Suppressive Effect of an Isoflavone Fraction on Tumor Necrosis Factor-α-Induced Interleukin-8 Production in Human Intestinal Epithelial Caco-2 Cells

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Summary This study demonstrates the effect of soybean components on the tumor necrosis factor-α (TNF-α)-induced production of interleukin-8 (IL-8), one of the major inflammatory chemokines, in intestinal epithelial-like Caco-2 cells. Among the soybean components, an isoflavone fraction (IFF) suppressed the TNF-α-induced IL-8 secretion by Caco-2 cells in a dose-dependent manner, whereas a soyasaponin fraction and soypeptide fraction had no significant effect on TNF-α-induced IL-8 secretion. The IL-8 secretion induced by hydrogen peroxide and by IL-1β was not suppressed by IFF, suggesting that the inhibitory effect of isoflavone was specific for the TNF-α-induced regulation of IL-8. The increased expression of IL-8 mRNA by TNF-α was almost completely suppressed by IFF. Furthermore, the transcriptional activity of the human IL-8 promoter was increased by the TNF-α treatment, and IFF significantly suppressed the IL-8 promoter activity. These results indicate that IFF suppressed TNF-α-induced IL-8 production at the transcriptional level in human intestinal Caco-2 cells, suggesting IFF of soybean as a promising food component for preventing intestinal inflammation such as inflammatory bowel disease.

Key Words isoflavone, interleukin-8, Caco-2, tumor necrosis factor-α, inflammatory bowel disease

The intestinal epithelium is an organ for absorbing nutrients, as well as playing a key role in the gut immune system for defense against pathogens and xenobiotics (1). It acts as a physical barrier to the external environment and also as a sensor monitoring any invasion by xenobiotics. In response to external stimuli such as bacteria, hydrogen peroxide, and inflammatory cytokines, the intestinal epithelial cells secrete inflammatory cytokines and such chemokines as interleukin-8 (IL-8) (2–4). IL-8 is a member of the C-X-C chemokine family and plays an essential role in the recruitment and activation of neutrophils, thereby initiating an inflammatory response (5). An appropriate inflammatory response is essential to protect against infection and to repair damaged tissue (6). However, an excessive inflammatory response will disturb the homeostasis of intestinal mucosa and destroy the intestinal epithelial monolayers (7, 8). In particular, any abnormal activation of such immune cells as macrophages beneath the intestinal epithelial monolayer overproduces inflammatory cytokines, including tumor necrosis factor-α (TNF-α). In intestinal mucosa, TNF-α has been reported to induce apoptosis in intestinal epithelial cells and also to produce inflammatory chemokines, including IL-8 (3, 5, 9). It has been assumed that, under intestinal inflammatory conditions, TNF-α secreted from abnormally activated immune cells would affect the intestinal epithelial cells which extensively increase the secretion of IL-8 in response to TNF-α. The secreted IL-8 from intestinal epithelial mucosa recruits and activates neutrophils to the intestinal mucosa, and these recruited neutrophils further secrete hydrogen peroxide and inflammatory cytokines, which again stimulate IL-8 secretion by the intestinal epithelial cells. This vicious loop, which is called the intestinal inflammatory loop, therefore makes the inflammatory condition worse. The intestinal inflammatory loop further leads to inflammatory bowel diseases (IBDs), including Crohn’s disease (CD) and ulcerative colitis (UC) in the intestinal mucosa (10, 11). It is therefore important to regulate and maintain the secretion of IL-8 by intestinal epithelial cells to prevent the intestinal inflammatory loop from developing. It has recently been discovered by using intestinal epithelial cell lines that such food components as flavonoids and amino acids suppressed stimulant-induced IL-8 secretion (12–15). We focused the present study on the effect of food-derived components on stimulant-induced IL-8 production by intestinal epithelial cells. Soybean ingredients were selected from various food components for this study. Although soybean has been reported to have such beneficial effects as anti-atherosclerosis progression and inflammation (16–18), there has been no study on the effect of soybean ingredients on IL-8 secretion by intestinal epithelial cells. We report

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in this paper the effect of soybean ingredients on TNF-α-induced IL-8 production by using a Caco-2 cell line as a model of human intestinal epithelial cells.

**Materials and Methods**

**Materials.** The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Sigma (St. Louis, MO, USA). Penicillin-streptomycin (10,000 U/mL and 10 mg/mL in 0.9% sodium chloride, respectively) and non-essential amino acids (NEAA) were purchased from Gibco (Gaithersburg, MD, USA). The monoclonal antihuman IL-8 antibody, biotinylated antihuman IL-8 antibody, and recombinant human TNF-α were purchased from Genzyme Technne (Cambridge, MA, USA), and recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human interleukin-1β (IL-1β) was purchased from Pepro Tech (London, UK). Isogen was from Nippon Gene (Tokyo, Japan), and hydrogen peroxide was from Kanto Chemicals Co. (Tokyo, Japan). The QuantaBlu fluorogenic peroxidase substrate kit was purchased from Pierce (Rockford, IL, USA), and the ExScript RT reagent kit and SYBR Premix Ex Taq for the real-time polymerase chain reaction (PCR) were from Takara Bio (Osaka, Japan). All the other chemicals used were of reagent grade. Such soybean ingredients as the isoflavone, soyasaponin, and soypeptide fractions were all provided by Fuji Oil Co. (Osaka, Japan). The gross composition of these fractions is shown in Table 1.

**Cell culture.** Caco-2 cells were cultured in 78.5-cm² plastic dishes with a culture medium consisting of DMEM, 10% FBS (v/v), 1% NEAA, 100 U/mL of penicillin, 100 μg/mL of streptomycin and an appropriate amount of sodium bicarbonate. The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air, the culture medium being renewed on alternate days. After they had reached confluence, the cells were trypsinized with 0.1% trypsin and 0.02% EDTA in PBS, and then subcultured. The cells were seeded at a density of 2×10⁵ cells/well on a 24-well plate and then cultured for 2 wk, before their use in the subsequent experiments. All cells were used between passages 40 and 60.

**ELISA for measuring IL-8 secretion.** The confluent monolayers of Caco-2 cells in the 24-well plate were exposed for 24 h to a culture medium containing each stimulant (TNF-α (10 ng/mL), IL-1β (1 ng/mL) and hydrogen peroxide (2 mM)) in the presence or absence of the soybean components. The culture medium was then collected, and the level of IL-8 secretion was determined by a sandwich enzyme-linked immuno-sorbent assay (ELISA) according to the following procedure. A 96-well plate was coated overnight at 4°C with 1 μg/mL of monoclonal antihuman IL-8. The plate was then treated with a blocking buffer (1% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.5% Tween 20) for 2 h at room temperature. A 100 μL amount of each sample was added to the wells and incubated overnight at room temperature, before 100 ng/mL of the biotinylated antihuman IL-8 antibody was added to each well. The plate was incubated for 1 h at room temperature, the HRP-streptavidin conjugate was added to the wells, and the plate was incubated again for 1 h at room temperature. The peroxidase activity was detected by using the QuantaBlu fluorogenic peroxidase substrate (Pierce) and with a plate reader, Fluoroskan Ascent CF (Labsystems, Helsinki, Finland).

**Real-time PCR for IL-8 mRNA.** Total RNA was extracted from the cell lysate by using Isogen according to the manufacturer’s instructions. The steady-state levels of IL-8 and β-actin were determined by real-time PCR. Total RNA (5 μg) was reverse-transcribed with an ExScript reverse transcription reagent kit. The obtained cDNA (1 μL) was amplified by a SYBR Green real-time PCR kit, using the Lightcycler system (Roche Diagnostics). The reaction mixture was incubated for 15 min at 95°C and then subjected to 40 amplification cycles consisting of denaturing at 95°C for 15 s, annealing at 57°C for 15 s, and extension at 72°C for 15 s. The primer sequences were as follows: human IL-8, 5’-AGA GTG ATT GAG AGT GGA CC-3’ (forward) and 5’-ACT TCT CCA CAA CCC TCT G-3’ (reverse); and β-actin, 5’-CCA CGA AAC TAC CTG GAA C-3’ (forward) and 5’-GAT CCT CAT TGT GCT GGG-3’ (reverse). Their specificity was verified by analyzing the melting curve for each product and by agarose gel electrophoresis. mRNA was quantified by the ΔΔCT comparative method (19). The gene expression levels of IL-8 were finally normalized by using β-actin as a housekeeping gene. To detect any contamination by genomic DNA and/or by the preparation of real-time PCR, RT minus the control and nontemplate control were included in all.

**Transfection and luciferase reporter assay.** Caco-2 cells were transiently transfected with lipofectamine plus reagent according to the manufacturer’s instructions in 12-well plates at 80% confluency with the pGL3-basic vector (Promega, Tokyo, Japan) containing the IL-8-promoter region (-300 to +50 bp) upstream of the luciferase gene. This IL-8 promoter region contains NFκB responsive element that is essential for transcriptional induction of IL-8 promoter by TNF-α. Cells

### Table 1. Composition of isoflavone, soyasaponin, and soypeptide fractions.

| Ingredient (%) | Fraction |
|----------------|----------|
|                | Isoflavone | Soyasaponin | Soypeptide |
| Isoflavones    | 50        | 3–6         | —          |
| Saponin        | 10–15     | 58          | —          |
| Protein (MW>500) | 15        | 15          | 30         |
| Peptide (MW<500) | —         | —           | 60         |
| Ash, others    | 20–25     | 21–24       | 10         |

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treated with TNF-α alone or with a combination of TNF-α and a soybean component for 24 h were washed with PBS and lysed with a passive lysis buffer. The luciferase activity was determined by using the Dual-Luciferase Reporter assay (Promega) with an LB 9507 Lumet luminometer (Berthold Technologies).

**Statistical analyses.** Each data value is expressed as the mean±SD (n=3 or 4). The data were analyzed with Tukey’s multiple-range test when significant differences (p<0.05) had been obtained by one-way ANOVA.

**Results and Discussion**

The effect of each soybean ingredient on TNF-α-induced IL-8 secretion was examined by using human intestinal epithelial Caco-2 cells. Caco-2 cells were incubated with 100 ng/mL of TNF-α in the presence or absence of 1 mg/mL of a soybean ingredient (isoflavone fraction (IFF), or the soyasaponin or soypeptide fraction). After 24 h, the amount of IL-8 in the supernatant was measured by sandwich-ELISA as described in “Materials and Methods.” Each value is the mean±SD (n=4). Values not sharing a common superscript letter are significantly different at p<0.05.

![Fig. 1](image1.png)

**Fig. 1.** Effect of soybean ingredients on TNF-α-induced IL-8 secretion by human intestinal Caco-2 cells. Caco-2 cells were treated with 100 ng/mL of TNF-α and 1 mg/mL of IFF, or the soyasaponin or soypeptide fraction. After 24 h, the amount of IL-8 in the supernatant was measured by sandwich-ELISA as described in “Materials and Methods.” Each value is the mean±SD (n=4). Values not sharing a common superscript letter are significantly different at p<0.05.

Each data value is expressed as the mean±SD (n=3 or 4). The data were analyzed with Tukey’s multiple-range test when significant differences (p<0.05) had been obtained by one-way ANOVA.

![Fig. 2](image2.png)

**Fig. 2.** Effect of IFF on various stimulant-induced IL-8 secretion by human intestinal Caco-2 cells. Caco-2 cells were treated with various concentrations of IFF and (A) TNF-α (100 ng/mL), (B) IL-1β (1 ng/mL), and (C) hydrogen peroxide (2 mM) for 24 h. Each culture medium was collected, and the IL-8 level was determined by ELISA as described in “Materials and Methods.” Each value is the mean±SD (n=4). Values not sharing a common superscript letter are significantly different at p<0.05.

These results suggest that the suppressive effect of IFF was specific to TNF-α-induced IL-8 secretion.

Although IFF is rich in isoflavones (50%), it also contains saponins (10–15%) and proteins (15%) (Table 1). It cannot therefore be excluded that components other than isoflavones might have suppressed the TNF-α-induced IL-8 secretion. However, the soyasaponin fraction, which contained 58% saponin and 15% protein, had little effect on IL-8 secretion (Fig. 1). The only difference between the composition of IFF and that of the soyasaponin fraction is in the amount of isoflavones and soyasaponins in each fraction. Considering the composition of the isoflavone and soyasaponin frac-
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It has been reported that TNF-α-induced IL-8 secretion via NFκB activation at the transcriptional level (20). It is therefore expected that IFF suppressed TNF-α-induced IL-8 secretion by inhibiting NFκB activation. However, Fig. 2C shows that IFF did not affect hydrogen peroxide-induced IL-8 induction, although hydrogen peroxide also induces IL-8 secretion via NFκB activation (22). It has been reported that such food components as chlorogenic acid, caffeic acid, and histidine suppressed not only TNF-α-induced IL-8 secretion but also hydrogen peroxide-induced IL-8 secretion (13, 15). However, there have been no reports about food components which can suppress TNF-α-induced IL-8 activation, but not hydrogen peroxide-induced activation. In respect of the regulatory mechanism, the specific suppression of TNF-α-induced IL-8 suppression by IFF is considered to be quite interesting, being different from the cases of other food factors. It is assumed that IFF inhibited the TNF-α signaling cascade upstream of NFκB activation, possibly being specific to TNF-α signaling close to the TNF-α receptor. Revealing the precise regulatory mechanism for IL-8 suppression by IFF is one of the most crucial aspects remaining to be elucidated.

In conclusion, IFF could suppress TNF-α-induced IL-8 production in human intestinal Caco-2 cells. Although further experiments are necessary to identify the active isoflavones which suppress IL-8 production and to reveal the unique regulatory mechanism specific to the TNF-α cascade, the present findings suggest that isoflavones in soybean ingredients could be a promising food component for preventing intestinal inflammation such as IBD.

Fig. 3. Effect of IFF on (A) the TNF-α-induced IL-8 mRNA expression and (B) TNF-α-induced transcriptional activity of the 5′-flanking region of the human IL-8 gene in human intestinal Caco-2 cells. (A) Total RNA from the cells was extracted after incubating with IFF and TNF-α (100 ng/mL) for 3 h. First-strand cDNA was prepared from 5 mg of total RNA. A real-time PCR analysis was performed with SYBR Green. β-actin being used as a stable housekeeping gene. (B) Caco-2 cells cotransfected with the pGL3-basic vector containing the IL-8 promoter region and pRL-CMV were treated with IFF and TNF-α (100 ng/mL) for 24 h. The transcriptional activity was estimated by a luciferase assay. Each value is the mean±SD (n=3). Values not sharing a common superscript letter are significantly different at p<0.05.
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REFERENCES

1) Bourlioux P, Koletzko B, Guarnier F, Bracero V. 2003. The intestine and its microra are partners for the protection of the host: Report on the Danone Symposium “The Intelligent Intestine,” held in Paris, June 14, 2002. *Am J Clin Nutr* **78**: 675–683.

2) Yamamoto K, Kushima R, Kikawa O, Fujiiya M, Okabe H. 2003. Combined effect of hydrogen peroxide induced oxidative stress and IL-1β on IL-8 production in Caco-2 cells (a human colon carcinoma cell line) and normal intestinal epithelial cells. *Inflammation* **27**: 123–128.

3) Eckmann L, Jung HC, Schurer-Maly C, Panja A, Morzycka-Wroblewska E, Kagnoff MF. 1993. Differential cytokine expression by human intestinal epithelial cell lines: Regulated expression of interleukin 8. *Gastroenterology* **105**: 1689–1697.

4) Eckmann L, Kagnoff MF, Fierer J. 1993. Epithelial cells secrete interleukin-8 in response to bacterial entry. * Infect Immun* **61**: 4569–4574.

5) Baggiolini M, Loetscher P, Moser B. 1995. Interleukin-8 and the chemokine family. *Int J Immunopharmacol* **17**: 103–108.

6) Nathan C. 2002. Points of control in inflammation. *Nature* **420**: 846–852.

7) Bouma G, Strober W. 2003. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* **3**: 521–533.

8) MacDonald TF, Monteleone G. 2005. Immunity, inflammation, and allergy in the gut. *Science* **307**: 1920–1925.

9) Schulze JD, Bojarski C, Zeissig S, Heller F, Fromm M. 2006. Disrupted barrier function through epithelial cell apoptosis. *Ann NY Acad Sci* **1072**: 288–299.

10) Gerard C, Rollins BJ. 2001. Chemokines and disease. *Nat Immunol* **2**: 108–115.

11) Gijbers K, Asche GV, Joossens S, Struyf S, Proost P, Rutgeerts P, Geboes K, Damme JV. 2004. CXCR1-binding chemokines in inflammatory bowel diseases: down-regulated IL-8/CXCL8 production by leukocytes in Crohn’s disease and selective GCP-2/CXCL6 expression in inflamed intestinal tissue. *Eur J Immunol* **34**: 1992–2000.

12) Liboni K, Li N, Neu J. 2004. Mechanism of glutamine-mediated amelioration of lipopolysaccharide-induced IL-8 production in Caco-2 cells. *Cytokine* **26**: 57–65.

13) Zhao Z, Shin HS, Satsu H, Totsuka M, Shimizu M. 2008. 5-Caffeoylquinic acid and caffeic acid down-regulate the oxidative stress- and TNF-α-induced secretion of interleukin (IL)-8 from Caco-2 cells. *J Agric Food Chem* **56**: 3863–3868.

14) Zhao Z, Satsu H, Fujisawa M, Hori M, Ishimoto Y, Totsuka M, Nambu A, Kakuta S, Osaki Y, Shimizu M. 2008. Attenuation by dietary taurine of dextran sulfate sodium-induced colitis in mice and of THP-1-induced damage to intestinal Caco-2 cell monolayers. *Amino Acids* **35**: 217–224.

15) Son DO, Satsu H, Shimizu M. 2005. Histidine inhibits oxidative stress- and TNF-alpha-induced interleukin-8 secretion in intestinal epithelial cells. *FEBS Lett* **579**: 4671–4677.

16) Wagner JD, Schwenke DC, Greaves KA, Zhang L, Anthony MS, Blair RM, Shadoan MK, Williams JK. 2003. Soy protein with isolavones, but not an isoflavone-rich supplement, improves arterial low-density lipoprotein metabolism and atherogenesis. *Arterioscler Thromb Vasc Biol* **23**: 2241–2246.

17) Fair DE, Ogborn MR, Weiler HA, Bankovic-Calic N, Nitschmann EP, Fitzpatrick-Wong SC, Aukema HM. 2004. Dietary soy protein attenuates renal disease progression after 1 and 3 weeks in Han:SPRD-cy weanling rats. *J Nutr* **134**: 1504–1507.

18) Komatsu W, Nagata J, Kaneko M, Yamada T, Moriya D, Miura Y, Yagasaki K. 2008. Effect of dietary soy protein on tumor necrosis factor productivity in macrophages from nephritic and hepatoma-bearing rats. *J Nutr Sci Vitam* **54**: 435–439.

19) Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* **30**: e36.

20) Jobin C, Holt L, Bradham CA, Streetz K, Brenner DA, Sartor RB. 1999. TNF receptor-associated factor-2 is involved in both IL-1 beta and TNF-alpha signaling cascades leading to NF-kappa B activation and IL-8 expression in human intestinal epithelial cells. *J Immunol* **162**: 4447–4454.

21) Romier B, Van De Walle J, During A, Larondelle Y, Schneider YJ. 2008. Modulation of signalling nuclear factor-kappaB activation pathway by polyphenols in human intestinal Caco-2 cells. *Br J Nutr* **100**: 542–551.

22) Shi XZ, Lindholm PE, Sarra SK. 2003. NF-kappa B activation by oxidative stress and inflammation suppresses contractility in colonic circular smooth muscle cells. *Gastroenterology* **124**: 1369–1380.