available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Epithelial cell survival by activating transcription factor 3 (ATF3) in response to chemical ribosome-inactivating stress

Hyun Yang, Seong Hwan Park, Hye Jin Choi, Yuseok Moon *

Department of Microbiology and Immunology, Medical Research Institute, Pusan National University School of Medicine, 1-10 Ami-dong Seo-Ku, Busan 602-739, Republic of Korea

ARTICLE INFO

Article history:
Received 17 September 2008
Accepted 26 November 2008

Keywords:
Ribotoxic stress
Activating transcription factor 3 (ATF3)
Epithelial cell survival
p38 MAP kinase
Double-stranded RNA (dsRNA)-activated protein kinase (PKR)

ABSTRACT

Ribotoxic stress responses lead to the expression of genes important for cellular homeostasis by modulating cell survival, proliferation and differentiation. ATF3 was investigated for its modulation of the epithelial cellular integrity in response to mucosal ribotoxic stress. ATF3 expression was up-regulated by chemical agents causing ribotoxic stress such as deoxynivalenol and anisomycin in different types of intestinal epithelial cells. Moreover, reduction of ATF3 gene expression promoted ribotoxic stress-triggered programmed cell death, implicating a protective role of ATF3 in epithelial cell survival. Mechanistically, stabilization of ATF3 messenger RNA and protein played a critical role in maintaining enhanced levels of ATF3 production in response to the ribotoxic chemical agent. For ATF3 mRNA stability, p38 specific inhibitor SB203580 was the most efficient agent for suppression, suggesting the involvement of the p38 MAP kinase in ATF3 mRNA stabilization. In addition, the p38 MAP kinase as well as its downstream mediator glycogen synthase kinase 3β (GSK3β) was involved in ATF3 protein stabilization caused by chemical ribotoxic stress in human epithelial cells. As another separate signaling cascade, double-stranded RNA (dsRNA)-activated protein kinase (PKR) was demonstrated to translationally modulate ATF3 expression and contribute to the epithelial cell survival. PKR interference caused cells to be more susceptible to cell death caused by the chemical ribotoxic stress. The results of this study showed that enhanced ATF3 production was associated with cellular defenses by maintaining the epithelial survival after ribotoxic mucosal insults.

1. Introduction

Specific ribosome-directed xenobiotics have the capacity to damage 28S ribosomal RNA by interfering with its functioning during gene translation. This can lead to what has been labeled as ribotoxic stress responses that stimulate intra-cellular sentinel signaling pathways. This process results in the expression of genes important in cellular homeostasis as well as a variety of pathogenic processes involved in the modulation of cell survival, proliferation and differentiation [1,2]. Mechanistically, anisomycin, UV radiation, ricin and a variety of sesquiterpenoid trichothecene fungal metabolites including deoxynivalenol (DON), have been reported to be effective triggers of the ribotoxic stress response in cells because these xenobiotics bind to the 28S ribosomal RNA peptidyltransferase site, block protein synthesis and can activate stress signals [2]. Although many different stress-related mediators have been reported to be involved in ribotoxic stress responses, signaling cascades of mitogen-activated protein kinases (MAP kinase) have been considered to be the main stress-signaling clusters [3,4].

* *Corresponding author*. Tel.: +82 51 240 7711; fax: +82 51 243 2259.
E-mail address: moon@pusan.ac.kr (Y. Moon).
0006-2952/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.bcp.2008.11.028

ATF3 is a transcription factor of the ATF/cyclic AMP response element-binding family that contains a basic region/leucine zipper DNA-binding motif and binds to the cyclic AMP response element consensus sequence [5]. In most cases, the protein is induced by external stress signals such as ischemic injuries, mutagens, carcinogens, mitogenic cytokines, or endoplasmic reticulum (ER) stresses from abnormal protein processing [6,7]. It functions as homodimer or a complex with the CCAAT/enhancer binding protein (C/EBP) family such as C/EBP homologous protein (CHOP). Moreover, several other stress inducible transcription factors such as AP-1 and early growth response gene 1 (EGR-1) are also induced as the stress mediators in the cluster with ATF3 [8]. Studies on cancer progression have shown that ATF3 plays dichotomous roles in the cancer development [7]. It can either promote or suppress the cellular growth depending on the endogenous or exogenous texture of disease conditions. One example of growth stimulation role of ATF3 is when the epithelium triggers its genetic re-programming in response to external skin insults and induces ATF3 expression as a wound healing-associated mitogenic mediator [9].

Ribotoxic stress responses have been associated with diverse pathological events such as apoptotic cell death and inflammation [10,11]. Several epidemiological studies have suggested a link between ribotoxic deoxynivalenol intoxication and human mucosal epithelial abnormalities including gastroenteritis and airway disruption [12,13]. DON has been shown to disrupt the epithelial integrity by interfering with nutritional uptake or eliciting epithelial atrophy and inflammation [14–16]. Recently, we demonstrated that the chemical ribotoxic stress can induce G2/M cell cycle arrest via p21 expression in human epithelium [17]. Other chemical or physical ribotoxic stresses have also been shown to stimulate p53- or p21-dependent cell cycle arrest and apoptosis [18,19]. The primary toxic actions of ribotoxic trichothecenes are generally recognized for their functional inhibition of global protein synthesis in rapidly dividing tissues such as lymphoid tissues and gastrointestinal epithelium, which are the most susceptible targets of these toxins. Acute exposure to high doses of trichothecenes causes radiomimetic syndromes in the gastrointestinal tract and bone marrow apoptosis, which are also observed after high levels of ribotoxic UV radiation. However, whereas the leukocytes easily undergo apoptotic cell death, recent reports demonstrated that ribotoxic stress does not induce cellular apoptosis in human intestinal epithelial cells [16,17]. Instead of the cell death response, ribotoxic insults can induce G2/M cell cycle arrest via p21 gene regulation and extend the epithelial cell survival.

The purpose of this study was to identify the decision mechanisms involved in epithelial survival or death. The growth-modulating molecule ATF3 was studied to investigate its activity in maintaining the mucosal integrity in response to the ribotoxic stresses. Moreover, mechanistically we evaluated how a ribotoxic insult can affect the genetic expression of ATF3. ATF3 modulation in response to external ribotoxic insults may contribute to gut homeostasis by enhancing cell survival in a stressful mucosal environment.

2. Materials and methods

2.1. Cell culture conditions and reagents

For human epithelial cell line, HCT-8 cells obtained from human ileocecal tissues were purchased from the American Type Culture Collection (Rockville, MD, USA). They were maintained in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma Chemical Company, St. Louis, MO, USA), 50 units/ml penicillin (Sigma Chemical Company), and 50 μg/ml streptomycin (Sigma Chemical Company) in a 5% CO_2 humidified incubator at 37 °C. The cell number and viability were assessed by trypan blue (Sigma Chemical Company) dye exclusion using a hemacytometer. Deoxynivalenol with a purity of 97.6 ± 2.4% was isolated from *Fusarium graminearum* (Sigma Chemical Company). DON was used as the standard chemical agent triggering the ribotoxic stress responses. Additional ribotoxic stress agents such as anisomycin (Sigma Chemical Company) and DON derivatives (Sigma Chemical Company) were compared with regard to ATF3 gene expression. All the inhibitors were purchased from Sigma Chemical Company.

2.2. Cell cycle analysis and apoptosis quantification by flow cytometry

Trypsinized cells (1×10^6) were prepared and re-suspended in 0.2 ml PBS. Following addition of 0.2 ml heat-inactivated fetal bovine serum, the cells were immediately fixed by slow drop-wise addition of 1.2 ml ice-cold 70% (v/v) ethanol with gentle mixing, and then held at 4 °C overnight. The cells were washed and incubated in 1 ml propidium iodide (PI) DNA staining reagent (PBS containing 50 μg/ml PI, 50 μg/ml RNase A, 0.1 mM EDTA, and 0.1% [v/v] Triton X-100) on ice until analyzed. The cell cycle distribution for single cells was measured with a Becton Dickinson FACS Calibur (San Jose, CA, USA). Data from 10,000 cells were collected in the list mode. The 488 line of an argon laser was used to excite PI, and fluorescence was detected at 615–645 nm. The cell cycle of individual cells was studied using a doublet discrimination gating to eliminate doublets and cell aggregates based on the DNA fluorescence. The gate was drawn to include hypofluorescent cells. Cells in the DNA histogram with hypofluorescent DNA were designated as apoptotic. All other cells distributed themselves in a normal cell cycle profile.

2.3. DNA fragmentation analysis

DNA was extracted from colon epithelial cell line. In brief, cells (2×10^6) in PBS were centrifuged for 5 min (2000 rpm) at 4 °C, and the pellet was suspended in 0.1 ml hypotonic lysis buffer (10 mM Tris, pH 7.4, 10 mM EDTA, pH 8.0, 0.5% [v/v] Triton X-100). Cells were incubated for 15 min at 4 °C. The resultant lysate was centrifuged for 30 min (13,000 rpm) at 4 °C. The supernatant containing fragmented DNA was digested for 1 h at 37 °C with 0.04 mg/ml of RNase A (Bio Basic Inc., Ontario, Canada) and then incubated for an additional 1 h at the same temperature with 0.04 mg/ml of

proteinase K (Sigma Chemical Company). DNA was precipitated in 50% (v/v) isopropanol in 0.4 M NaCl at −20 °C overnight. The precipitate was centrifuged at 13,000 rpm for 30 min at 4 °C. The resultant pellet was air dried and resuspended in TE buffer. An aliquot equivalent to 2×10^6 cells was electrophoresed at 40 V for 3 h in a 2% (w/v) agarose gel in 90 mM Tris–glacial acetic acid buffer containing 2 mM EDTA (pH 8.0). After electrophoresis, the gel was stained with ethidium bromide (1 μg/ml), and the nucleic acids were visualized with a UV transilluminator. A 1Kb+ DNA ladder (SolGent Co., Ltd., Korea) was used for molecular sizing.

2.4. Construction of plasmids

The entire coding region of ATF3 including the TATAA region was generated by reverse transcription-polymerase chain reaction (RT-PCR) using RNA from HCT-8 cells with the following primers: forward CGTGAGTCCTCGGTGCTC, reverse GACAGCTCTCCAATGGCTTC. The resulting 721 bp construct was cloned using TopCloner TA kit (Enzynomics, Daejeon, Korea) followed by excision at the HindIII/NotI sites, then transferred in the sense and antisense orientations into the expression plasmid pcDNA3.1Zeo+/− (Invitrogen) using T4 DNA ligase (NEB, Beverly, MA, USA), and then confirmed by DNA sequencing. The ATF3 promoter constructs (−2062/+45) and (−74/+45) were amplified using the *pfu* turbo polymerase chain reaction with human genomic DNA (HCT-8) and cloned into the pGL3 basic vector (Promega, Madison, WI, USA). The 3′-untranslated region (UTR) of the human ATF3 gene (+647/+1811) was cloned into the pGL3 control vector at the Xba I site. The construct of dominant negative human PKR (dnPKR) expression vector (PKR-K296R) was kindly provided from Dr. Chen, Pei-Jer (College of Medicine, National University of Taiwan).

2.5. Western immunoblot analysis

The levels of protein expression were compared using Western immunoblot analysis with rabbit polyclonal anti-human actin antibody and rabbit polyclonal anti-ATF3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p-eIF2a (Assay Designs, Ann Arbor, MI, USA), rabbit polyclonal anti-p-MAPKs, anti-p-GSK3β (Ser9), and anti-p-AKT antibody (Cell signaling technology, Beverly, MA, USA). 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. The lysates containing proteins were quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA). Fifty micrograms of protein were separated by Bio-Rad gel mini electrophoresis. The proteins were transferred onto a PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and the blots were blocked for 1 h with 5% skim milk in Tris-buffered saline plus Tween 0.05% (TBST) and probed with each antibody for a further 2 h at room temperature or overnight at 4 °C. After washing three times with TBST, the blots were incubated with horseradish-conjugated secondary antibody for 1 h and washed with TBST a further three times. The proteins were detected by ECL Chemi-luminescent substrates (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.6. Traditional reverse transcription-polymerase chain reaction

RNA was extracted with RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNA (100 ng) from each sample was transcribed to cDNA by BD Sprint PowerScript (Clontech, Mountain View, CA, USA). The amplification was performed with Takara HS ExTaq DNA Polymerase (Takara Bio Inc., Shiga, Japan) in *Mycycler* Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the following parameters: denaturation at 94 °C for 2 min and 25 cycles of denaturation reactions at 98 °C for 10 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 45 s. An aliquot of each PCR product was subjected to 1.2% (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide. The 5′ forward and 3′ reverse-complement PCR primers for amplification of each gene were as follows: human ATF3 (5′-CTC CTG GGT CAC TGG TGT TT-3′ and 5′-AGG CAC TCC GTC TTC TCC TT-3′), and human GAPDH (5′-TCA ACG GAT TTG GTC GTA TT-3′ and 5′-CTG TGG TCA TGA GTC CTT CC-3′).

2.7. Transient and stable transfection

Cells were transfected with mixture of plasmids using the Trans-LT1 transfection reagent (Mirus, Madison, WI, USA) according to the manufacturer's protocol. For transfection of the luciferase reporter gene, a mixture of 1.5 μg firefly luciferase reporter and 0.15 μg renilla luciferase, pRL-null vector (Promega, Madison, WI, USA) per 4.5 μl of Trans-LT1 reagent was applied to a 6-well culture plate. For the luciferase assay, 18 h after the transfection, the cells were exposed to chemicals for a further 24 h and lysed for dual-luciferase reporter assay system (Promega). The tranfection efficiency was maintained at around 50–60% for all assays, which was confirmed with pMX-enhanced GFP vector. To create the dnPKR-expressing stable cell lines, cells were transfected using CarriGene reagent (Kinovate Life Sci., Oceanside, CA, USA). After 48 h the cells were subjected to selection for stable integrants by exposure to 400 μg/ml hygromycin B (Invitrogen, Carlsbad, CA, USA) in complete medium containing 10% fetal bovine serum. Selection was continued until monolayer colonies were formed. The transfectants were then maintained in medium supplemented with 10% fetal bovine serum and 200 μg/ml hygromycin B.

2.8. Luciferase assay

The cells were washed with cold PBS, lysed with passive lysis buffer (Promega, Madison, WI, USA) and then centrifuged at 12,000 × *g* for 4 min. The supernatant was collected isolated and stored at −80 °C until assessment for luciferase activity. Luciferase activity was measured with a dual-mode luminometer (Model TD-20/20, Turner Designs Co., Sunnyvale, CA, USA) after briefly mixing the supernatant (10 μl) with 50 μl firefly luciferase assay substrate solution, followed by 50 μl stopping renilla luciferase assay solution (Promega, Madison, WI, USA). The firefly luciferase activity was normalized against renilla luciferase activity using the following formula: firefly luciferase activity/renilla luciferase activity.

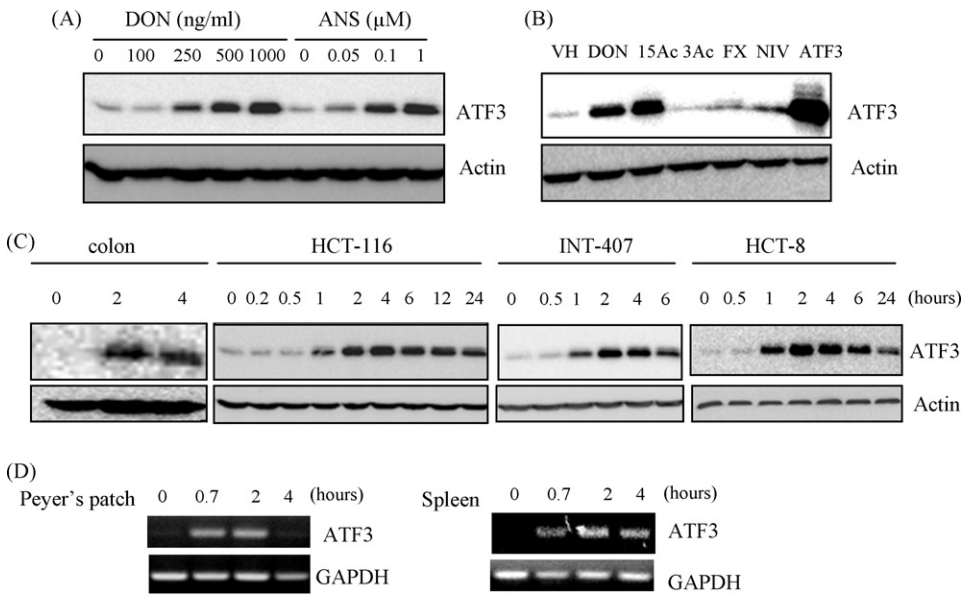

Fig. 1 – Effects of ribotoxic stress agents on ATF3 gene expression in the intestinal epithelial cells. (A and B) HCT-8 cells were treated with ribotoxic agents (vehicle (VH), anisomycin (ANS), deoxynivalenol (DON), 15-acetyldeoxynivalenol (15Ac), 3-acetyldeoxynivalenol (3Ac), fuxarenone (FX), and nivalenol (NIV)) for 2 h. Chemical dose in (B) was 250 ng/ml. (C) Mice (male B57BL/6J) were treated with 25 mg/kg DON via gavage. Protein from the colonic cells was analyzed. Other intestinal cell lines were treated with 500 ng/ml DON for the indicated time and the cellular lysate was analyzed by Western blot. (D) mRNA from the Peyer's patch or spleen of the DON-gavaged mice was analyzed using RT-PCR. All results are representative of three independent experiments.

2.9. *Statistical analyses*

Data were analyzed using SigmaStat for Windows (Jandel Scientific, San Rafael, CA, USA). For comparative analysis of two groups of data the Student's *t*-test was performed. For comparative analysis of multiple groups the data were subjected to analysis of variance (ANOVA) and pairwise comparisons using the Student–Newman–Keuls (SNK) method. Data not meeting normality assumptions were subjected to the Kruskal–Wallace ANOVA on ranks and then pairwise comparisons were made by the SNK method.

3. Results

3.1. *ATF3 gene expression was up-regulated by ribotoxic stress agents in different types of intestinal epithelial cells*

The tissue-damaging stress of ischemia-reperfusion, epithelial wound, or neuronal injuries activates ATF3 gene expression to change the intrinsic growth state and can trigger the regeneration machinery [9,20,21]. The ribotoxic stresses disrupt the epithelial integrity [22,23] and we assessed the ribotoxic stress-induced effects on expression of ATF3 gene in the gastrointestinal epithelial cells in terms of cell survival and injury. Several representative ribotoxic agents including trichothecenes and anisomycin were tested for their effect on ATF3 gene expression. Trichothecenes and ribotoxin anisomycin induced ATF3 expression in a dose-dependent manner (Fig. 1A). Moreover, when the structurally similar 8-keto trichothecenes were compared, the rank of potency of ATF3 induction was 15-acetyl–DON > DON > NIV (nivalenol), suggesting a structural preference for ATF3 induction by trichothecenes (Fig. 1B). Among the orally exposed trichothecenes in the daily diet, deoxynivalenol is the most frequently and ubiquitously detected. It was used here as a model chemical of ribotoxic stresses in the following experiments. When mice were exposed to the mucosal ribotoxin deoxynivalenol, ATF3 expression was induced in colon epithelial cells as well as other human intestinal epithelial cell lines such as HCT-116 (colon), INT-407 (embryonic intestine), and HCT-8 (ileocecum) (Fig. 1C). Since lymphoid tissues are also important targets of the ribotoxic stress agents, we also observed the ATF3 induction in the spleen and Peyer's patch after oral gavage with the ribotoxic deoxynivalenol (Fig. 1D). Taken together, ribotoxic chemical agents increased the induction of the ATF3 gene in the intestinal epithelial cells, which was next assessed for its growth-modulating activity.

3.2. *Interference of ATF3 expression triggered the ribotoxic stress-induced apoptosis*

ATF3 has been extensively studied for its diverse role in the growth regulation of cells. To elucidate the relationship between ATF3 expression and cell cycle modulation, genetic suppression using antisense ATF3 was performed in the human intestinal epithelial cells. A mild dose of the ribotoxic stress agent did not change the sub-G1 phase apoptotic pattern and maintained cell survival. However, it caused cell cycle arrest particularly in the G2/M and S phased cells (Fig. 2A). The antisense ATF3-transfected cells were much more susceptible to the ribotoxic stress and demonstrated an increase in cell

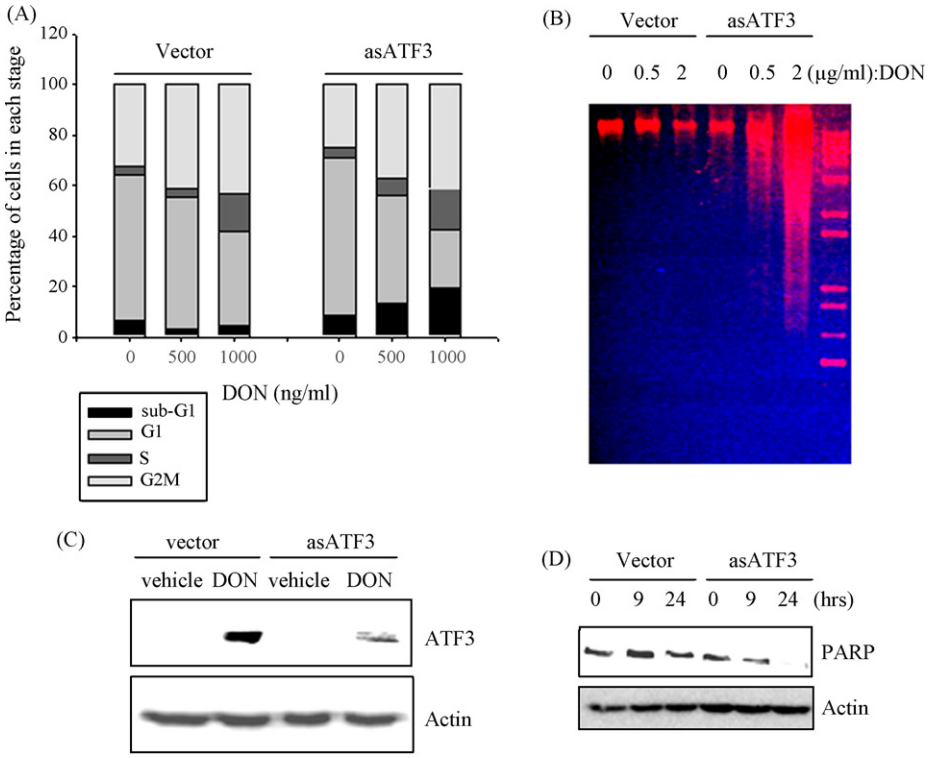

Fig. 2 – Effects of interference of ATF3 expression on cell viability. (A) Control vector- or antisense ATF3 (asATF3) vector-transfected HCT-8 cells were treated with DON for 48 h and stained with propidium iodide to analyze the cell cycle. (B–D) Control vector- or antisense ATF3 (asATF3) vector-transfected HCT-8 cells were treated with DON. Fragmented DNA (B) or protein (C and D) was analyzed. All results are representative of three independent experiments.

death at mild doses of the chemical ribotoxin. The enhanced sub-G1 apoptotic pattern was again confirmed using the apoptotic DNA fragmentation analysis (Fig. 2B). The antisense ATF3 was also shown to suppress the ribotoxin-induced ATF3 production in the epithelial cells (Fig. 2C). Induction of apoptotic DNA fragmentation in ATF3-interfered cells in response to the chemical ribotoxic stress was also assessed by detecting poly (ADP-ribose) polymerase (PARP) degradation (Fig. 2D). Taken in sum, ATF3 genetic abrogation increased the cellular responses of the ribotoxic stress-induced programmed cell death, suggesting protective roles of ATF3 gene induction in the epithelial cellular survival. We next investigated the inner molecular mechanism of ATF3 gene expression in terms of ribotoxic stress-mediated signaling pathways.

3.3. *Messenger RNA and protein stabilization play a critical role in maintaining enhanced levels of ATF3 production*

ATF3 messenger RNA (mRNA) levels were measured in the presence of the ribotoxic agents in a time-dependent manner. Both deoxynivalenol (Fig. 3A) and anisomycin (Fig. 3B) transiently elevated ATF3 mRNA expression in the human epithelial cells. To assess the contribution of ATF3 promoter activity to its gene expression, the ATF3 luciferase reporter assay was performed. The ribotoxic stress agent induced a slight increase in the reporter activity using luciferase vectors including fragments of the ATF3 promoter regions (−2062/+45 or −74/+45) (Fig. 3C). However, the fold increase was relatively smaller than folds of ATF3 mRNA induction. As another aspect of gene regulation, the post-transcriptional regulation of mRNA stability of ATF3 expression in response to the chemical ribotoxic stress was investigated. It was observed that the chemical ribotoxic stress caused by deoxynivalenol enhanced the ATF3 mRNA half life after transcriptional arrest (Fig. 4A). When inhibitors of diverse signaling cascades such as MAP kinase, NF-κB, and PI3 kinase were screened for their effects on the ATF3 mRNA stability, the p38 specific inhibitor SB203580 was found to be the most efficient suppressing agent, suggesting the involvement of p38 MAP kinase in ATF3 mRNA stabilization (Fig. 4A). We constructed constitutively expressing luciferase reporter plasmids tagged with the 3′-untranslated region of human ATF3 gene. Ribotoxic deoxynivalenol enhanced the stability of the reporter activity and p38 MAP kinase inhibition attenuated the elevated luciferase activity (Fig. 4B). When the ATF3 protein level was compared, p38 blocking also suppressed ribotoxin-induced ATF3 protein expression whereas the PI3 kinase inhibition enhanced the ATF3 protein levels (Fig. 4C). Since the PI3 kinase pathway was negatively involved in ATF3 expression in our result, its downstream glycogen synthase kinase 3β was assessed for its involvement in ATF3 induction. Inhibition of GSK3 activity by AR-A014418 suppressed ATF3 protein induction by chemical ribotoxic stress, suggesting positive regulation of ATF3 expression by the GSK3 signaling pathway (Fig. 4D). However, GSK3 was

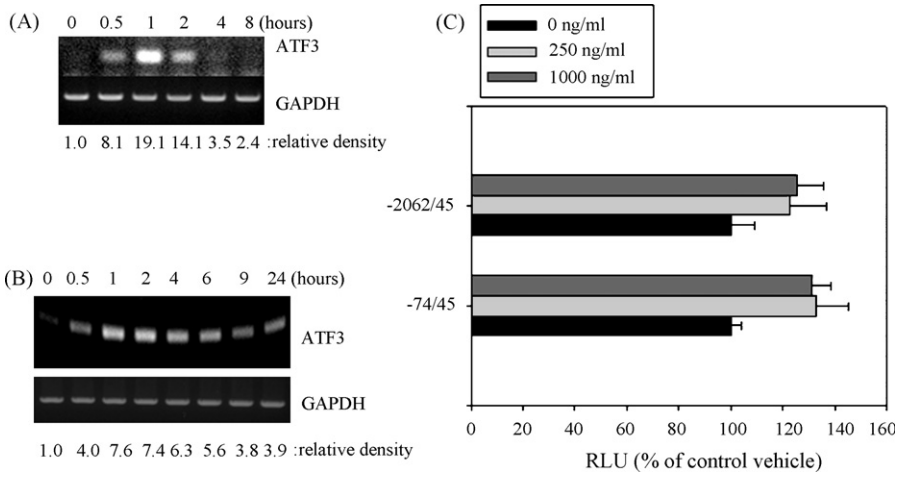

Fig. 3 – Effects of the ribotoxic agent on ATF3 mRNA expression and promoter activity. (A and B) Human epithelial HCT-8 cells were treated with 500 ng/ml DON (A) or 1 μM anisomycin (B) for the indicated time and mRNA was analyzed by RT-PCR. (C) Human epithelial HCT-8 cells were transiently transfected with ATF3 promoter (−2062/+45 bp or −74/+45 bp) luciferase plasmid and then treated with DON for 4 h. The RLU represents the relative luciferase unit of activity. All results are representative of three independent experiments.

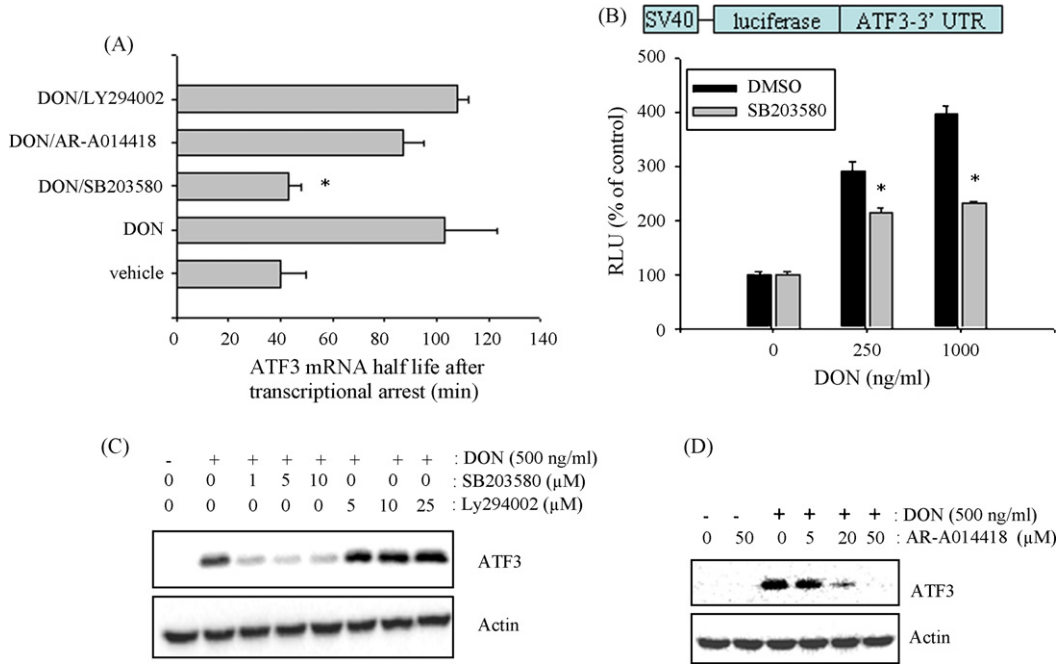

Fig. 4 – Effects of the ribotoxic agent on ATF3 mRNA stability. (A) HCT-8 cells were treated with 500 ng/ml DON for 1 h to reach the maximum level and then transcription was monitored by adding 5 μM actinomycin D in the presence of vehicle or inhibitors (50 μM AR-A014418, 5 μM SB203580, or 10 μM LY294002). The average half lives of ATF3 mRNA after the translational arrest were calculated from three measured experiments using the RT-PCR method. The marker '*' indicates a significant difference from only the DON-treated group ($p < 0.05$). (B) HCT-8 cells were transfected with the constructed luciferase reporter vector plus the pRL-null vector. At 12 h after transfection, cells were pre-treated with DMSO or 5 μM SB203580 for 2 h and then 500 ng/ml DON was administered for 6 h. The marker '*' indicates a significant difference from cells at each dose of DON-treated group in the absence of SB203580 ($p < 0.05$). (C and D) HCT-8 cells were pre-treated with vehicle or each signal-inhibiting chemicals for 2 h and then 500 ng/ml DON was administered for 2 h to measure ATF3 protein levels using Western blot analysis. Results are representative of three independent experiments.

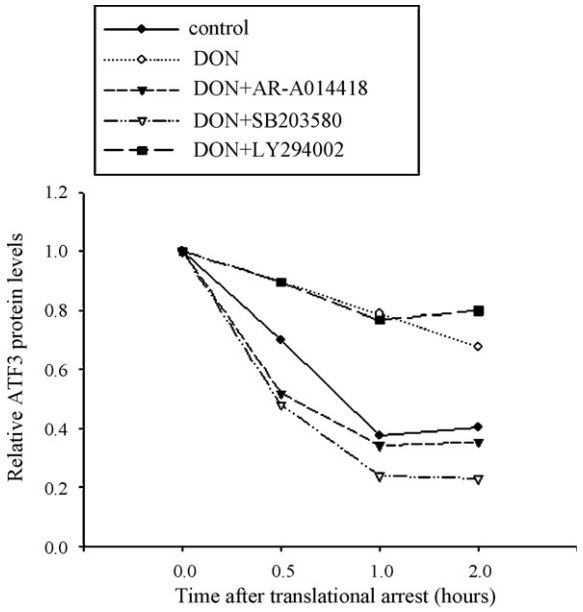

Fig. 5 – Effects of the ribotoxic agent on ATF3 protein stability. HCT-8 cells were treated with 500 ng/ml DON for 2 h to reach the maximum protein level and then translation was measured after adding 50 μM cycloheximide in the presence of vehicle or inhibitors (50 μM AR-A014418, 5 μM SB203580, or 10 μM LY294002). The relative amount of ATF3 protein was calculated from three measured experiments using Western blot analysis. Results are representative of three independent experiments.

little involved in ribotoxin-induced ATF3 mRNA stabilization (data not shown) and we further observed gene expression at the level of post-translation. Effects of signaling inhibitors on ATF3 protein stabilization were measured in the presence of the translational arresting agent cycloheximide D. Chemical ribotoxic stress also enhanced ATF3 protein stability which was reduced by each inhibitor of GSK3 or p38 MAP kinase (Fig. 5). Taken together, p38 MAP kinase and GSK3 pathways are involved in mRNA or protein stabilization by chemical ribotoxic stress in the human epithelial cells.

3.4. *p38 MAP kinase and double-stranded RNA-activated protein kinase (PKR) were the main upstream modulators of ribotoxic stress-induced ATF3 production*

We next addressed the interaction flow between p38 MAP kinase and PI3 kinase cascade. Although p38 MAP kinase inhibitor, SB203580, inhibits the kinase activity of p38, its binding to p38 MAP kinase also suppresses levels of the phosphorylated p38 [24]. The level of kinase-active p38 MAPK was decreased by SB203580 treatment, demonstrating that SB203580 inhibited p38 MAPK (Fig. 6A). Blocking of p38 MAP kinase almost completely suppressed phosphorylation of AKT and GSK3 of PI3 kinase downstream as well as ATF3, suggesting the positive regulation of PI3 kinase cascade by p38 MAP kinase signals in ATF3 induction by ribotoxic stress agent (Fig. 6A). Since ribotoxic stress responses are generally known to be associated with the translational suppression and cellular activation via activated double-stranded RNA (dsRNA)-activated protein kinase (PKR) [25,26], the effects of PKR suppression were assessed for the modulation of ATF3-inducing signaling pathways. Although PKR inhibition by dominant negative PKR induction suppressed ATF3 induction, it did not affect either MAP kinases or PI3 kinase cascades, suggesting new pathways of ATF3 production via the PKR pathway in the human epithelial cells (Fig. 6B). When the ATF3 protein induction kinetics was compared between the control and the dominant negative PKR-expressing cells, the control cells more rapidly induced ATF3 production (Fig. 7A). However, the levels of ATF3 mRNA and the promoter activity were not significantly different in comparisons between the two cell lines of the control and dnPKR-expressing cells (Fig. 7B and C). Moreover, the stabilization of the ATF3 mRNA and protein by

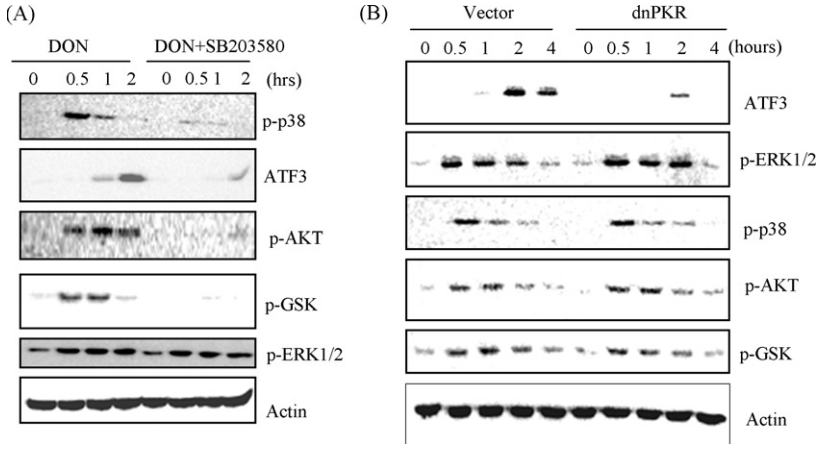

Fig. 6 – Involvement of p38 MAP kinase and PKR in ATF3 induction signaling pathway. (A) Human intestinal epithelial HCT-8 cells were pre-treated with DMSO or 5 μM SB203580 for 2 h and then 500 ng/ml DON was added for the indicated time. The total cellular protein lysate was analyzed using Western blot analysis. (B) Cells stably transfected with the empty vector or dominant negative PKR expression vector (dnPKR) were treated with 500 ng/ml DON for each time and cellular lysate was analyzed. Results are representative of three independent experiments.

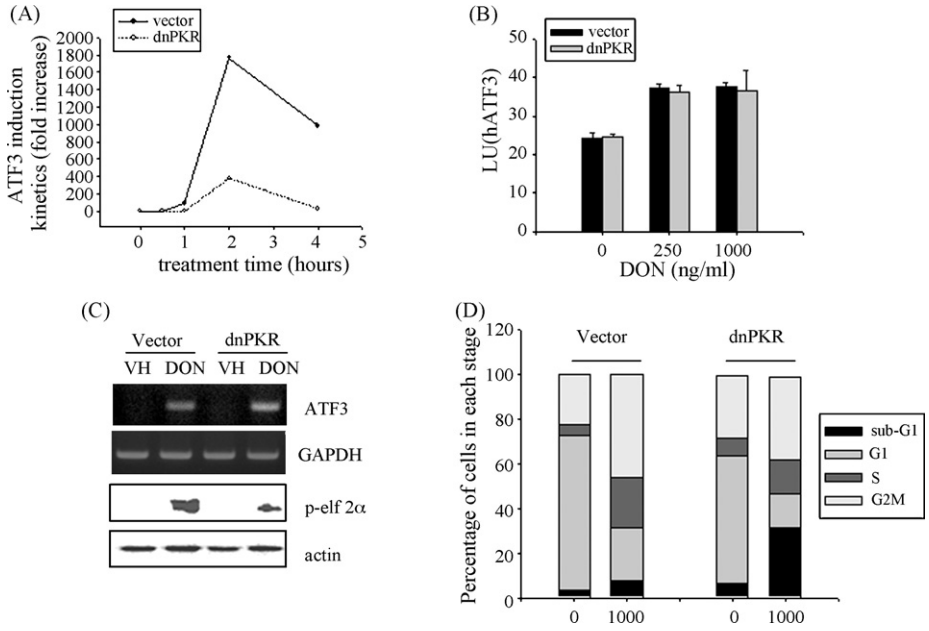

Fig. 7 – Role of ATF3-inducing PKR in the chemical ribotoxin-mediated apoptosis. (A) Cells stably transfected with the empty vector or dominant negative PKR expression vector (dnPKR) were treated with 500 ng/ml DON for each time and cellular protein lysate was analyzed. (B) Cells stably transfected with the empty vector or dnPKR were transfected with ATF3 promoter (−2062/+45) reporter plasmid plus pRL-null and then treated with 500 ng/ml DON for 4 h. (C) Cells stably transfected with the empty vector or dnPKR were treated with 500 ng/ml DON for 2 h or 30 min. Cellular protein or mRNA was analyzed using Western blot analysis or RT-PCR method, respectively. (D) Cells stably transfected with the empty vector or dnPKR were treated with 500 ng/ml DON for 48 h and stained with propidium iodide to analyze the cell cycle. Results are representative of three independent experiments.

the chemical ribotoxin was not influenced by PKR (data not shown). Since ATF3 has been demonstrated to be a critical molecule for the survival of the cells after the ribotoxic stress, the ATF3-regulating PKR was tested for its contribution to the cell survival. It was observed that PKR-interfered cells showed more susceptible response to the cell death by the chemical ribotoxic stress (Fig. 7D). Therefore, translational up-regulation of ATF3 protein production was modulated by PKR, which also was associated with the cell survival after the ribotoxic stress in human intestinal epithelial cells.

4. Discussion

Intestinal epithelial survival depends on the resistance to the mucosal toxic insults from the gut environment. This is the first report that ATF3 plays a critical role in cell survival in the context of death signals from the chemical ribotoxic stress. The present study suggests the decision mechanisms involved in epithelial survival or death in response to the ribosome-inactivating stresses. ATF3 modulation in response to external ribotoxic insults may contribute to gut homeostasis by enhancing cell survival in a stressful mucosal environment. Mucosal epithelial cells triggered the protective ATF3 production which counteracted the ribotoxic stress-triggered apoptotic cell death.

Depending on features of the regulated gene profile, functions of ATF3 could be protective or detrimental in diverse pathogenic environments. ATF3 functions both as an inducer and suppressor of specific gene expression involved in cell growth and apoptosis [7,27,28]. Therefore, the modulated targets of the ATF3 transcription factor need to be addressed. Some reports have suggested that ATF3 has apoptosis-promoting effects but it can also protect cells from the physical and chemical cytotoxic stresses by suppressing the induction of cell death factors such as p53 proteins [29]. ATF3-suppressed cells show enhanced level of p53 that probably makes the epithelial cells more sensitive to the cytotoxic effects. In an unpublished study from our laboratory, the p53 protein was also assessed to determine whether ATF3 can function together with it in response to the ribotoxic stress. p53 is known as a survival-death checker. Suppression of ATF3 induction enhanced p53 expression which might make cells more sensitive to the death signals from the ribotoxic stress agent in the epithelial cells. In response to the injuries, ATF3 up-regulates a survival factor, the heat shock protein HSP27 as well as the c-Jun/Fos protein without which cells will undergo a retarded regeneration after injury [20].

Levels of ATF3 were enhanced by the ribotoxic stress and its triggered signaling pathways including p38 MAP kinase, glycogen synthase kinase 3β, and double-stranded RNA-activated protein kinase (PKR). These signaling networks characterize the pattern of ATF3 up-regulation in response to the ribotoxic insults in the mucosal epithelia. The contributions of each signaling pathway to the ATF3 regulation will be discussed one by one. Stimulation of ATF3

induction was mediated by the p38 MAP kinase signaling pathway which enhanced the stability of ATF3 mRNA. The mRNA stability of a specific gene is modulated by many factors. Among these, AU-rich elements (AREs), located in the 3′-untranslated region of unstable early inducible genes, such as cytokines or chemokines, promote rapid decay of mRNA and may mediate selective mRNA stabilization in response to mitogenic or pro-inflammatory factors. The chemical ribotoxic agent elevated the ATF3 mRNA stability and three AU-rich element-containing 3′-untranslated region was also an important determinant of ATF3 mRNA stabilization. Moreover, the mRNA stabilization was mediated by the p38 MAP kinase pathway. Therefore, ribotoxic stress-mediated p38 MAP kinase activation can affect the *cis*-activation of the AU-rich element of ATF3 gene. In addition, transcription activation of ATF3 can contributes to the enhanced ATF3 production in response to the ribotoxic stress. Recent report demonstrated that anisomycin induced transcriptional activation of ATF3 which was also regulated by p38 MAP kinases [30]. However, deoxynivalenol, a representative ribotoxin used in our experiment, caused a slight increase in ATF3 transcription which was only a minor contributor to ATF3 induction. Thus more careful observation is required to provide a generalized mechanism of ATF3 induction by ribotoxic stresses. In addition to the gene regulatory effects, p38 also has been associated with cell survival and regeneration [31,32]. The enhanced survival chance could be attributed to the p38 MAP kinase-mediated suppression of the death factor or induction of regenerating factors other than ATF3.

The chemical ribotoxic agent also activated the PI3 kinase signaling cascade which was also involved in otherwise ATF3 protein stabilization via GSK3β. Active GSK3β has been demonstrated to modulate protein turnover by phosphorylation and subsequent ubiquitin-dependent proteasomal degradation of specific proteins such as beta-catenin and Smad proteins [33–35]. GSK3β may reduce or increase the protein stability depending on the complex binding interaction or the modification patterns [36,37]. Activated GSK3β protein was shown to up-regulate ATF3 protein stability in the present study. Moreover, further investigation is needed to determine how the activated GSK3β mediates ATF3 induction in response to the chemical ribotoxic agent. In our study, p38 MAP kinase pathway was shown to modulate PI3 kinase/AKT/GSK3β pathway, whose relationship was also described in the previous reports [38–40]. Mechanistically, AKT translocation from cytoplasm to plasma membrane is regulated by PI3 kinase-mediated p38 kinase activity in intact cells. Moreover, AKT is activated by forming a stable complex with p38 MAP kinase. In terms of pharmacological regulation, pyridinyl imidazole inhibitors, particularly SB203580, inhibited phosphorylation of AKT as well as P38 MAP kinase. The concentrations of SB203580 required to block AKT phosphorylation (IC_{50} 3–5 μM) are only approximately 10-fold higher than those required to inhibit p38 MAP kinase (IC_{50} 0.3–0.5 μM) [38].

As another critical signaling modulator for ATF3 induction, double-stranded RNA-activated protein kinase (PKR) was also found to be involved in the survival response, in the presence of the ribotoxic stress agent. PKR is a serine/threonine protein kinase ubiquitously expressed in mammalian cells [41,42]. PKR was initially identified as an interferon-induced protein that is activated in virus-infected cells by dsRNA and produced during the virus life cycle. PKR is a component of signal transduction pathways mediating many important cellular functions, such as survival, proliferation, differentiation, apoptosis, and stress responses [43,44]. In addition to dsRNA, PKR can be activated by cytokines, growth factors, serum deprivation, bacterial products, or physiochemical stress [41,42,44]. Several reports have demonstrated that the chemical ribotoxic stresses can also activate PKR and subsequent pathogenic processes [45–47]. In our study, PKR played a critical role in cell survival from the ribotoxic stress responses. Although PKR activation generally phosphorylates eIF2a and subsequently induces apoptotic pathway, PKR also can enhance cell survival by delaying the late programmed cell death [48]. PKR first triggers the NF-κB survival responses by binding to the IKKβ independent of its kinase or dsRNA-binding activities. Activated NF-κB induces survival- or antiapoptosis-associated gene products such as caspase inhibitor c-IAPs. When the PKR induction was blocked by dominant negative expression in our study, the ribotoxic

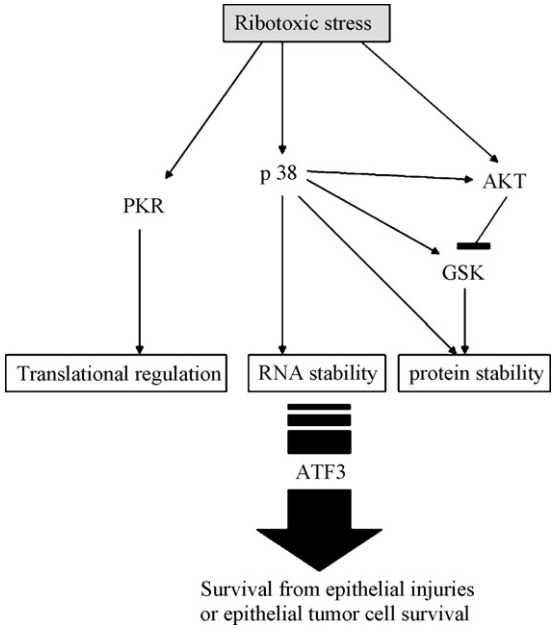

Fig. 8 – A putative mechanism of ribotoxic stress agent-mediated ATF3 induction and its role in the intestinal epithelial cell death. The schematic signaling patterns illustrate that chemical ribotoxic stress-induced ATF3 protein is modulated at diverse levels of gene expression including transcription, mRNA stability, translational activity, and protein stability. Ribotoxic agents activate p38 MAP kinase signals and PI3 kinase signaling cascade. p38 MAP kinase can increase ATF3 mRNA stability as well as protein stability. However, AKT signals in association with PI3 kinase had a negative regulatory effect by suppressing p38-mediated GSK3β activation, which otherwise enhances ATF3 protein stability. Up-regulated ATF3 expression may be involved in the epithelial cell survival after the ribotoxic insult in the human gut mucosa. This survival process can promote epithelial tumor cell survival when exposed to the anti-cancer ribotoxic agents.

agent promoted the cell death responses at the early exposure time in the epithelial cells. The protective function of PKR requires further investigation to determine the downstream targets such as survival factors in response to the ribotoxic insults in the gut mucosal environment.

Although the survival-promoting effect of ATF3 gene regulation can provide some resistance to cytotoxicity by the ribotoxic stress agents in the normal gut epithelium, these events can be harmful during the epithelial cancer progression. Enhanced survival induced by ATF3 may facilitate the tumor cells to pass through the cytotoxic environment and provide a growth advantage over the normal cells. ATF3 increases the expression of several genes implicated in the malignant tumor metastasis such as TWIST1, FN-1, Snail, and Slug genes [7]. In particular, the ATF3 gene copy number and protein levels are significantly higher in the malignant breast tumors [7]. However, some ribotoxic agents such as anisomycin, flavopiridol, and trichostatin are currently being investigated for the suppression of the tumor cell growth and the application in the clinical setting [11,49]. Like the chemical ribotoxic agents in our study, ATF3 genes are induced by agents of anti-tumoric activities such as NSAID, anti-inflammatory polyphenol curcumin and green tea catechins. Therefore, the oncogenic activity of ATF3 implicates the potential adverse effects of these anti-tumor agents on the tumor chemotherapy. In the further study, it is also warranted to address the toxicological safety issues of the ribotoxic agents and ATF3-modulating chemicals for the cancer treatment, despite of positive roles of ATF3 in the injured epithelial tissues as depicted in Fig. 8.

Acknowledgement

This work was supported by grant no. (R01-2006-000-10564-0) from the Basic Research Program of the Korea Science & Engineering Foundation.

REFERENCES

[1] Laskin JD, Heck DE, Laskin DL. The ribotoxic stress response as a potential mechanism for MAP kinase activation in xenobiotic toxicity. Toxicol Sci 2002;69(2):289–91.
[2] Shifrin VI, Anderson P. Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. J Biol Chem 1999;274(20):13985–92.
[3] Iordanov MS, Pribnow D, Magun JL, Dinh TH, Pearson JA, Chen SL, et al. Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. Mol Cell Biol 1997;17(6):3373–81.
[4] Iordanov MS, Pribnow D, Magun JL, Dinh TH, Pearson JA, Magun BE. Ultraviolet radiation triggers the ribotoxic stress response in mammalian cells. J Biol Chem 1998;273(25):15794–803.
[5] Liang G, Wolfgang CD, Chen BP, Chen TH, Hai T. ATF3 gene. Genomic organization, promoter, and regulation. J Biol Chem 1996;271(3):1695–701.
[6] Jiang HY, Wek SA, McGrath BC, Lu D, Hai T, Harding HP, et al. Activating transcription factor 3 is integral to the eukaryotic initiation factor 2 kinase stress response. Mol Cell Biol 2004;24(3):1365–77.
[7] Yin X, Dewille JW, Hai T. A potential dichotomous role of ATF3, an adaptive-response gene, in cancer development. Oncogene 2008;27(15):2118–27.
[8] Duggan SP, Gallagher WM, Fox EJ, Abdel-Latif MM, Reynolds JV, Kelleher D. Low pH induces co-ordinate regulation of gene expression in oesophageal cells. Carcinogenesis 2006;27(2):319–27.
[9] Harper EG, Alvares SM, Carter WG. Wounding activates p38 map kinase and activation transcription factor 3 in leading keratinocytes. J Cell Sci 2005;118(Pt 15):3471–85.
[10] Brigotti M, Carnicelli D, Ravanelli E, Vara AG, Martinelli C, Alfieri RR, et al. Molecular damage and induction of proinflammatory cytokines in human endothelial cells exposed to Shiga toxin 1, Shiga toxin 2, and alpha-sarcin. Infect Immun 2007;75(5):2201–7.
[11] Xia S, Li Y, Rosen EM, Laterra J. Ribotoxic stress sensitizes glioblastoma cells to death receptor induced apoptosis: requirements for c-Jun NH_2-terminal kinase and Bim. Mol Cancer Res 2007;5(8):783–92.
[12] Bhat RV, Beedu SR, Ramakrishna Y, Munshi KL. Outbreak of trichothecene mycotoxicosis associated with consumption of mould-damaged wheat production in Kashmir Valley, India. Lancet 1989;1(8628):35–7.
[13] Pestka JJ, Smolinski AT. Deoxynivalenol: toxicology and potential effects on humans. J Toxicol Environ Health B Crit Rev 2005;8(1):39–69.
[14] Li M, Harkema JR, Cuff CF, Pestka JJ. Deoxynivalenol exacerbates viral bronchopneumonia induced by respiratory reovirus infection. Toxicol Sci 2007;95(2):412–26.
[15] Maresca M, Mahfoud R, Garmy N, Fantini J. The mycotoxin deoxynivalenol affects nutrient absorption in human intestinal epithelial cells. J Nutr 2002;132(9):2723–31.
[16] Sergent T, Parys M, Garsou S, Pussemier L, Schneider YJ, Larondelle Y. Deoxynivalenol transport across human intestinal Caco-2 cells and its effects on cellular metabolism at realistic intestinal concentrations. Toxicol Lett 2006;164(2):167–76.
[17] Yang H, Chung DH, Kim YB, Choi YH, Moon Y. Ribotoxic mycotoxin deoxynivalenol induces G2/M cell cycle arrest via p21Cip/WAF1 mRNA stabilization in human epithelial cells. Toxicology 2008;243(1–2):145–54.
[18] Reimertz C, Kogel D, Rami A, Chittenden T, Prehn JH. Gene expression during ER stress-induced apoptosis in neurons: induction of the BH3-only protein Bbc3/PUMA and activation of the mitochondrial apoptosis pathway. J Cell Biol 2003;162(4):587–97.
[19] Zhang F, Hamanaka RB, Bobrovnikova-Marjon E, Gordan JD, Dai MS, Lu H, et al. Ribosomal stress couples the unfolded protein response to p53-dependent cell cycle arrest. J Biol Chem 2006;281(40):30036–45.
[20] Seijffers R, Mills CD, Woolf CJ. ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. J Neurosci 2007;27(30):7911–20.
[21] Yoshida T, Sugiura H, Mitobe M, Tsuchiya K, Shirota S, Nishimura S, et al. ATF3 protects against renal ischemia-reperfusion injury. J Am Soc Nephrol 2008;19(2):217–24.
[22] Smith WE, Kane AV, Campbell ST, Acheson DW, Cochran BH, Thorpe CM. Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. Infect Immun 2003;71(3):1497–504.
[23] Wang L, Li T, Lu L. UV-induced corneal epithelial cell death by activation of potassium channels. Invest Ophthalmol Vis Sci 2003;44(12):5095–101.
[24] Lin Z, Crockett DK, Jenson SD, Lim MS, Elenitoba-Johnson KS. Quantitative proteomic and transcriptional analysis of the response to the p38 mitogen-activated protein kinase

inhibitor SB203580 in transformed follicular lymphoma cells. Mol Cell Proteomics 2004;3(8):820–33.
[25] Iordanov MS, Paranjape JM, Zhou A, Wong J, Williams BR, Meurs EF, et al. Activation of p38 mitogen-activated protein kinase and c-Jun NH(2)-terminal kinase by double-stranded RNA and encephalomyocarditis virus: involvement of RNase L, protein kinase R, and alternative pathways. Mol Cell Biol 2000;20(2):617–27.
[26] Iordanov MS, Wong J, Bell JC, Magun BE. Activation of NF-kappaB by double-stranded RNA (dsRNA) in the absence of protein kinase R and RNase L demonstrates the existence of two separate dsRNA-triggered antiviral programs. Mol Cell Biol 2001;21(1):61–72.
[27] Lu D, Wolfgang CD, Hai T. Activating transcription factor 3, a stress-inducible gene, suppresses Ras-stimulated tumorigenesis. J Biol Chem 2006;281(15):10473–81.
[28] Yan C, Lu D, Hai T, Boyd DD. Activating transcription factor 3, a stress sensor, activates p53 by blocking its ubiquitination. Embo J 2005;24(13):2425–35.
[29] Kawauchi J, Zhang C, Nobori K, Hashimoto Y, Adachi MT, Noda A, et al. Transcriptional repressor activating transcription factor 3 protects human umbilical vein endothelial cells from tumor necrosis factor-alpha-induced apoptosis through down-regulation of p53 transcription. J Biol Chem 2002;277(41):39025–34.
[30] Lu D, Chen J, Hai T. The regulation of ATF3 gene expression by mitogen-activated protein kinases. Biochem J 2007;401(2):559–67.
[31] Alvarado-Kristensson M, Melander F, Leandersson K, Ronnstrand L, Wernstedt C, Andersson T. p38-MAPK signals survival by phosphorylation of caspase-8 and caspase-3 in human neutrophils. J Exp Med 2004;199(4):449–58.
[32] Colombara M, Antonini V, Riviera AP, Mainiero F, Strippoli R, Merola M, et al. Constitutive activation of p38 and ERK1/2 MAPKs in epithelial cells of myasthenic thymus leads to IL-6 and RANTES overexpression: effects on survival and migration of peripheral T and B cells. J Immunol 2005;175(10):7021–8.
[33] Guo X, Ramirez A, Waddell DS, Li Z, Liu X, Wang XF. Axin and GSK3-control Smad3 protein stability and modulate TGF-signaling. Genes Dev 2008;22(1):106–20.
[34] Ha NC, Tonozuka T, Stamos JL, Choi HJ, Weis WI. Mechanism of phosphorylation-dependent binding of APC to beta-catenin and its role in beta-catenin degradation. Mol Cell 2004;15(4):511–21.
[35] Topol L, Jiang X, Choi H, Garrett-Beal L, Carolan PJ, Yang Y. Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation. J Cell Biol 2003;162(5):899–908.
[36] Doble BW, Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci 2003;116(Pt 7):1175–86.
[37] Kockeritz L, Doble B, Patel S, Woodgett JR. Glycogen synthase kinase-3—an overview of an over-achieving protein kinase. Curr Drug Targets 2006;7(11):1377–88.
[38] Lali FV, Hunt AE, Turner SJ, Foxwell BM. The pyridinyl imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation, and retinoblastoma hyperphosphorylation in interleukin-2-stimulated T cells independently of p38 mitogen-activated protein kinase. J Biol Chem 2000;275(10):7395–402.
[39] Park CM, Park MJ, Kwak HJ, Lee HC, Kim MS, Lee SH, et al. Ionizing radiation enhances matrix metalloproteinase-2 secretion and invasion of glioma cells through Src/epidermal growth factor receptor-mediated p38/Akt and phosphatidylinositol 3-kinase/Akt signaling pathways. Cancer Res 2006;66(17):8511–9.
[40] Rane MJ, Coxon PY, Powell DW, Webster R, Klein JB, Pierce W, et al. p38 kinase-dependent MAPKAPK-2 activation functions as 3-phosphoinositide-dependent kinase-2 for Akt in human neutrophils. J Biol Chem 2001;276(5):3517–23.
[41] Garcia MA, Gil J, Ventoso I, Guerra S, Domingo E, Rivas C, et al. Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. Microbiol Mol Biol Rev 2006;70(4):1032–60.
[42] Williams BR. Signal integration via PKR. Sci STKE 2001;2001(89):RE2.
[43] Barber GN. The dsRNA-dependent protein kinase, PKR and cell death. Cell Death Differ 2005;12(6):563–70.
[44] Williams BR. PKR; a sentinel kinase for cellular stress. Oncogene 1999;18(45):6112–20.
[45] Dewitte-Orr SJ, Zorzitto JR, Sutton LP, Bols NC. Preferential induction of apoptosis in the rainbow trout macrophage cell line, RTS11, by actinomycin D, cycloheximide and double stranded RNA. Fish Shellfish Immunol 2005;18(4):279–95.
[46] Gray JS, Bae HK, Li JC, Lau AS, Pestka JJ. Double-stranded RNA-activated protein kinase mediates induction of interleukin-8 expression by deoxynivalenol, Shiga toxin 1, and ricin in monocytes. Toxicol Sci 2008;105(2):322–30.
[47] Zhou HR, Lau AS, Pestka JJ. Role of double-stranded RNA-activated protein kinase R (PKR) in deoxynivalenol-induced ribotoxic stress response. Toxicol Sci 2003;74(2):335–44.
[48] Donze O, Deng J, Curran J, Sladek R, Picard D, Sonenberg N. The protein kinase PKR: a molecular clock that sequentially activates survival and death programs. EMBO J 2004;23(3):564–71.
[49] Ruller S, Stahl C, Kohler G, Eickhoff B, Breder J, Schlaak M, et al. Sensitization of tumor cells to ribotoxic stress-induced apoptotic cell death: a new therapeutic strategy. Clin Cancer Res 1999;5(10):2714–25.