Activating Transcription Factor 3-mediated Chemo-intervention with Cancer Chemokines in a Noncanonical Pathway under Endoplasmic Reticulum Stress*

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Background: Cancer-favoring ER stress responses drive proinflammatory programs including cancer chemokine production.

Results: ER stress-induced cancer chemokines are regulated by preventive exposure to a natural flavone apigenin via ATF3.

Conclusion: ATF3 epigenetically suppresses expression of EGR-1, a noncanonical proinflammatory transcriptional modulator crucial to cancer chemokine induction.

Significance: ATF3-mediated chemokine suppression implicates a novel chemo-intervention with ER stress response-related tumorigenesis.

The cell-protective features of the endoplasmic reticulum (ER) stress response are chronically activated in vigorously growing malignant tumor cells, which provide cellular growth advantages over the adverse microenvironment including chemotherapy. As an intervention with ER stress responses in the intestinal cancer cells, preventive exposure to flavone apigenin potentiated superinduction of a regulatory transcription factor, activating transcription factor 3 (ATF3), which is also known to be an integral player coordinating ER stress response-related gene expression. ATF3 superinduction was due to increased turnover of ATF3 transcript via stabilization with HuR protein in the cancer cells under ER stress. Moreover, enhanced ATF3 caused inhibitory action against ER stress-induced cancer chemokines that are potent mediators determining the survival and metastatic potential of epithelial cancer cells. Although enhanced ATF3 was a negative regulator of the well known proinflammatory transcription factor NF-κB, blocking of NF-κB signaling did not affect ER stress-induced chemokine expression. Instead, immediately expressed transcription factor early growth response protein 1 (EGR-1) was positively involved in cancer chemokine induction by ER stressors. ER stress-induced EGR-1 and subsequent chemokine production were repressed by ATF3. Mechanistically, ATF3 directly interacted with and recruited HDAC1 protein, which led to epigenetic suppression of EGR-1 expression and subsequent chemokine production. Conclusively, superinduced ATF3 attenuated ER stress-induced cancer chemokine expression by epigenetically interfering with induction of EGR-1, a transcriptional modulator crucial to cancer chemokine production. Thus, these results suggest a potent therapeutic intervention of ER stress response-related cancer-favoring events by ATF3.

Endoplasmic reticulum (ER) is a protein biosynthesis organelle in which newly synthesized proteins are accurately folded into their proper conformation. However, the folding process may occur improperly or proteins may unfold under pathological stress, subsequently triggering a severe stress response known as the ER stress response (1). Phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) is a highly conserved point of convergence for the distinct signaling pathways that adapt eukaryotic cells to a wide variety of stressful conditions, including ER stress (2, 3). Phosphorylation of eIF2α provides stress resistance via global protein translational arrest and induction of numerous homeostasis-modulating genes. eIF2α that has been phosphorylated by eIF2 kinases such as RNA-like endoplasmic reticulum kinase (PERK) triggers the expression of various stress response genes such as CHOP (CCAAT/enhancer-binding protein homologous protein), ATF4, ATF3, and GADD34 (growth arrest and DNA damage 34) (4, 5). In particular, ATF3 plays an integral role in the coordination of gene expression induced by eIF2 kinases in response to a more diverse set of stress conditions, including ER stress (6). ER stress response represents an adaptive mechanism to support cellular survival in response to a great variety of detrimental conditions, such as low nutrient levels, hypoxia, or accumulation of misfolded proteins in vigorously growing tumor cells (7, 8). Although excessive ER stress can turn on the pro-apoptotic process, the tumor-promoting features such as the mainte-

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2 The abbreviations used are: ER, endoplasmic reticulum; ATF, activating transcription factor; EGR-1, early growth response protein 1; eIF, eukaryotic translation initiation factor; PERK, RNA-like endoplasmic reticulum kinase; TG, thapsigargin; TM, tunicamycin; HDAC, histone deacetylase.
nance of inflammatory microenvironment are chronically acti-
vated by the ER stress responses, thus supporting aggressive prolifera-
tion and survival of epithelial cancer cells (9, 10). Mechanistically, ER stress causes signals to be transduced to NF-κB, which mediates inflammatory response by inducing cytokines. Toll-like receptor 4 (TLR4) activation via endotoxin LPS induces ER-resident chaperones including GRP94 (glu-
cose-regulated protein 94) and GRP78 to correct misfolded or malfolded protein conformations (11). However, the induction of chaperones is not sufficient to manage TLR4-triggered signaling molecules in cells, which promotes unfolded protein responses associated with ER stress (11). Moreover, ER stress-sentinel proteins such as IRE1, PERK, and ATF6 can initiate NF-κB signaling, which modulates the induction of proinflammatory mediators (12, 13). In particular, NF-κB activated by ER stress mediates inflammation-driven tumor promotion by inducing expression of “tumor-promoting cytokines” (14, 15).

As a representative negative regulator of proinflammatory signals, ATF3 is induced as part of the negative feedback loop that modulates TLR4-stimulated inflammatory responses (16–18). Moreover, ATF3 suppresses tumor growth and metastasis in epithelial cancers including colon cancer via diverse mecha-
nism, indicating ATF3 down-regulation as the prognostic marker (19, 20). In particular, ATF3 can regulate the expression of proinflammatory cytokines. Not surprisingly, ATF3-defi-
cient macrophages produce elevated levels of IL-6 and IL-12p40 cytokines in response to LPS (16, 18). Mechanisti-
cally, the negative regulation of transcription by ATF3 may occur indirectly via inhibition of CCAAT/enhancer-binding protein 6, a positive regulator of cytokine gene induction (21). Recent studies have also shown that ATF3 mediates epigenetic regulation of proinflammatory cytokines (16, 22).

Dietary flavonoids, which comprise the most common group of plant polyphenols, have anti-inflammatory or anticancer effects in various disease models, including gastrointestinal cancers (23, 24). Apigenin, one of the most common flavonoids, is widely distributed in fruits and vegetables and is particularly abundant in parsley, celery, garlic, and Chinese cabbage (25, 26). In the current study, ATF3 and apigenin as a potent ATF3 inducer were assessed for their inhibitory action against ER stress-mediated proinflammatory signaling in the intestinal cancer cells based on the assumption that ATF3 can regulate ER stress-triggered inflammatory signals. The results presented herein will provide new insights into ATF3 as a promising tar-
goal of therapeutics against the intestinal cancers and its proin-
flammatory tumor microenvironment promoted by ER stress responses.

MATERIALS AND METHODS

Cell Culture Conditions and Reagents—Human intestinal cancer cell lines HCT-8 and HCT-116 and mouse colon cancer cell line CMT-93 were purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (Welgene, Daegu, South Korea) for HCT-8 and HCT-
116 and DMEM (Welgene) for CMT-93 supplemented with 10% (v/v) heat-inactivated FBS (Welgene), 50 units/ml penicil-
lin, and 50 μg/ml streptomycin (Welgene) in a 5% CO2 humid-
ified incubator at 37 °C. Cell number was assessed by exclusion of trypan blue dye (Sigma-Aldrich) using a hemacytometer. Thapsigargin (TG) was purchased from Assay Design (Enzo Life Sciences, Farmingdale, NY), and trichostatin A was pur-
chased from Wako Pure Chemical Industries (Osaka, Japan). LPS, Tunicamycin and all other chemicals were acquired from Sigma-Aldrich.

Construction of Plasmid—The entire coding region of ATF3, including the TATAA region, and of EGR-1 were generated by RT-PCR using RNA from HCT-8 cells with the following prim-
ers: forward, 5′-CGT GAG TCC TCG GTG CTC-3′, and reverse, 5′-GAC AGC TCT CCA ATG GCT TC-3′ for ATF3; and forward, 5′-ATG GCC GCG GCC AAG GCC GA-3′, and reverse, 5′-TTA GCA AAT TTC AAT TGT CC-3′ for EGR-1. The resulting 721-bp construct for ATF3 and 1732-bp con-
struct for EGR-1 were cloned using a TopCloner TA kit (Enzy-
nomics, Daejeon, Korea) followed by excision at the HindIII/NotI sites, after which they were transferred in the sense orientation into the pcDNA3.1Zeo+ expression plasmid (Invitrogen) using T4 DNA ligase (New England Biolabs, Bever-
ley, MA) and then confirmed by DNA sequencing. These vec-
tors were named ATF3-SC and EGR-1 overexpression, respecti-

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116 and DMEM (Welgene) for CMT-93 supplemented with 10% (v/v) heat-inactivated FBS (Welgene), 50 units/ml penicillin, and 50 μg/ml streptomycin (Welgene) in a 5% CO2 humidified incubator at 37 °C. Cell number was assessed by exclusion of trypan blue dye (Sigma-Aldrich) using a hemacytometer. Thapsigargin (TG) was purchased from Assay Design (Enzo Life Sciences, Farmingdale, NY), and trichostatin A was purchased from Wako Pure Chemical Industries (Osaka, Japan). LPS, Tunicamycin and all other chemicals were acquired from Sigma-Aldrich.

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Transient Transfection—HCT-8 and HCT-116 cells were transfected with combinations of plasmids using jetPRIME™ (Polyplus Transfection SA, Illkirch, France) according to the manufacturer’s protocols. All transfection efficiencies were maintained ~50–60% and confirmed by expression of a pMX-enhanced GFP vector. jetPRIME™ was used to transfect vehicle, ATF3-SC, EGR-1 overexpression, shEGR-1, shATF3, shHDAC1, shHuR, shATF6, shGRP78, or shCHOP transiently. After 4 h, the medium was changed, and transfected cells were incubated for 48–120 h.

Luciferase Assay—Cells were washed with cold PBS, lysed with passive lysis buffer (Promega), and then centrifuged at 13,800 × g once for 10 min. The supernatant was collected, isolated, and stored at −80 °C until measurement. Luciferase activity was measured with a model TD-20/20 dual mode lumi-
ATF3-attenuated Cancer Chemokine Production

nomet (Turner Designs, Sunnyvale, CA) after briefly mixing the supernatant (10 μl) with 40 μl of firefly luciferase assay substrate solution, followed by stopping with 50 μl of Renilla luciferase stop solution (Promega). The firefly luciferase activity was normalized against Renilla luciferase activity by dividing firefly luciferase activity by Renilla luciferase activity.

Reverse Transcription and Real Time PCR—RNA was extracted with RiboEx (GeneAll Biotech, Seoul, South Korea) according to the manufacturer's instructions. The RNA (3 μg) from each sample was transcribed to cDNA by Prime RT premix (Genetbio, Nonsan, South Korea). During real time PCR, 6-carboxyl fluorescein was used as the fluorescent reporter dye to detect amplified cDNA. Real time PCR was conducted using an iCycler Thermal Cycler (Bio-Rad) to subject the samples to denaturation at 95 °C for 15 min followed by cycles of denaturation at 95 °C for 20 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 30 s. Each sample was tested in triplicate to ensure statistical significance. Quantification of the relative gene expression was performed using the comparative Ct method. The Ct value is defined as the point where a statistically significant increase in the fluorescence has occurred. The number of PCR cycles (Ct) required for the 6-carboxylfluorescein intensities to exceed a threshold just above the background level was calculated for the test and reference reactions. In all experiments, GAPDH was used as the endogenous control. The 5' forward and 3' reverse-complement PCR primers for amplification of each gene were as follows: human ATF3, 5'-CTG CTC CTG GGT CAC TGG TT-3' and 5'-AGG CAC TCC GTC TTC TCC TT-3'; human EGR1, 5'-CAG TGG CCT AGT GAG CAT GA-3' and 5'-CCG CAA GTG GAT CTT GGT AT-3'; human GRP78, 5'-TGG ATT TGT CAC TGT TCA GCA-3' and 5'-GGG CTC TGG TCA AAG TCT TCT CC-3'; human MCP1, 5'-CTG ACC AGC TTG GAT GAC AC-3' and 5'-GGG CTC CAG CCA CTT TC-3' and 5'-TG GGC TGG AAC ACT GGT TTG-3'; human CXCL1, 5'-CAG ACG TTG GTC TTC TCC TT-3' and 5'-TTG ATT TGT CAC TGG TCA GCA-3'; human GAPDH, 5'-CAG ATG TTT GTC GTA TTA-3' and 5'-CTG TGG TCA TGA GTC TTC CC-3'; mouse monoclonal anti-hnRN P (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-phospho-p65, monoclonal anti-HDAC1, and polyclonal anti-phospho-eIF2α (Cell Signaling Technology, Beverly, MA). The cells were washed with ice-cold phosphate buffer, lysed in boiling lysis buffer (1% (w/v) SDS, 1.0 mM sodium ortho-vanadate, and 10 mM Tris, pH 7.4), and sonicated for 5 s. Lysates containing proteins were quantified using a BCA protein assay kit (Wegene). Next, 15 μg of protein was separated by Bio-Rad mini gel electrophoresis, after which they were transferred to a PVDF membrane (Pall Corporation, Port Washington, NY), and the blots were blocked for 1 h with 5% skimmed milk in Tris-buffered saline plus Tween 0.1% (TBST). The samples were then probed with each antibody for an additional 2 h at room temperature or overnight at 4 °C. After washing three times with TBST, blots were incubated with horseradish-conjugated secondary antibody for 1 h and then washed with TBST three times. Finally, protein was detected by pico enhanced peroxidase detection (ELPIS Biotech. Inc., Taejon, South Korea).

Isolation of Nuclear Extracts and Immunoprecipitation Assay—To isolate the nuclear proteins, cells were collected from culture plates by scraping in ice-cold PBS and centrifuged at 200 × g once for 3 min. The cell pellet after centrifugation was then resuspended in a lysis buffer containing 10 mM HEPES, 10 mM KCL, 1.5 mM MgCl2, 0.5 mM DTT, 0.5 mM PMSF, 0.1% Nonidet P-40, and protease inhibitor mixture (Sigma-Aldrich); incubated for 10 min on ice; and centrifuged at 9000 × g once for 15 min. Next, the supernatant (cytosolic fraction) was collected, and the remaining pellet was resuspended in a buffer containing 20 mM HEPES, 1 mM MgCl2, 400 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 25% glycerol, and protease inhibitor mixture. After 10 min of incubation on ice, samples were centrifuged at 13,800 × g once for 30 min, and the supernatants (nuclear proteins) were collected, divided into aliquots, and stored at −80 °C until analysis. Next, mouse monoclonal anti-HDAC1 was added to the supernatant to obtain the nuclear extracts, and the antibody nuclear extracts were rotated overnight at 4 °C. Protein G PLUS-agarose (30 μl; Santa Cruz Biotechnology) was then added to the antibody nuclear extracts, after which the samples were rotated at 4 °C for 3 h. The antibody nuclear extracts were then washed three times, after which 6 × SDS sample buffer was added. Finally, immunoprecipitates were collected by centrifugation and subjected to SDS-PAGE. The nuclear (hnRNP) and cytoplasmic (β-actin) markers verified the identity and purity of the fractions.

Confocal Microscopy—Cells were incubated in a glass-bottomed culture dish. Following pretreatment with vehicle or 25 μg/ml apigenin for 48 h, HCT-8 cells were treated with vehicle, 2 μg/ml LPS, or 0.1 μg/ml TG for 1 h, after which they were fixed with 4% paraformaldehyde (Biosesang. Inc., Sungnam, South Korea). Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min, after which they were blocked for 2 h with 3% BSA in PBS and then incubated with a 1:200 dilution of buffer (3% BSA in PBS) and rabbit polyclonal anti-ATF3 antibody (Santa Cruz Biotechnology) and mouse monoclonal anti-HDAC1 at room temperature for 2 h and then washed with PBS. The cells were then incubated with Alexa Fluor 488
Chromatin Immunoprecipitation Assay—Cells were cross-linked for 10 min in 1% formaldehyde, after which the reaction was stopped by the addition of glycine to 125 mM, and the cells were washed twice with 1/3 PBS. Chromatin was then fragmented by sonication for 10 s nine times to yield 1000–2000-bp fragments in lysis buffer (1% (w/v) SDS, 10 mM EDTA, pH 8.0, 50 mM Tris-HCl, pH 8.0, and protease inhibitor mixture) using VibraCell (Sonics & Materials, Inc., Newtown, CT). The soluble chromatin was immunoprecipitated with 2 μg of rabbit polyclonal anti-HDAC1 antibody in a mixture of nine parts dilution buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, pH 8.0, 20 mM Tris, pH 8.0, and protease inhibitor mixture) and one part lysis buffer. After rotating overnight at 4 °C, protein G PLUS-agarose (30 μl; Santa Cruz Biotechnology) was added to 100 μl of a 9:1 mixture of dilution buffer and lysis buffer containing 100 μg/ml BSA (Promega, Madison, WI) and 500 μg/ml salmon sperm DNA (Invitrogen) per sample. Following centrifugation of the protein G PLUS-agarose mixture, each sample was washed twice in dilution buffer, after which the chromatin was resuspended in the 9:1 dilution buffer/lysis buffer solution and incubated at 37 °C with proteinase K and RNase A (500 μg/ml for each sample). Chromatin was purified using a LaboPass™ gel and PCR clean-up kit (Cosmogenetech, Seoul, South Korea). Collected chromatin amplification was performed with HS Prime Taq DNA Polymerase (Genet Bio) in a Mycycler Thermal Cycler (Bio-Rad). The 5′ forward and 3′ reverse-complement PCR primers for amplification of each gene were as follows: human EGR-1 promoter, 5′-CTA GGG TGCAGG ATG GAG GT-3′ and 5′-GAA CAC TGA GAA GCG TGC AG-3′.
RNA Immunoprecipitation—Immunoprecipitation of protein-RNA complexes was performed using a modified protocol for chromatin immunoprecipitation (29). After cellular treatment, interacting protein and mRNA were cross-linked with 1% formaldehyde for 10 min at room temperature. The cytoplasmic extract was incubated overnight at 4 °C with 5 μg/gof either goat anti-mouse IgG (nonspecific control) or anti-HuR antibody. The antibody-bound complexes were precipitated with protein G PLUS-agarose beads and then sequentially washed in low salt, high salt, LiCl, and TE buffers (5 min/wash). The protein-RNA complexes were eluted from the protein G PLUS-agarose beads with 250 μl elution buffer at 37 °C for 15 min. RNA in the immunoprecipitated complexes was released by reversing the cross-linkage by incubating at 65 °C for 4–5 h in 200 mM NaCl and 20 μg of proteinase K. RNA was then extracted with RiboEx reagent (GeneAll, Seoul, South Korea) and subjected to RT real time PCR.

**FIGURE 2.** Potentiating effects of apigenin on ER stress-induced ATF3 expression in human intestinal cancer cells. A, HCT-8 cells were treated with vehicle or 25 μM apigenin for the indicated times. Cellular lysate was subjected to Western blot analysis. B and C, HCT-8 cells pre-exposed to vehicle or 25 μM apigenin for 12 h (B) or 24 h (C) were treated with vehicle or 0.1 μM TG for 2 h. Protein samples were subjected to Western blot analysis. D–F, HCT-8 cells pretreated with vehicle or 25 μM apigenin for 48 h were exposed to vehicle, 2 μg/ml LPS (D), 0.1 μM TG (E), or 1 μg/ml TM (F) for the indicated times. Cellular lysate was subjected to Western blot analysis. Each lower panel shows relative intensity of ATF3 protein per actin. *, significant difference from the LPS, TG, or TM treatment group without apigenin pre-exposure (p < 0.05). G–I, HCT-116 cells pretreated with vehicle or 25 μM apigenin for 48 h were exposed to vehicle, 2 μg/ml LPS (G), 0.1 μM TG (H), or 1 μg/ml TM (I) for the indicated times. Cellular lysate was subjected to Western blot analysis. J and K, HCT-8 cells pretreated with vehicle or each dose of apigenin for 48 h were exposed to vehicle, 2 μg/ml LPS (J) or 0.1 μM TG (K) for 2 h. Cellular lysate was subjected to Western blot analysis. Pre-Api, apigenin pre-exposure.

**RNA Immunoprecipitation**—In this experiment, we aimed to identify protein-RNA complexes using a modified protocol for chromatin immunoprecipitation. After cellular treatment, protein and mRNA complexes were cross-linked with 1% formaldehyde, allowing for the precipitation of protein-RNA complexes. The complexes were then sequentially washed with low salt, high salt, LiCl, and TE buffers. Subsequently, the complexes were eluted with 250 μl elution buffer at 37 °C, followed by the release of RNA through reversal of the cross-linkage by incubating at 65 °C. RNA was extracted using RiboEx reagent and subjected to RT real time PCR.

**In Vivo Tumor Growth**—In this section, we describe the in vivo tumor growth experiment. Thirteen-week-old male C57BL/6 mice were obtained from the Hyo Channing Science (Daegu, South Korea). All procedures conformed to the university committee’s guidelines for animal care and use. Animals were acclimated for a week before experiments and maintained at 25 °C with 45–55% relative humidity and 12-h light/dark cycles. Mice were housed three per a cage and provided with sufficient food and water.

For tumor inoculation, 5 × 10^5 CMT-93 mouse tumor cells were subcutaneously injected into the flank of 14-week-old male C57BL/6 mice. The mice were sacrificed at day 7 after tumor cell injection. Tumor tissues were surgically excised, suspended in RiboEX solution (GeneAll, Seoul, South Korea), and immediately frozen for homogenization. RNA was extracted using RiboEx reagent and subjected to RT real time PCR.
Statistical Analysis—Data were analyzed using SigmaPlot for Windows (Jandel Scientific, San Rafael, CA). Student’s t tests were used for comparative analysis of two groups of data, whereas analysis of variance was used for unpaired matched comparative analysis of multiple groups. Data that did not meet normality assumptions were subjected to Kruskal-Wallis analysis of variance on ranks, after which pairwise comparisons were made using the Student-Newman-Keuls method.

RESULTS

Preventive Exposure to Apigenin Alters Cancer ER Stress-related Genes and Signals—Endotoxin LPS as another ER stress trigger was assessed for its ability to alter eIF2α phosphorylation, which is a central biomarker of ER stress-induced translational arrest in HCT-8 and HCT-116 intestinal cancer cells that are frequently used as a model of inflammatory diseases and carcinogenesis (30, 31) (Fig. 1, A and B). LPS and a representative chemical ER stress inducer, TG, enhanced eIF2α phosphorylation, which was suppressed by genetic ablation of a representative eIF2α kinase, RNA-like endoplasmic reticulum kinase (PERK), using its shRNA. Moreover, the effects of apigenin on eIF2α activation were also tested based on the assumption that plant-derived flavonoid apigenin can suppress ER stress-linked translational arrest. To simulate the preventive exposure to dietary apigenin, cultured cancer cells were pretreated with apigenin for 48 h and then exposed to ER stress. Preventive exposure to apigenin attenuated eIF2α activation by chemical ER stressors, including TG and endotoxin LPS (Fig. 1, C and D). Apigenin was also assessed for its effects on the expression of ER stress-linked signaling molecules. As expected from previous

![Figure 3](https://example.com/fig3.png)

**FIGURE 3.** Effects of apigenin on stability of ATF3 mRNA in human intestinal cancer cells. A, HCT-8 (left panel) and HCT-116 (right panel) cells pre-exposed to the vehicle or 25 μM apigenin for 48 h were treated with 0.1 μM TG for 1 h. The cellular transcription was then arrested by adding 5 μM actinomycin D, and cellular RNA was extracted at each indicated time. Each mRNA was measured using RT real time PCR. *, significant difference from the TG group without apigenin pre-exposure (p < 0.05). B, plasmid containing CMV promoter-linked reporter gene tagged with 3′-UTR of the human ATF3 gene was constructed. HCT-8 cells transfected with the plasmid were exposed to the vehicle or 25 μM apigenin for 48 h. The cells were then treated with vehicle or 0.1 μM TG for 6 h. The different letters (a–c) over each bar represent significant differences between groups (p < 0.05). C, HCT-8 cells were treated with 25 μM apigenin for each indicated time. Cytosolic or nuclear fractions were subjected to Western blot analysis. D and E, HCT-8 (D) and HCT-116 (E) cells transfected with the empty vector (control) or shHuR were pretreated with vehicle or 25 μM apigenin for 48 h. The cells were then treated with vehicle or 0.1 μM TG for 2 h. Total lysates were subjected to Western blot analysis. F, HCT-8 cells pre-exposed to the vehicle or 25 μM apigenin for 48 h were treated with 0.1 μM TG for 1 h. Cytosol-extracted ATF3 mRNA bound to HuR protein was measured using RT real time PCR. *, significant difference from the control group PCR (p < 0.05); #, significant difference from the group treated with TG alone (p < 0.05). Pre-Api, apigenin pre-exposure.
investigations of ER stress-linked signals (6, 32). LPS, like TG, 
enhanced ATF3, ATF4, ATF6, and GRP78 (Fig. 1, 
E and 
F), all 
of which generally play critical roles in induction of genes 
essential for cellular homeostasis in response to translational 
blocking. Along with eIF2α activation, the gene induction of ER 
stress-related signaling molecules was suppressed in cancer 
cells pre-exposed to apigenins other than ATF3. In contrast, ER 
stress-induced ATF3 expression was more enhanced by 
chronic treatment with apigenin in human intestinal cancer 
cells. These superinduction patterns in ATF3 mRNA were 
consistently observed in the protein levels (Fig. 2). Because apige-
nin itself also can trigger ATF3 expression, a time course exper-
iment was performed in Fig. 2A. ATF3 was induced at 12 h and 
then shut down. We also assessed ER stress-induced ATF3 
pre-exposure to apigenin (12 h-24 h) (Fig. 2, B and C). Synergy 
was observed and increased with times of apigenin pre-exposure. 
In the 48-h pre-exposure to apigenin, the superinduction 
of ATF3 was more obvious, although the effects of apigenin 
itself on ATF3 expression are marginal. Compared with the 
patterns in HCT-8 cells (Fig. 2, D–F), ATF3 superinduction was 
more persistent in the HCT-116 cells (Fig. 2, G–I). Because the 
present study simulates the chemo-preventive effects of apige-
nin during ER stress-induced inflammation, all the following 
experiments related to ATF3 superinduction were performed 
after pre-exposure to apigenin for 48 h. Moreover, ATF3 super-
induction was almost maximal with 10–25 μM apigenin in the 
cancer cells under ER stress (Fig. 2, J and K), and all the follow-
ing experiments were performed using 25 μM apigenin as ATF3 
superinducer in the presence of ER stressor. Taken together, 
endotoxin LPS modulated crucial genes under ER stress in
human intestinal cancer cells, whereas pre-exposure to apigenin as a chemo-preventive agent against ER stress response attenuated eIF2α phosphorylation and subsequent ER stress response-related signaling molecules other than ATF3. In contrast, ATF3 was superinduced by apigenin pre-exposure; therefore, subsequent experiments focused on elucidating the details of the molecular mechanism of ATF3 superinduction and its roles in the regulation of cancer chemokines in cancer cells under ER stress.

Apigenin Potentiates ATF3 Superinduction via Post-transcriptional Regulation—Because the transcriptional activation alone cannot account for superinduction of ATF3 expression (33), its mRNA stability was assessed as a potent mechanism as shown in previous reports on superinduction of specific genes (34–36). As expected, apigenin pre-exposure increased the stability of ATF3 mRNA in the intestinal cancer cells (Fig. 3A). To provide other evidence of ATF3 mRNA stabilization, we assessed effects of apigenin pre-exposure on the relative expressions of reporter gene tagged with the 3′-UTR of ATF3 gene (Fig. 3B). We constructed constitutively expressing luciferase reporter plasmids tagged with the 3′-UTR of human ATF3 gene. Pre-exposure to apigenin increased the production of the luciferase reporter, which was more enhanced under ER stress (Fig. 3B), suggesting that reduced
turnover of ATF3 mRNA may be associated with the regulations of 3′-UTR of ATF3.

Although many RNA binding proteins regulate mRNA stability, only HuR is considered as a definite enhancer of mRNA turnover (33). HuR is an RNA-binding protein that regulates both the stability and cytoplasm-nucleus localization of mRNA species containing AU-rich elements (33), and ATF3 transcript also has three AU-rich elements in the 3′-UTR depicted in Fig. 2B. In the present study, apigenin treatment promoted cytosolic translocation of HuR protein in the intestinal cancer cells (Fig. 3C). Moreover, when HuR expression was genetically ablated using its shRNA, apigenin-potentiated ATF3 expression was almost suppressed (Fig. 3, D–E), suggesting that HuR is a positive regulator of ATF3 superinduction. To address the direct binding of HuR protein with ATF3 transcript, RNA immunoprecipitation assay was performed. ER stress increased the binding of HuR protein to ATF3 transcript, which was more enhanced in the cancer cells pre-exposed to apigenin (Fig. 3F).

Taken together, pre-exposure to apigenin triggered translocation of HuR protein that bound to ATF3 mRNA, which was stabilized in the intestinal cancer cells under ER stress. Apigenin-enhanced ATF3 Mediates the Suppression of ER Stress-induced Chemokine Expression—Because ATF3 is known to regulate cancer cytokine expression (16−18), we tested whether ATF3 overexpression altered cancer chemokines such as MCP-1 (monocyte chemotactic protein-1) and CXCL-1 (chemokine (CXC motif) ligand 1). Functionally, apigenin-potentiated ATF3 may suppress the tumor-favoring inflammatory responses. In in vivo tumor growth assay, apigenin treatment suppressed the ER stress response (indicated as a representative biomarker, GRP78) and expression of proinflammatory chemokines in tumor mass (Fig. 4, A−D) but elevated ATF3 expression (Fig. 4, E and F). In the cell culture model, cancer ATF3 overexpression suppressed both endotoxin LPS- and TG-induced chemokine expression (Fig. 5, A−D), indicating the negative regulation of cancer cytokines by ATF3. When ATF3 expression was blocked by its shRNA, TG-induced chemokines was thus enhanced (Fig. 5E), supporting negative regulatory action of ATF3 in chemokine induction by ER stress. Therefore, it can be assumed that apigenin-enhanced ATF3 can also regulate ER stress-induced chemokines. As expected, ATF3-enhancing apigenin suppressed the induction of CXCL-1 and MCP-1 (Fig. 5, F−I). In contrast, ATF3 suppression using shRNA reinstated chemokine induction, even in the presence of the ATF3 inducer, apigenin (Fig. 5, F−I). Similar patterns were also observed in the cancer cells under ER stress by another trigger, tunicamycin (TM) (Fig. 5, J and K). Therefore, it can be concluded that apigenin-induced ATF3 negatively regulates ER stress-induced chemokine induction.

**FIGURE 6. Regulation of ATF3 for ER stress-activated NF-κB signal in human intestinal cancer cells.** A–D, HCT-8 (A and B) and HCT-116 (C and D) cells transfected with the empty vector (control) or ATF3-SC were treated with vehicle, 2 μg/ml LPS, or 0.1 μM TG for the indicated times. Total lysates were subjected to Western blot analysis. E–G, HCT-8 (E) and HCT-116 (F) cells transfected with the empty vector (control) or SR-IκB were exposed to vehicle, 2 μg/ml LPS, or 0.1 μM TG for 1 h. Each mRNA was measured using RT real time PCR and protein samples (G) were subjected to Western blot analysis. The different letters (a−c) over each bar represent significant differences between groups (p < 0.05).
Induced ATF3 Regulates Transcription Factor Early Growth Response Protein 1, but Not the Conventional Proinflammatory Transcription Factor NF-κB in Response to Proinflammatory ER Stress—

NF-κB was assessed to address a potent target regulated by ATF3 because it is the key transcription factor in many inflammatory responses. Specifically, ATF3 was tested to determine whether it regulates proinflammatory transcription factor NF-κB activated by ER stressors in enterocytes. Overexpression of ATF3 suppressed p65 phosphorylation by LPS or TG treatment (Fig. 6, A–D), indicating negative regulation of NF-κB signaling by ATF3. We next tested whether NF-κB activation plays critical roles in chemokine production in response to ER stressors. However, blocking of NF-κB signaling using superrepressor mutant IκB (SR-IκB) did not affect ER stress-induced chemokine (MCP-1 and CXCL-1) expression (Fig. 6, E and F), even though the expression of SR-IκB specifically inhibited p65 phosphorylation (Fig. 6G). Therefore, other proinflammatory signaling mediators may contribute to chemokine induction by ER stress. In addition to NF-κB activation, gut epithelia have developed sentinel signal transduction in response to exogenous stress via elicitation of the acute expression of immediately induced transcription factors, including the EGR-1 (37, 38). The expression of EGR-1 can be induced by a range of proinflammatory stimuli, which in many cases overlap with those known to be capable of inducing NF-κB expression (39, 40). Both LPS and TG also enhanced EGR-1 expression, which was suppressed by ATF3 expression, suggesting a negative relationship between ATF3 and EGR-1 (Fig. 6, A–D). In contrast to the NF-κB signal, EGR-1 was positively involved in chemokine (MCP-1 and CXCL-1) induction by either TG or LPS (Fig. 7, A and B). In terms of signaling pathway, EGR-1 was positively regulated by PERK, a crucial upstream signaling mediator of ER stress response (Fig. 7, C and D) and thus suppression of PERK decreased EGR-1-associated chemokine expression in human enterocytes (Fig. 7, E and H). Moreover, treatment with pharmacological ATF3 inducer apigenin suppressed ER stress-induced chemokine expression, which was reinstated by EGR-1 overexpression (Fig. 8, A–F). Additionally, apigenin was shown to repress ER stress-induced EGR-1 expression (Fig. 8, G–L), which was reinstated by ATF3 suppression using its shRNA (Fig. 8 M). Taken together, the present results indicate that EGR-1 plays crucial roles in ER stress-induced cancer chemokines, including MCP-1 and CXCL-1 in intestinal cancer cells, and the regulation of cancer chemokines by apigenin occurred mechanistically via ATF3-suppressed EGR-1 expression. In addition to EGR-1, other crucial transcription factors in ER stress responses, including ATF6, GRP78, and CHOP, were assessed for their involvement in cancer chemokine regulation (Fig. 9). Genetic ablation of ATF6, GRP78, or CHOP, using each shRNA attenuated chemokine induction (Fig. 9, A–C) and EGR-1 expression (Fig. 9D), indicating positive regulation of ER stress-triggered EGR-1 and subsequently induced chemokines by ATF6, GRP78, and CHOP in intestinal cancer cells.
ATF3 Suppresses EGR-1 Expression via Epigenetic Regulation

As indicated in the introduction, ATF3 is a transcription repressor that regulates target gene expression by competition with other transcription factors or chromatin remodeling (16). The present study assessed the HDAC1-associated epigenetic modulation of EGR-1 expression and subsequent chemokine induction. ATF3 overexpression suppressed LPS-induced EGR-1, but HDAC1 inhibition using trichostatin A reinstated EGR-1 expression (Fig. 10A), suggesting the involvement of ATF3-recruited HDAC1 in epigenetic regulation of EGR-1. Similarly, ATF3-triggering apigenin attenuated EGR-1 expression via HDAC (Fig. 10B). Epigenetic regulation of ER stress-induced chemokine expression was also demonstrated by genetic ablation using HDAC1 shRNA (Fig. 10, C and D). Apigenin-suppressed CXCL-1 and MCP-1 expression were restored in HDAC1-suppressed cells, suggesting that apigenin-enhanced ATF3 regulates chemokine induction via epigenetic suppression of EGR-1.

ATF3 has a DNA-binding basic leucine zipper domain that can bind to the cyclic AMP response element consensus sequence (41) that is also present in EGR-1 promoter. Therefore, the specific binding of ATF3 to its binding motif in EGR-1 promoter was assessed. ER stress enhanced ATF3 binding to EGR-1 promoter, and the binding was enhanced by ATF3 overexpression (Fig. 10E). Moreover, ATF3-elevating apigenin enhanced the ER stress-triggered binding of ATF3 to its motif in EGR-1 promoter (Fig. 10F). In addition to ATF3 binding to EGR-1 promoter, ATF3-mediated epigenetic regulation of gene expression was assessed by monitoring the nuclear interaction of HDAC1 with ATF3 in cancer cells exposed to ER stress.
stressors. ER stress triggered the interaction of ATF3 with HDAC1, and the degree of the nuclear interaction between HDAC1 and ATF3 was enhanced in cells pre-exposed to apigenin as well as cells overexpressing ATF3 (Fig. 10, G and H). Similar patterns were also observed in the nuclear co-localization of ATF3 and HDAC1 protein in the intestinal cancer cells (Fig. 10, I and J). Taken together, ER stress increased the nuclear co-localization of ATF3 and HDAC1, and ATF3 induction enhanced the degree of the nuclear co-localization. All of the present results confirm the epigenetic recruitment of HDAC1 to ATF3-bound EGR-1 promoter in response to apigenin treatment.

DISCUSSION

Endotoxin LPS is a potent trigger of ER stress-linked signaling responses. Among the genes regulated by ER stressors, ATF3 was found to be located at an integral point of coordination for stress response and to play regulatory roles in ER stress-induced proinflammatory response in the present study. Moreover, ATF3 was superinduced by flavone apigenin and strongly interfered with cancer chemokine induction in response to ER stressors, including LPS, in human intestinal cancer cells. Mechanistically, elevated ATF3 blocked the expression of EGR-1, a crucial proinflammatory transcription factor in cancer chemokine induction by ER stress, via epigenetic regulation (Fig. 11). Although Toll-like receptor 4-linked NF-κB is a central transcription factor for LPS-induced proinflammatory mediators (42, 43), human intestinal cancer cells showed different signaling patterns for chemokine (MCP-1 and CXCL-1) induction, regardless of NF-κB. Cancer cell EGR-1 played key roles in the induction of MCP-1 and CXCL-1 in the present study, which would lead to recruitment of leukocytes to the hot inflamed tumor microenvironment. EGR-1 has been investigated as one of the proinflammatory transcription factors involved in the induction of cytokine production (44, 45). EGR-1-associated cytokine production has been examined in combination with three central proinflammatory transcription factors, NF-κB, CCAAT-enhancer-binding protein, and AP-1.
(activator protein 1) (46), and some of the target gene transcription occurs through synergistic interaction of EGR-1 with other transcription factors, such as nuclear factors of activated T cells, by forming heterodimers (47). Although a range of growth- or differentiation-related stimuli can promote the expression of EGR-1, bacterial products such as lipopolysaccharide and peptidoglycan also trigger EGR-1 gene expression during inflammation (48, 49). EGR-1 binds to the Sp1/EGR-1 overlapping sequence motif, which is located in the promoter region of TNF-α (50, 51). In unstimulated monocytes, Sp1 binds to this site to maintain basal levels of gene expression, whereas EGR-1 displaces Sp1 to mediate LPS-induced TNF-α promoter activity. The Sp1/EGR-1-overlapping binding motif is also present in human MCP-1 (50, 51) and CXCL-1 (52, 53, 54); thus, it can be speculated that upon ER stress, EGR-1 displaces Sp1 in chemokine expression in the intestinal cancer cells.
In the present study, EGR-1 was regulated by apigenin via ATF3, which subsequently contributed to cancer chemokine suppression, even in the presence of ER stressors. EGR-1 regulation by ATF3 was epigenetically mediated by HDAC1 protein. In addition to this mechanism, ATF3 could modulate target gene expression via other modes: namely, 1) binding to the target gene promoter directly as a transcriptional regulator, and 2) inhibition of recruitment of coactivator on the target gene promoter (52, 53). However, direct transcriptional regulation by ATF3 did not contribute to chemokine regulation by apigenin (data not shown). Instead, ATF3-recruited HDAC1 via direct interaction mediated chromatin remodeling and led to chemokine suppression. Flavones including apigenin have been shown to be associated with the anti-inflammatory activity of dietary herbal extracts from fruits and vegetables (23, 24). Although the link of apigenin to anti-inflammatory action that is usually investigated is the NF-κB-linked signal, the results of the present study suggest EGR-1 as a new target of intervention of inflammation-promoted cancers. Apigenin can alter the NF-κB signaling pathway but was not involved in chemokine MCP-1 and CXCL-1 expression in the intestinal cancer cells. Here, apigenin-enhanced ATF3 regulated EGR-1 and EGR-1-promoted cancer chemokines.

In terms of immune tolerance in proinflammatory responses, anti-inflammatory action by ATF3 prevented excessive production of proinflammatory chemokine induced by ER stressors, including endotoxins, in the present study. However, ATF3-mediated anti-inflammatory responses are not always beneficial to the host because they can weaken the defense against infection in the gut environment. Although chronic induction of ATF3 expression can offer protection against inflammatory insults, ATF3 increases susceptibility to secondary infections during sepsis-associated immune suppression (54). Moreover, the wound healing process during mucosal inflammation can be inhibited by increased ATF3 expression because epithelial NF-κB and EGR-1 expression promotes the reconstitution of injured mucosal monolayers (55, 56). The gastrointestinal tract is subjected to a wide variety of mucosal insults linked to several intestinal inflammatory diseases. Regardless of the cause of injuries, the mucosa usually responds rapidly by activating defense mechanisms that stimulate repair and restoration of the mucosal lining. If the production of early gene products including epithelial NF-κB and EGR-1 is suppressed by ATF3 in injured mucosal tissues, the compensatory response during wound healing would be inhibited. However, more studies are needed to determine whether ATF3-mediated gene regulation is beneficial under different pathogenic conditions.

In terms of normal tissue, ATF3 can be protective in response to mucosal insults by modulating features of the regulated gene profile (57, 58). Enhanced ATF3 production can be associated with cellular defenses by maintaining epithelial sur-
ATF3-attenuated Cancer Chemokine Production

vival after toxic insults (59). Although many reports have suggested that ATF3 has apoptosis-promoting effects (60, 61), it can also protect cells from physical and chemical cytotoxic stresses by suppressing the induction of cell death factors such as p53 proteins (62). ATF3-suppressed cells show an enhanced level of p53 that probably makes the cells more sensitive to cytotoxic effects. Additionally, ATF3 up-regulates a survival factor, heat shock protein HSP27, as well as the c-Jun/Fos protein, without which cells will undergo retarded regeneration after injury (63). However, these survival-promoting effects of ATF3 gene regulation can be harmful during epithelial cancer progression. Enhanced survival induced by ATF3 may help tumor cells pass through cytotoxic environments and provide a growth advantage over normal cells. ATF3 increases the expression of several genes implicated in malignant tumor metastasis such as TWIST1, FN-1, Snail, and Slug (58). The ATF3 gene copy number and protein levels are significantly higher in malignant breast tumors (58). Similar to apigenin in our study, ATF3 genes can be induced by other antitumoric agents such as nonsteroidal anti-inflammatory drug, anti-inflammatory polyphenol curcumin, and green tea catechins. Therefore, the oncogenic activity of ATF3 implicates the potential adverse effects of these anti-tumor agents, including apigenin, on tumor chemotherapy. Accordingly, future studies are warranted to address the toxicological safety issues associated with apigenin and ATF3-modulating chemicals for cancer treatment, despite the positive roles of ATF3 in tumor microenvironment-associated inflammation implicated in the present study. Overall, ATF3 inducer including apigenin attenuated ER stress-induced chemokine (MCP-1 and CXCL-1) expression in the intestinal cancer cells. Mechanistically, cancer chemokine-promoting EGR-1 gene expression was epigenetically interfered with by ATF3 protein via HDAC1. Thus, the present study provides new insights into NF-κB-independent modulation of tumor-associated inflammation via ATF3-regulated EGR-1. However, apigenin as a potent ATF3 modulator needs to be carefully investigated because ATF3 can also exert adverse effects depending on the dynamic pathogenic context.

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