Anti-Inflammatory Effect of Buckwheat Sprouts in Lipopolysaccharide-Activated Human Colon Cancer Cells and Mice

Satoshi ISHI, Takafumi KATSUMURA, Chikara SHIOZUKA, Keisuke OYAUCHI, Kunito KAWASAKI, Shigenobu TAKIGAWA, Tatsunobu FUKUSHIMA, Yoshihiko TOKUJI, Mikio KINOSHITA, Masao OHNISHI, Mika KAWAHARA, and Kiyoshi OHBA

1Department of Agricultural and Life Sciences, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
2National Agricultural Research Center for Hokkaido Region, Memuro, Hokkaido 082-0071, Japan
3Yokohama Research Laboratories, Mitsubishi Rayon Co., Ltd., Yokohama, Kanagawa 220-0053, Japan
4Hokkaido Tokachi Area Regional Food Processing Technology Center, Obihiro, Hokkaido 080-2462, Japan

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In conducting an in vitro screening of ethanol extracts from various natural foods using a human colon cancer cell line (CoLoTC cells), an extract of buckwheat sprouts (ExtBS) was found to express significant anti-inflammatory activity. The anti-inflammatory activity of ExtBS was confirmed by oral administration of lipopolysaccharide (LPS) to mice. Inflammatory cytokines (interleukin 6 and tumor necrosis factor alpha) were markedly up-regulated in the spleen and liver from LPS-administered mice, and combinatory treatment with LPS and ExtBS decreased up-regulation of them in both cytokines. Both serum cytokine levels corresponded to their gene expressions in tissues, but no anti-inflammatory effect in mice was observed when ExtBS was treated intraperitoneally. ExtBS oral administration also showed protective activity as to hepatic injury induced by galactosamine/LPS treatment. Based on these data, we suggest that ExtBS contains anti-inflammatory compounds.

Key words: anti-inflammatory effect; buckwheat sprouts; lipopolysaccharide-activated cells; cytokine expression

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, elicits inflammatory effects in the host through Toll-like receptor 4 (TLR4), which is known to be an LPS receptor. Host cells such as mononuclear phagocytes/macrophages initiate a rapid and massive immune response to LPS that includes the lethal function of endotoxin caused by the generation of cellular mediators such as interleukin 1 (IL-1), IL-6, tumor necrosis factor alpha (TNF-α), and chemokines such as IL-8. TNF-α has been identified as the major cytokine mediator in the expression of endotoxicity.

LPS-treated culture cells have been used to evaluate the anti-inflammatory effects of various materials, including folk medicines such as Polygonum tinctorium, Cyperus rotundus, Semecarpus anacardium, Ginkgo biloba, and Cryptolepis buchanani, and natural foods such as turmeric, ginger, chicory, and green tea. Although the mechanisms of the anti-inflammatory effects of those materials are different, a well-known mechanism has been described as inhibition of NF-κB activation and NO production caused by antioxidant active compounds.

Buckwheat (Fagopyrum esculentum) is a health food in that it is rich in flavonoids such as rutin. Rutin is an effective radical scavenger, and Mukoda et al. have reported that buckwheat hull extract showed strong antioxidant activities in vitro and in vivo. Human intestinal epithelial cells have been used to study the effects of health foods on the mucosal immune response. Particular human intestinal epithelial cells expressing TLR4 and MD-2 have been found to respond to LPS activation. Although we used both human colon carcinoma cell lines and human mononuclear cell line THP-1 cells to screen anti-inflammatory compounds in natural foods that might be effective through the mucosal immune response, we found that ethanol extract of buckwheat sprouts contains anti-inflammatory compounds that inhibit the up-regulation of inflammatory cytokines induced by LPS when we used CoLoTC cells.
but not THP-1 cells. We further confirmed its effect in an LPS-activated mouse model.

Materials and Methods

Flavonoid standards. A chlorogenic acid standard was obtained from LKT Laboratories (St. Paul, MN) and other flavonoid standards were from Extrasynthese (Genay Cedex, France).

Preparation of extracts from buckwheat sprouts. Dried powder (10 g) of buckwheat sprouts was soaked in 70% ethanol overnight with shaking at room temperature. After filtration through filter paper, the extract was concentrated in a rotary evaporator and dried in a freezing dryer. Dried extract (about 0.7 g) was dissolved in 70% ethanol overnight with shaking at room temperature. After filtration through filter paper, the extract was concentrated in a rotary evaporator and dried in a freezing dryer. Dried extract (about 0.7 g) was dissolved in 70% ethanol and designated extracts of buckwheat sprouts (ExtBS) for subsequent experiments.

Determination of flavonoids in ExtBS. To extract flavonoids from ExtBS, 10 mg of dried ExtBS was soaked in 1 ml of methanol containing 10% phosphoric acid for 3 h at 37 ℃. The extract, pooled by centrifugation (1,000 × g, 5 min), was passed through a disposable syringe filter (PTFE, 0.5 μm, hydrophobic; Advantec, Tokyo). The filtrate was analyzed by HPLC (class-VP chromatography data system; Shimadzu, Kyoto, Japan) in a Capcel PAC ODS column (250 mm × 4.6 mm, particle size, 5 μm; Shiseido, Tokyo), and quantification was performed using commercial external standards, as described previously.

Cell culture. Human colon carcinoma cell lines CoLoTC (RCB1984), LoVo (RCB1639), CACO-2 (RCB0988), and COLO-320 (RCB1193) cells and human mononuclear cell line THP-1 (RCB1189) cells were supplied by the RIKEN BioResource Center (Tsukuba, Japan). During the preparation of this manuscript, the provider informed us that the COLO205 cell line, derived from colon cancer, as determined short tandem repeat polymorphism analysis. The HeLa cell line (JCRB9004) was purchased from the Human Science Research Resources Bank (Osaka, Japan). CoLoTC, CACO-2, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma Chemicals, St. Louis, MO) supplemented with 10% fetal calf serum (FCS, Biological Industries, Haemek, Israel). LoVo, COLO-320 cells, and THP-1 cells were cultured in RPMI-1640 medium (Sigma) containing 10% FCS. The cultures were maintained in a humidified 5% CO₂ atmosphere at 37 ℃. To determine the effect of LPS treatment, cells (1 × 10⁶) were prepared in fresh medium and incubated in the presence of 100 ng/ml of LPS (Escherichia coli serotype O55:B5, Sigma) for 16h. CoLoTC cells were treated with ExtBS (final concentration, 1 mg/ml) in the presence and the absence of LPS for 16 h. The culture medium was collected for determination of IL-8 secretion, and then the cells were rinsed with phosphate-buffered saline (PBS) and precipitated by centrifugation at 2,000 × g for 5 min. The cells were stored at −20 ℃ until use. To quantify cell viability, we counted live cells under light microscopy after trypan blue staining.

Oral administration in mice. The animal experimental protocols in this study were approved by the Animal Experiments Committee of Obihiro University of Agriculture and Veterinary Medicine. Female C57Bl/6J mice (8 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). Food was withheld overnight before the experiments. LPS (20 mg/ml dissolved in water) was mixed with an equal volume of 70% ethanol or 100 mg/ml ExtBS in 70% ethanol and labeled LPS and LPS + ExtBS treatment material respectively. A 0.2-ml mixture of LPS and LPS + ExtBS treatment material was administered per mouse orally by gavage. The amounts of LPS and ExtBS were calculated to be approximately 100 mg and 500 mg respectively per kg of body weight. Control mice were administered 0.2 ml of 35% ethanol. At 2 h or 4 h after administration, the mice were sacrificed and blood was collected via cardiac puncture. The spleen and liver were quickly removed and rinsed with ice-cold PBS and used for RNA isolation.

Intraperitoneal injection of LPS and ExtBS. Eight-week-old female C57Bl/6J mice were intraperitoneally injected with 1 μg of LPS plus 5 μg or 500 μg of ExtBS in 0.2 ml of PBS. For intraperitoneal injection, LPS was dissolved in PBS and ExtBS-ethanol solution was diluted 10-fold with PBS. LPS (10 μg/ml) was mixed with an equal volume of 7% ethanol, 50 μg/ml, or 5,000 μg/ml of ExtBS in 7% ethanol for treatment with LPS, LPS + ExtBS or LPS + ExtBS(H), respectively. An aliquot of 0.2 ml of LPS, LPS + ExtBS, or LPS + ExtBS(H) was intraperitoneally injected into each mouse. Control mice were given 0.2 ml of 3.5% ethanol in PBS. At 4 h after administration, the mice were sacrificed and blood was collected by cardiac puncture, then the spleen and liver were quickly removed and rinsed with ice-cold PBS and used in RNA isolation.

Measurement of cytokine mRNA by semi-quantitative RT-PCR. Cells were suspended with RNAiso (Takara Bio, Shiga, Japan) by micropipette, and mouse tissues were homogenized in RNAiso using a microhomogenizer (Phycsottron, Niti-on, Chiba, Japan). Total RNA was prepared according to the manufacturer’s protocol. The OD260 and OD260/280 values were measured with a spectrophotometer to determine the RNA concentrations. After the quantity of RNA was carefully adjusted, reverse transcriptase (RT)-PCR was performed with a Takara RNA PCR Kit (AMV) Ver.3.0 (Takara Bio) with specific oligonucleotide primers, as summarized in Table 1. The RT reaction was performed at 30 ℃ for
Table 1. Primer Sequences and Product Sizes for RT-PCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (forward/reverse)</th>
<th>Size of amplified products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hTLR4</td>
<td>5'-agcactgagacattcag-3/5'-cctctagtccagatgga-3</td>
<td>305</td>
</tr>
<tr>
<td>hTLR2</td>
<td>5'-tacgagcagccagtttta-3/5'-agtctcctcagagga-3</td>
<td>336</td>
</tr>
<tr>
<td>hMD-2</td>
<td>5'-actgaagcagacacgtaa-3/5'-gtttatctcagctcttctc-3</td>
<td>302</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-acacactgctacatca-3/5'-caccggtgtttagc-3</td>
<td>447</td>
</tr>
<tr>
<td>hL-1P</td>
<td>5'-aacacagggcttgc-3/5'-cttcaacagacagag-3</td>
<td>405</td>
</tr>
<tr>
<td>hL-6</td>
<td>5'-atgaacctctcttcaacag-3/5'-aatctctgtcagagctact-3</td>
<td>398</td>
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<tr>
<td>hL-8</td>
<td>5'-atgactctcaagctgtt-3/5'-gaatctctcaggtctcag-3</td>
<td>356</td>
</tr>
<tr>
<td>hL-12</td>
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<td>384</td>
</tr>
<tr>
<td>hL-18</td>
<td>5'-aagtgtctgctgaac-3/5'-tactaggagagtcgctc-3</td>
<td>382</td>
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<td>316</td>
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<tr>
<td>mTNF-α</td>
<td>5'-gtctctcgttcagc-3/5'-ctgccccttaggaa-3</td>
<td>302</td>
</tr>
</tbody>
</table>

10 min, and then at 42°C for 15 min, followed by incubation at 99°C for 5 min. PCR amplification was performed using the following conditions: initial denaturation (94°C for 5 min), followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, elongation at 72°C for 30 s, and further elongation at 72°C for 2 min. PCR products were run on 2% agarose gels, stained with ethidium bromide, and photographed. Cytokine mRNA expression levels were densitometrically quantified by AE-6920M (Atto, Tokyo), and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. The long exposure time was used for the detection of low-expression genes (IL-6 and TNF-α).

Cytokine immunoassays. The amounts of IL-8 in the culture medium and of TNF-α and IL-6 in mouse serum were determined with Quantikine ELISA kits (R&D Systems, Minneapolis, MN) for human IL-8 and for mouse TNF-α and IL-6 respectively. The cytokine level was quantified by comparison with standard curves run concurrently using recombinant cytokines according to the manufacturer’s protocol.

Focused DNA array analysis. The Genopal™ Allergy chip (Mitsubishi Rayon, Tokyo, http://www.mrc.co.jp/genome/e/index.html) containing probes for 209 genes related to inflammation and immune response, including 49 cytokine and cytokine-related genes was used in gene expression analysis. Genopal™ is composed of hollow plastic fibers (oligonucleotide DNA probes are immobilized to a hydrophilic gel within the interior of each hollow fiber). Hybridization signal acquisition was performed using a DNA chip reader using multi-beam excitation technology. (Yokogawa Electric, Tokyo) The DNA chips were scanned at multiple exposure times, and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. The long exposure time was used for the detection of low-expression genes (IL-6 and TNF-α).

Biotinylated antisense RNA was synthesized and amplified from 1 µg of total RNA from each sample using the MessageAmp™ II biotin enhanced amplification kit (Ambion, Austin, TX), according to the manufacturer’s instructions, and then column purified and eluted in nuclease-free water. Biotinylated aRNA (5 µg) was fragmented using 10X fragmentation reagents (Ambion) and incubation at 70°C for 7.5 min. Hybridization was carried out with the DNA chips in 150 µl of hybridization buffer (0.12 M Tris HCl/0.12 M NaCl/0.05% Tween-20 and 5 µg of fragmented biotinylated aRNA) at 65°C overnight. After hybridization, the DNA chips were washed twice in 0.12 M Tris HCl/0.12 M NaCl/0.05% Tween-20 at 65°C for 20 min, followed by washing in 0.12 M Tris HCl/0.12 M NaCl for 10 min. Then the DNA chips were washed, and labeled with streptavidin-Cy5 (GE Healthcare Bio-Science, Piscataway, NJ). After fluorescent labeling, the DNA chips were washed 4 times in 0.12 M Tris–HCl/0.12 M NaCl/0.05% Tween-20 at room temperature for 5 min each. Hybridization signal acquisition was performed using a DNA chip reader using multi-beam excitation technology. DNA chip images and gene expression values were calculated automatically. Gene expression values were normalized using housekeeping genes (GAPDH, β-actin), so that each of the DNA chips compared in an experiment would have the same mean value.

Acute liver-failure models. A model experiment of acute liver failure with t-galactosamine/LPS was performed by the method described by Nakama et al. with some modifications. Eight-week-old female C57Bl/6J mice were intraperitoneally injected with 0.2 ml of 50 mg/ml D-galactosamine (GalN, Wako Pure Chemical Industries, Osaka, Japan) in PBS. In addition, the mice were orally administered LPS (2 mg/mouse) or LPS (2 mg) + ExtBS (10 mg) by gavage. The survival of the mice was observed for 5 d.

For histopathologic analysis, the mice were sacrificed 24 h after treatment, and liver tissues were fixed in 10% phosphate-buffered formalin (Wako) and embedded in paraffin. Sections (5 µm) were stained with hematoxylin-eosin for morphologic examination under microscopy.

Results

LPS-Induced response in CoLoTC cells and effect of ExtBS on IL-8 expression

Expression of cytokine genes in four human colon cancer cell lines (CoLoTC, LoVo, CACO-2, and COLO-320) was determined by RT-PCR, and the response to LPS-treatment was studied (Fig. 1A). IL-1β, IL-8, and IL-18 were expressed in LoVo cells, but no expression level of these cytokines was changed by treatment with LPS. IL-8 and IL-18 expression was observed in CACO-2 and CoLoTC cells, and only IL-8 was increased by treatment with LPS in the cell lines. In contrast with these cell lines, only IL-12 expression was detected in COLO-320 cells on these data, we chose...
CoLoTC cells for the screening of anti-inflammatory compounds because of the good response against LPS-treatment, a good proliferation curve, and ease of handling the subcultures. To determine the inflammatory response of CoLoTC cells against LPS, we assayed the expression of TLR4 and MD-2 (Fig. 1B). The expression level of TLR4 was high but that of TLR2 was low in CoLoTC cells. This expression pattern in CoLoTC cells was different from THP-1 cells, in which the expression of both TLR4 and TLR2 was high (data not shown). Significant expression was observed for MD-2, which is necessary for the receptor function of TLR4.22) In the screening of anti-inflammatory compounds using LPS-treated cells, we used IL-8 as a biomarker because it was the most inducible cytokine expressed in CoLoTC cells upon treatment with LPS in our preliminary experiment. While conducting an in vitro screening (semi-quantitative RT-PCR) of ethanol extracts from natural foods (22 samples including potatoes, beans, and sprouts) using CoLoTC cells, we found ExtBS to express significant anti-inflammatory activity (Fig. 1C). In our screening method, a rather high concentration (1 mg/ml) was used to detect obvious changes in the anti-inflammatory response in the first screening of natural foods. The IL-8 expression level was significantly reduced in a dose-dependent manner with the addition of ExtBS (lanes 3 and 4, Fig. 1C).

**Effect of ExtBS on IL-8 production and cell viability in CoLoTC cells**

To confirm the anti-inflammatory effect of ExtBS, we determined IL-8 production by ELISA (Fig. 2A). We observed a 2.7-fold increase in IL-8 production post-treatment with LPS, and the IL-8 level was significantly reduced with the addition of ExtBS. No significant difference was observed in cell viability under any treatment (Fig. 2B). These data indicate that ExtBS reduced IL-8 production in LPS-activated CoLoTC cells without toxicity.

**Anti-inflammatory effect of flavonoid standards in LPS-induced CoLoTC cells**

Total flavonoids (49.5 mg) were detected by HPLC in 1 g of ExtBS, which contained 10.8 mg of vitexin, 8.3 mg of isovitexin, 6.4 mg of orientin, 12.6 mg of isoorientin, 2.0 mg of chlorogenic acid, and 9.4 mg of rutin. Therefore, about 50 mg/ml flavonoids were present in 1 mg/ml of ExtBS solution. Twice as high a concentration (100 μg/ml) of each flavonoid standard was used in the determination of the anti-inflammatory effect in LPS-activated CoLoTC cells, but no flavonoid showed an effect as obvious as ExtBS, and we observed only a weak effect with the addition of rutin (Fig. 3). In our preliminary experiment, ExtBS was applied to an
Amberlite ER-120B column which almost all flavonoids fail to bind. The column binding fraction eluted with 1 M NH₄OH had stronger anti-inflammatory activity than that of pass-through fraction. These data indicate that the anti-inflammatory effect of ExtBS is due to an unknown compound not a flavonoid.

Anti-inflammatory effect of oral administration of ExtBS in mice

To confirm further the anti-inflammatory effect of ExtBS, an oral administration study of ExtBS was performed in LPS-induced mice (Fig. 4). In our preliminary experiment, higher induction of inflammatory cytokines (IL-1β and TNF-α) in the mouse spleen was observed with a higher concentration of LPS, but not in mouse intestinal tissues (data not shown). We set the LPS concentration at 2 mg because inflammatory cytokines, interferon γ (IFN-γ), IL-1β, IL-6 and TNF-α, in both spleen and liver were markedly induced by treatment with it. Although four inflammatory cytokines were markedly induced, we chose IL-6 and TNF-α as biomarkers of the inflammatory response because both cytokines are known as pathogenic factors. Treatment with 2 mg of LPS per mouse caused a marked increase in the gene expression of IL-6 and TNF-α (lanes 4–6, Fig. 4A) in the spleen and liver, but the addition of ExtBS (10 mg/mouse) prevented the induction of both inflammatory cytokine genes (lanes 7–9, Fig. 4A). The relative expression levels of IL-6 and TNF-α were calculated from the band intensity of cytokine genes in comparison with gene expression of them observed in the LPS-treated group (Fig. 4B–E). Although the expression levels of IL-6 and TNF-α were low and hard to detect in the control mice, expression levels significantly changed in the spleen and liver under the LPS treatment, except for TNF-α in the spleen (a 13.0-fold and a 31.7-fold increase in IL-6 in the spleen and liver respectively, and a 1.1-fold and a 22.9-fold increase in TNF-α in the spleen and liver respectively). Induction of both inflammatory cytokines under LPS treatment was strongly prevented by the addition of ExtBS (0.8-fold and 2.1-fold of the control level of IL-6 in the spleen and liver respectively, and 0.9-fold and 6.6-fold of the control level of TNF-α in the spleen and liver respectively).

To confirm the anti-inflammatory effect of ExtBS, we determined serum IL-6 and TNF-α by ELISA (Fig. 5). Both were markedly increased by oral treatment with LPS (65.7-fold and 16.9-fold respectively of the control level), and were prevented by the addition of ExtBS (8.0-fold and 3.8-fold respectively of control). These data on cytokine production correlated well with the change in cytokine gene expression in the liver, and indicate that ExtBS has strong anti-inflammatory activity.
Effect of intraperitoneal treatment of ExtBS on the anti-inflammatory response in mice

In the intraperitoneal treatment, 1 μg of LPS/mouse, a 2,000-fold lower dose than used in oral treatment, caused marked induction of inflammatory cytokines in the spleen and liver (Fig. 6). The relative expression levels of IL-6 and TNF-α changed significantly in both the spleen and liver upon intraperitoneal injection of LPS (a 25.0-fold and a 26.4-fold increase in IL-6 in the spleen and liver respectively, and a 1.8-fold and a 34.6-fold increase in TNF-α in the spleen and liver respectively). For the intraperitoneal treatment with ExtBS, we prepared two mixtures: one contained LPS and ExtBS in a ratio of 1:5, the same ratio used in oral treatment, and the other was a 1:500 ratio that contained a 100-fold excess amount of ExtBS. Intraperitoneal treatment with either concentration of ExtBS did not show significant anti-inflammatory activity, but rather induced IL-6 in the spleen (82.9-fold and 77.5-fold of control by treatment with LPS + ExtBS and LPS + ExtBS(H) respectively).

Focused array analysis of the anti-inflammatory activity of ExtBS

To determine the details of the anti-inflammatory action of ExtBS, we performed a focused array analysis and determined the effects on the expression of various cytokines 2 h and 4 h after treatment. Up-regulated cytokine, which showed a more than 4-fold increase in two independent determinations under treatment with LPS, was distinctive between the spleen and the liver 2 h after administration, but up-regulated cytokines at 4 h in the spleen were similar to those in the liver. In the spleen at 2 h, IL-1β was the only up-regulated cytokine, and at 4 h marked increases were detected in CCL2/MCP1, CCL4, CCL5/RANTES, IFI204, IFI35, IFI47, and ISG15. In contrast, CCL2/MCP1 and CXCL1 were markedly increased in the liver at 2 h, and CCL4, CCL5/ RANTES, IFI204, IFI35, IFI47, and ISG15 were up-regulated at 4 h after LPS-administration. Additional treatment with ExtBS resulted in inhibition of all the cytokine induction caused at 2 h and 4 h. Although the Genopal™ Allergy chip contains 209 genes related to inflammation, including cytokine receptor proteins, no marked increase in other genes was observed under LPS-treatment.

Effect of ExtBS on hepatic injury produced by GalN/LPS treatment

GalN/LPS-induced liver injury is a well-known experimental model of acute hepatic failure. 25) In our present study, GalN was intraperitoneally injected, and LPS and ExtBS were given orally. The lethality of GalN/LPS was 40% (4 of 10 mice), and that of GalN/LPS and ExtBS into Mice.

LPS and ExtBS in 0.2 ml of PBS containing 3.5% ethanol were administered to a female C3H/6J 8-week-old mouse by intraperitoneal injection. Control, ethanol; LPS, 5 μg/ml LPS; LPS + ExtBS, 5 μg/ml LPS + 25 μg/ml ExtBS; LPS + ExtBS(H), 5 μg/ml LPS + 2.5 mg/ml ExtBS. Four h after administration, three mice in each group were sacrificed, and the spleens and livers were removed. A. RT-PCR of total RNA of spleens and livers from control and treated mice was performed as described in the legend to Fig. 4. The relative intensity in comparison with the LPS-treated group represents the expression levels of IL-6 in the spleen (B), of TNF-α in the spleen (C), of IL-6 in the liver (D), and of TNF-α in the liver (E) normalized by GAPDH expression. The values are means ± SD for three mice. The statistical significance of the difference was determined by Student’s t-test; *p < 0.05 vs. LPS-treated mice.
LPS + ExtBS was 20% (2 of 10 mice). Next we examined the histopathology of the mice (two mice per group). Severe liver damage, including massive necrosis with intralobular hemorrhage, was observed even in the surviving mice after 24 h of treatment with GalN/LPS (Fig. 7). In contrast, the livers from the mice treated with GalN/LPS + ExtBS showed light damage, similar to the control (GalN/vehicle) livers. The percentage of damaged area was calculated from five independent tissue sections (300 × 300 μm square) from two mice. The damaged area for GalN/LPS was 38%, about 4-fold larger than that for GalN/vehicle (5%). In contrast, the damaged area for GalN/LPS + ExtBS (10%) was markedly smaller than that for GalN/LPS. These data suggest that ExtBS protected against liver injury induced by GalN/LPS treatment.

**Effects of ExtBS on inflammatory cytokine expression in HeLa cells**

To determine the direct effects of ExtBS on inflammatory cytokine expression, HeLa cells, which show high-level expression of IL-6 and IL-8 under the usual culture conditions without LPS treatment, were treated with 1 μg/ml of ExtBS for 16 h, and the gene expression level was determined by RT-PCR (Fig. 8). A marked decrease in cytokine expression was observed under treatment with ExtBS, and reached 18.9% and 37.9% of IL-6 and IL-8 respectively in comparison with untreated cells. These data indicate that the anti-inflammatory activity of ExtBS is caused by signaling regulation of inflammatory cytokine genes and not by blockage of the LPS receptor.

**Discussion**

In our research, we have used a human intestinal epithelial cell line (CoLoTC cells) for determination of the anti-inflammatory activity of ethanol extracts from local products such as sugar beets, adzuki beans, potatoes, and buckwheat sprouts. Recent studies of natural products with anti-inflammatory activity have been reported, and many of them had been done using macrophage-like cell lines, RAW264.7 cells and THP-1 cells. However, we chose human colon carcinoma cells for screening because we were focusing on anti-inflammatory compounds in dietary products which might be effective through the mucosal immune response. CoLoTC cells showed a good response to LPS treatment and marked induction of IL-8 expression and production due to significant expression of TLR4 and the MD-2 gene (Fig. 1). Although we received information from the provider of these cells that they might be the same as COLO205 cells (which do not show any response to LPS without IFN-γ treatment due to low expression of MD-2), The CoLoTC cells in our culture condition showed an LPS-response without IFN-γ treatment. We do not know exactly why the CoLoTC cells originating from COLO205 cells became reactive...
to LPS, but during passage they may be transformed. ExtBS, which was selected by our CoLoTC-screening system, showed significant anti-inflammatory activity in mice when administered orally. This screening system might be useful in identifying other orally effective anti-inflammatory compounds.

Six flavonoids (vitexin, isovitexin, orientin, isoorientin, chlorogenic acid, and rutin) were detected in ExtBS, but anti-inflammatory activity of ExtBS was not explained by the presence of any of these flavonoids using our CoLoTC cell protocol (Fig. 3). Although a weak anti-inflammatory activity was observed for rutin, the ethanol-extract from another kind of buckwheat (‘Hokkai T10’) that contains higher rutin and flavonoid content did not show a greater effect than ExtBS in mice (data not shown). These data indicate that the anti-inflammatory activity in ExtBS is caused by a compound other than flavonoids.

Although the active component in ExtBS is still unknown, ExtBS has significant anti-inflammatory activity. In our present study, we established an in vivo assay system for anti-inflammatory activity by both oral and intraperitoneal LPS-treatment in mice. Significant induction of inflammatory cytokines (IL-6 and TNF-α) was observed 4 h after oral treatment with 2 mg of LPS (Figs. 4 and 5). In our preliminary experiment, we tried to detect changes in cytokine expression in intestinal tissues, but their expression level was too low to be detected in those tissues. We used IL-6 and TNF-α gene expression in the spleen and liver as biomarkers of inflammatory response because they quickly responded to orally-treated LPS dose-dependently. IL-6 is a major pathogenic factor causing rheumatoid arthritis, \(^{29,30}\) and anti-IL-6 receptor antibodies are now available for clinical treatment of this disease. \(^{31}\) TNF-α is also known as a pathogenic factor in various diseases, such as inflammatory bowel disease, \(^{32}\) diabetes, \(^{33}\) acute hepatic injury, \(^{34}\) and rheumatoid arthritis. \(^{35}\)

ExtBS was orally effective in preventing induction of IL-6 and TNF-α (Figs. 4 and 5) and it was suggested that mouse survival was increased by treatment with ExtBS in the acute hepatic injury model caused by GalN/LPS. These data indicate that ExtBS and its components is a useful candidate for a medicine or a supplement to prevent disease.

In our present study, the Genopal™ Allergy chip containing probes for 209 genes related to inflammation was used in further study on the immune response due to oral treatment with LPS. Forty-nine of the 209 genes were cytokine and cytokine-related genes in this chip, and time-dependent gene expression (more than a 2-fold increase) was observed in 11 genes in both the spleen and liver, but some genes, such as IL-6 and TNF-α, were hard to detect correctly and we could not see any correlation with the results of RT-PCR due to their low expression and RNA purity. RNA sample preparation is one of the crucial issues in the correct determination of low-expression genes. The effects of oral administration of LPS were not many, and this is the first report on the cytokine gene expression sequence after treatment in the spleen and liver. Induction of IL-1β in the spleen and CCL2/MCP1 and CXCL1 in the liver after 2 h of LPS administration was observed. It was rather low at 4 h after treatment. These cytokines might be responsible for the initial inflammatory response against LPS in tissues. Based on data, ExtBS blocked almost all cytokine gene induction, and ExtBS can affect at the initiation step of the LPS-activated inflammatory response.

Intraperitoneal treatment with ExtBS did not show any anti-inflammatory activity in the mice (Fig. 6). LPS might have been trapped by peritoneal macrophages when it was treated intraperitoneally. It was interesting that ExtBS did not prevent IL-8 induction by LPS in human mononuclear cell line THP-1 cells, which are macrophage-like cells (data not shown). The anti-inflammatory activity of ExtBS can be seen in a cell-type specific manner, because it was observed in oral treatment in mice and in human intestinal epithelial cell line CoLoTC cells. One of the possible explanations of the anti-inflammatory effect of ExtBS in LPS-stimulated cells is that ExtBS causes a simple blocking of LPS-binding to TLR4 by a mechanism like the masking of LPS by ExtBS. However, the anti-inflammatory effect of ExtBS was probably not caused by a simple blocking of LPS-binding to TLR4, because intraperitoneal delivery of ExtBS did not show any anti-inflammatory activity even when it was used at a 100-fold excess concentration. The effect of intraperitoneal injection of ExtBS was observed rather as an inducer of the inflammatory response in the spleen.

In the present study, an anti-inflammatory effect of ExtBS was observed under oral treatment, but the mechanism of its effect is still unclear. The simplest explanation is that intake of LPS is decreased by combinatory treatment with ExtBS, because ExtBS blocked almost all the gene expression induced by LPS treatment. However, the degree of decrease in the LPS-induced cytokine gene was distinctive for different cytokine genes. Further experimentation on the LPS-induced cytokine expression of ExtBS in detail is needed to clarify its mechanism of operation. Since ExtBS directly affected IL-6 and IL-8 gene expression in HeLa cells (Fig. 8), cell-type specific anti-inflammatory effect is another possible mechanism of ExtBS.

In conclusion, ExtBS showed significant anti-inflammatory activity in vitro and in vivo, and it may be a useful material for treatment or prevention of the progress of inflammatory diseases.

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References


