production through not only TLR4 signaling, but also other signaling. On the other hand, the IL-6 product

Fig. 5 Effect of dried bonito extract (DBE) on signaling pathways involved in macrophage activation. J774.1 cells were treated with RPMI-1640 medium containing 10% fetal bovine serum and 8.0 mg protein/mL of DBE, 10 mM sodium phosphate buffer (pH 7.4) as control, or 100 ng/mL of lipopolysaccharide (LPS) as a positive control at 37 °C for 15 min. **a** The protein levels of ERK, JNK, and p38 were evaluated by immunoblot analysis. The p-ERK, p-JNK, and p-p38 represent phosphorylated ERK, phosphorylated JNK, and phosphorylated p38, respectively. A representative blot from

three independent experiments is shown. The result of densitometric analysis is expressed as the ratio of (phosphorylated protein amount)/(whole protein amount). **b** The protein levels of IκBα and NF-κB were evaluated by immunoblot analysis. The p-IκBα represents phosphorylated IκBα. A representative blot from three independent experiments is shown. The result of densitometric analysis is expressed as the ratio of (phosphorylated protein amount)/(whole protein amount). Data are expressed as the mean ± SD ($n = 3$). No statistical significance was found by the Tukey–Kramer test

ion stimulatory activity of DBE was inhibited about 80% by treatment with TAK-242, whereas that of LPS was almost completely inhibited. There was no significant difference in the inhibition rate between LPS and DBE under TAK-242 treatment condition, suggesting that DBE enhances IL-6 production through TLR4 signaling.

Next, we investigated whether the DBE-induced activity is due to contamination of LPS. Polymyxin B binds to lipid A motif of LPS and suppresses the biological activities of LPS. J774.1 cells were incubated with the polymyxin B-treated DBE for 6 h, and the cytokine concentration in the culture media was measured by ELISA. As shown in Fig. 6b, polymyxin B treatment completely inhibited both TNF-α and IL-6 production stimulatory activities of LPS. On the other

hand, TNF-α production stimulatory activity was inhibited about 40% by polymyxin B treatment of DBE, whereas IL-6 production stimulatory activity of DBE was inhibited about 80% by polymyxin B treatment. Since there is a significant difference ($P < 0.01$) in the effect of polymyxin B treatment between LPS and DBE, it was suggested that the activity of DBE is not only due to contamination of LPS, but also due to the active substance contained in DBE.

Effect of proteolytic enzyme-treated DBE on cytokine production by J774.1 cells

To estimate the bioactive substance in DBE, 8.0 mg protein/mL of DBE was treated with 500 μg/mL of proteinase K at 37 °C for 15 h. As shown in Fig. 7,

(A) TAK-242 treatment **(B) Polymyxin B treatment**

Fig. 6 Effects of Toll-like receptor 4 (TLR4) or lipopolysaccharides (LPS) inhibitors on macrophage activation induced by dried bonito extract (DBE). **a** J774.1 cells were treated with 0.2 μ M TAK-242 for 1 h. The cells were subsequently treated with RPMI-1640 medium containing 10% fetal bovine serum and 8.0 mg protein/mL of DBE, 10 mM sodium phosphate buffer (pH 7.4) as control, or 10 ng/mL of LPS as a positive control at 37 °C for 6 h. After incubation, the concentrations of TNF- α and IL-6 in culture media were measured by enzyme-linked immunosorbent assays. *Solid bars*, non-treatment with TAK-242; *open bars*, treatment with TAK-242. **b** DBE (8.0 mg/

mL) and LPS (10 ng/mL) were treated with 10 μ g/mL of polymyxin B at room temperature for 30 min. Then, J774.1 cells were incubated in RPMI-1640 medium containing 10% fetal bovine serum and each polymyxin B-treated sample at 37 °C for 6 h. After incubation, the concentrations of TNF- α and IL-6 in culture media were measured by enzyme-linked immunosorbent assays. *Solid bars*, non-treatment with polymyxin B; *open bars*, treatment with polymyxin B. Data are expressed as the mean $\pm$ SD ($n = 3$). ** $P < 0.01$ by the Tukey–Kramer test; n.s. indicates no statistical significance by the Tukey–Kramer test

TNF- α production was significantly suppressed by the proteinase K-treated DBE ($P < 0.01$ against non-treated DBE), whereas IL-6 production was not affected by the proteinase K treatment. These results indicated that DBE includes multiple bioactive substances and a substance that stimulates TNF- α production is a protein.

Effect of oral administration of DBE on peritoneal macrophages

We finally investigated the effect of oral administration of DBE. Mice were orally administered with 10 mM sodium phosphate buffer for the control group, with DBE at 16 mg protein/kg body weight for the

Fig. 7 Effect of proteolytic enzyme-treated dried bonito extract (DBE) on cytokine production by J774.1 cells. DBE was treated with 500 μ g/mL of proteinase K at 37 °C for 15 h. After heating the samples to inactivate the protease, J774.1 cells were treated with RPMI-1640 medium containing 10% fetal bovine serum and the proteinase K-treated DBE or 10 mM sodium phosphate buffer (pH 7.4) as control. After incubation at 37 °C for 6 h, the

concentrations of TNF- α and IL-6 in culture media were measured by enzyme-linked immunosorbent assays. *Solid bars*, 10 mM sodium phosphate buffer as control; *open bars*, DBE. Data are expressed as the mean $\pm$ SD ($n = 3$). ** $P < 0.01$ by Tukey–Kramer test; n.s. indicates no statistical significance by the Tukey–Kramer test

high-dose group, or with DBE at 1.6 mg protein/kg body weight for the low-dose group for 14 consecutive days. Mice were then sacrificed, and peritoneal macrophages were collected and cultured for 24 h to evaluate phagocytosis activity. As shown in Fig. 8, oral administration of DBE significantly increased the phagocytosis activity of peritoneal macrophages collected from the mice in the high-dose group ($P < 0.01$ against control). The result suggested that DBE could potentially activate macrophages *in vivo* by oral administration.

Discussion

In this study, we found that DBE stimulates the production of TNF- α and IL-6 by J774.1 cells and peritoneal macrophages by enhancing the expression levels of these cytokine genes (Figs. 1, 2). These cytokines regulate cell death in inflamed tissues, modify vascular endothelial permeability, recruit blood cells to inflamed tissues, and induce the production of acute-phase proteins (Takeuchi and Akira 2010). Therefore, promoting the production of these cytokines leads to enhancing the immune system. DBE also stimulated NO production by enhancing the gene expression of iNOS in RAW

Fig. 8 Effect of oral administration with dried bonito extract (DBE) on peritoneal macrophages. Mice were orally administered with 10 mM sodium phosphate buffer (pH 7.4), DBE at 16 mg protein concentration/kg body weight/day, or DBE at 1.6 mg protein concentration/kg body weight/day for 14 consecutive days, and peritoneal macrophages were collected and cultured for 24 h. The phagocytosis activity of the cells was measured on a flow cytometer. Data are expressed as the mean $\pm$ SD ($n = 5$). ** $P < 0.01$ against control by Dunnett's test

264.7 cells (Fig. 3). Because NO produced by macrophages exhibits bactericidal activity against microbial pathogens (Moncada et al. 1991; Nathan 1992), DBE might help immune cells to efficiently kill the microbial pathogens in the body. Furthermore, macrophages recruited to the site of infection ingest microbial pathogens into their vesicles, fragment the pathogens, and present them to T cells as an antigen. DBE enhanced the phagocytosis activity of the J774.1 cells (Fig. 4). Therefore, our findings suggest that DBE contributes to activating host defense against pathogens by macrophages.

There are two signaling pathways involved in macrophage activation, namely, MAPK and NF- κ B cascades. There are mainly three families of MAPKs in mammals: ERK1/2, JNK, and p38 kinases. They are activated by specific MAPK kinases (MAPKKs) and eventually lead to the synthesis of transcription factors such as c-Fos, c-Jun, activating transcription factor 2 (ATF2), and cyclic AMP responsive element-binding protein (CREB) (Su and Karin 1996; Zhang and Dong 2005). The synthesized transcription factors form the AP-1 dimers, which bind to the AP-1-binding site on DNA after translocating into the nucleus, thereby promoting the transcriptional activity of target genes (Karin 1995; Karin et al. 1997). As shown in Fig. 5a, DBE increased the phosphorylation levels of JNK and p38 in the J774.1 cells, whereas that of ERK was not affected. These results suggest that DBE activates AP-1 activity through upregulated phosphorylation of JNK and p38 among MAPK cascades, thereby promoting the expression levels of cytokine genes. In addition, DBE promoted the phosphorylation and degradation of I κ B α and NF- κ B translocation into the nucleus (Fig. 5b). The NF- κ B family consists of five members in mammals: NF- κ B1 (p105/p50), NF- κ B2 (p100/p52), RelA (p65), RelB, and c-Rel (Bonizzi and Karin 2004). NF- κ B proteins form homodimers or heterodimers with specific biological responses. The NF- κ B dimers become an inactive form by binding to I κ Bs, which are specific NF- κ B inhibitors and retained in the cytoplasm. In the classical NF- κ B pathways, stimuli such as LPS result in activation of I κ B kinase (IKK) complex (Bonizzi and Karin 2004). The activated IKK phosphorylates I κ Bs, and then the phosphorylated I κ B is rapidly degraded by the ubiquitin–proteasome system, thereby freeing NF- κ B (Ghosh and Karin 2002). The released NF- κ B dimers translocate into the nucleus, bind to the κ B motif sequences

found in promoter and enhancer regions of target genes, and promote gene expression (Matthews and Hay 1995). Consequently, DBE is considered to promote cytokine gene expression by upregulating the phosphorylation and degradation of I κ B α and subsequent translocation of NF- κ B into the nucleus. MAPK and NF- κ B cascades are also involved in NO production and the phagocytosis activity of macrophages. LPS-induced iNOS expression has been reported to be reduced by a specific p38 inhibitor (Chen et al. 1999). It has also been reported that NF- κ B/Rel is involved in inducing not just iNOS-derived reporter constructs but the iNOS gene itself in LPS-treated macrophages (Xie et al. 1994). Furthermore, specific p38 and NF- κ B inhibitors impair the TLR-induced phagocytosis activity of macrophages (Blander and Medzhitov 2004; Suzuki and Umezawa 2006). Therefore, the DBE-stimulated NO production and phagocytosis activity of macrophages are considered to be due to the activation of MAPK and NF- κ B cascades.

In this study, DBE was extracted with distilled water, heated to 100 °C, and dialyzed using a dialysis membrane with MWCO of 14,000. The active substances in DBE are estimated to be water-soluble and heat-stable with molecular weight equal to or higher than 14,000. Therefore, DBE is thought to act on cell surface receptors such as TLRs, because DBE is unlikely to pass through a plasma membrane and be active inside the cells. The TLR family is one of the characterized PRR families in macrophages; TLR2 recognizes lipoproteins from bacteria and mycoplasma, and TLR4 recognizes LPS from gram-negative bacteria together with myeloid differentiation factor 2 (MD2) on the cell surface (Takeuchi and Akira 2010). Their stimulation recruits Toll/IL-1 receptor (TIR) domain-containing adaptor protein (TIRAP) and MyD88, TIR domain-containing adaptor inducing interferon β (TRIF), and TRIF-related adaptor molecule (TRAM) to the TLR. MyD88 interacts with IL-1 receptor-associated kinase-4 (IRAK-4), and the IRAK-4 activates other IRAK family members. The activated IRAKs interact with TNF receptor associated factor 6 (TRAF6) after dissociation from MyD88 and activate a complex of transforming growth factor β -activated kinase 1 (TAK1), TAK1-binding protein (TAB) 1, and TAB 2/3, thereby resulting in the phosphorylation of IKK complex and MAPKKs (Aderem and Ulevitch 2000; Takeuchi and

Akira 2010). In this study, we examined whether DBE acts on TLR4 using TAK-242, which blocks interactions between TLR4 and its adaptors TIRAP and TRAM. As shown in Fig. 6a, TNF- α production enhanced by DBE was partially inhibited by treatment with TAK-242, whereas IL-6 production enhanced by DBE was almost inhibited. These results suggest that DBE strongly stimulates the TLR4 signaling pathway for macrophage activation, and its activation is also involved in other signaling pathways. However, Fig. 6b showed the contamination of LPS in DBE. Moreover, since there is a correlation between the results in Fig. 6a, b, it was suggested that LPS contained in DBE activates macrophages through TLR4 signaling. On the other hand, since DBE treated with polymyxin B showed the remained activity, it was suggested that DBE also contains other active substances, which are involved in signaling other than TLR4. We previously found that spinach aqueous extract (SAE) activates macrophages (Ishida et al. 2016), and its activity is similar to that of DBE because both extracts seem to stimulate the TLR4 signaling pathway for macrophage activation. The active substance in SAE seems to be a protein, because the activity is heat-unstable, and modified by protease treatment. On the other hand, the active substance in DBE is heat-stable, and at least one of them is assumed to be a protein because its activity was reduced by proteolytic enzyme treatment (Fig. 7). These results suggest that the active substance in SAE is different from that in DBE. In these experiments, sodium phosphate buffer was used as extraction solvent or control. However, we have confirmed that many proteins dissolved in sodium phosphate buffer have no effects on macrophages, for example lysozyme, suggesting that not all proteins contained in the buffer exhibit its activity. Because the sodium phosphate buffer itself has no such activity, the activity of DBE is thought to be due to a specific protein in DBE. To identify the active substance in DBE, we attempted to purify it; however, proteins contained in DBE were extensively degraded and indistinct bands were observed when DBE was run on SDS-PAGE (supplementary data). The reason is considered to be because DBE was prepared by extraction at 100 °C for 2 h. During the extraction process, proteins in DBE were randomly broken. In addition, the activity of DBE remained after proteinase K treatment (Fig. 7) or TAK-242 treatment (Fig. 6a). These results suggest

that the active substance contained in DBE has different characteristics from that of SAE and might act complexly. Because purification and identification of the active substance in DBE is extremely difficult, the active substance in DBE is not yet clear. Although further experiments are needed, our data suggest that DBE contains a certain substance that contributes to macrophage activation.

Finally, we investigated the effect of oral administration of DBE. As shown in Fig. 8, the oral administration of DBE significantly promoted the phagocytosis activity of peritoneal macrophages in the high-dose group, suggesting that the ingestion of DBE or dried bonito may be effective in enhancing the immune system *in vivo*. Ingestion of DBE is safe because it is a food-derived extract and exhibits no side effects in mice. Our findings suggest that daily ingestion of DBE contributes to the prevention or alleviation of infection in humans by enhancing the abilities of macrophages to remove and present invading pathogens. Taken together, our data strongly suggest that the extract of dried bonito shows the immunostimulatory effect on experimental animals as well as cell lines. Because the dried bonito sample, arabushi, used in this study is just smoked and commonly sold on the market, it is not much different than other commercial products. In addition, the dried bonito used here is commercially available. Thus, the arabushi used in this study is not a special product, and the activities of dried bonito indicated here could be generalized.

Conclusion

Although several functions of dried bonito on human health are known, no immunostimulatory effect of dried bonito on macrophages has been reported so far. This manuscript is therefore the first report describing that dried bonito has an immunostimulatory effect on macrophages. DBE significantly stimulated the production of TNF- α and IL-6 by both J774.1 cells and peritoneal macrophages by enhancing the expression levels of these cytokine genes. In addition, DBE stimulated NO production by enhancing the expression of iNOS in RAW264.7 cells. The phagocytosis activity of J774.1 cells was significantly increased by DBE. Immunoblot analysis revealed that DBE has an immunostimulatory effect on macrophages through

activation of MAPK and NF- κ B cascades. TNF- α production enhanced by DBE was partially inhibited by treatment with TAK-242, whereas IL-6 production enhanced by DBE was almost inhibited. Although DBE strongly stimulates the TLR4 signaling pathway for macrophage activation, its activation is also involved in other signaling. Finally, phagocytosis activity was significantly promoted by peritoneal macrophages from DBE-administered BALB/c mice. This result suggests that DBE has the potential to stimulate macrophage activity in vivo. Taken together, these data indicate that DBE would be a novel functional food for immunostimulation with academic significance and that quotidian intake of DBE contributes to the maintenance of human health with enhancement of the immunity due to macrophage activation.

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Author contributions MI, KN, KS, NK, and TS conceived and designed the experiments. MI and KS, and NK performed the experiments and analyzed the data. MI, KN, and TS wrote the manuscript.

Compliance with ethical standards

Conflict of interest Katsuhiko Osajima, Tomokazu Suemitsu, and Takuya Sugahara are inventors in a patent application filed by Ehime University, which disclose bioactive agents targeting immunostimulation described in the present article. The remaining authors declare no conflict of interest.

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