ER-Dependent Ca^{++}-mediated Cytosolic ROS as an Effector for Induction of Mitochondrial Apoptotic and ATM-JNK Signal Pathways in Gallic Acid-treated Human Oral Cancer Cells

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Abstract. *Release of calcium (Ca^{++}) from the endoplasmic reticulum (ER) has been proposed to be involved in induction of apoptosis by oxidative stress. Using inhibitor of ER Ca^{++} release dantrolene and inhibitor of mitochondrial Ca^{++} uptake Ru-360, we demonstrated that Ca^{++} release from the ER was associated with generation of reactive oxygen species (ROS), loss of mitochondrial membrane potential, and apoptosis of human oral cancer (OC) cells induced by gallic acid (GA). Small interfering RNA-mediated suppression of protein kinase RNA-like endoplasmic reticulum kinase inhibited tunicamycin-induced induction of 78 kDa glucose-regulated protein, C/EBP homologous protein, pro-caspase-12 cleavage, cytosolic Ca^{++} increase and apoptosis, but did not attenuate the increase in cytosolic Ca^{++} level and apoptosis induced by GA. Ataxia telangiectasia mutated (ATM)-mediated c-Jun N-terminal kinase (JNK) phosphorylation and apoptosis by GA was blocked by dantrolene. The specificity of ROS-mediated ATM-JNK activation was confirmed by treatment with N-acetylcysteine, a ROS scavenger. Blockade of ATM activation by specific inhibitor KU55933, short hairpin RNA, or kinase-dead ATM overexpression suppressed JNK phosphorylation but did not completely inhibit cytosolic ROS production, mitochondrial cytochrome c release, pro-caspase-3 cleavage, and apoptosis induced by GA. Taken together, these results indicate that GA induces OC cell apoptosis by inducing the activation of mitochondrial apoptotic and ATM-JNK signal pathways, likely through ER Ca^{++}-mediated ROS production.*

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The endoplasmic reticulum (ER) is a central organelle for the modulation of cytosolic Ca^{++} concentration and cell survival. Apoptotic stimuli known to act on cytosolic release of ER Ca^{++} induce Ca^{++} influx from the cytosol into the mitochondrial matrix. An increase in the Ca^{++} concentration within the mitochondrial matrix causes permeabilization of the mitochondrial membrane, which leads to release of pro-apoptotic factors from the intermembrane and intercristae spaces, such as cytochrome *c*, apoptosis-inducing factor, endonuclease G, and generation of reactive oxygen species (ROS) (1-3). Experimental evidence indicates that genotoxic stress-induced mitochondrial production of ROS is correlated with activation of ataxia telangiectasia mutated (ATM), a member of the phosphatidylinositol 3-kinase (PI3K)-like family involved in the regulation of cell-cycle progression and apoptosis (4, 5). In fact, high-level ROS activated-ATM triggers apoptosis of chemoresistant cancer cells by phosphorylating its downstream effector c-Jun *N*-terminal kinase (JNK) in response to genotoxic drug cisplatin (6). The silencing of antioxidant superoxide dismutase sensitizes human oral cancer (OC) cells to ROS-induced apoptosis through a nuclear factor-κB-dependent pathway (7). Immunohistochemical analysis of ATM protein level in paraffin-embedded section of human OCs indicated that absence or reduced ATM expression was significantly associated with lymph node metastasis and poor prognosis (8). Deficiency in ATM-dependent signaling was shown to correlate with chemoresistance to cisplatin in human OC cells (9). Mice lacking ATM exhibit high levels of oxidative damage and high incidence of lymphoma (10), suggesting that ROS-regulated ATM activity plays a critical role in

modulating oral cancer tumorigenesis. Thus, a potential agent in inducing OC cells toward ER Ca^{++}-dependent ROS-mediated ATM activation would be desirable.

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is a naturally-occurring phenolic compound that is found in grapes, tea, and berries (11-13). It has been pre-clinically documented to possess a variety of pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticancer activities (14-16). Incubation of human cancer cell lines (*e.g.* colon adenocarcinoma, hepatocellular carcinoma, leukemia, lung, prostate carcinoma, and stomach cancer cells) with GA was shown to selectively induce apoptosis (17-19). In addition to the finding that GA potently induced apoptosis of human prostate carcinoma cell line through the induction of ATM activity (19), a link between GA and ATM in OC cells has emerged in a recent study suggesting that the effect of GA on apoptosis takes place through an ATM signaling pathway (20). However, it is not clear how GA treatment relates to ATM activation. In this study, we investigated the molecular mechanism by which GA induces apoptotic effects on human OC SCC-4 cells and how these effects are modulated by ER Ca^{++} and ROS in ATM activation.

Materials and Methods

Cell culture. The human oral cancer SCC-4 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cell line was cultured routinely in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (both from Gibco BRL, Grand Island, NY, USA) and grown in 10-cm tissue culture dish at 37˚C in a humidified incubator containing 5% CO_2.

Chemicals, reagents and plasmids. Crystal violet, dantrolene, GA, 2-(4-morpholinyl)-6-(1-thianthrenyl)-4*H*-pyran-4-one (KU55933), *N*-acetyl-L-cysteine (NAC), propidium iodide (PI), Tris-HCl, Triton X-100, and tunicamycin were obtained from Sigma-Aldrich (St. Louis, MO, USA). GA was dissolved in and diluted with methanol (21), and then stored at –20˚C as a 100 mM stock solution. Methanol and potassium phosphate were purchased from Merck (Darmstadt, Germany). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA). FBS, trypsin-EDTA, and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). Caspase-3 activity assay kit was purchased from OncoImmunin (Gaithersburg, MD, USA). pcDNA3.1(+) FLAG-wild type (wt) ataxia telangiectasia mutated (ATM), pcDNA3.1(+) FLAG-kinase-dead (kd) ATM, and pATM short hairpin (sh) RNA vectors were obtained from Addgene (Cambridge, MA, USA). Western blot luminol reagent and Ru-360 (μ-oxo) *bis* (*trans*-formatotetramine ruthenium) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Antibodies. An antibody against JNK was purchased from BD PharMingen (San Diego, CA, USA). Antibodies to phospho (p)-JNK (Thr 183/Tyr 185), 78 kDa glucose-regulated protein (GRP78), and C/enhancer-binding protein homologous protein (CHOP) were provided by Santa Cruz Biotechnology. Antibodies against caspase-3 and caspase-12 were obtained from Calbiochem (San Diego, CA, USA). Anti-ATM, p-ATM (Ser 1981), cytochrome *c* oxidase subunit II (COX2), cytochrome *c*, protein kinase RNA-like endoplasmic reticulum kinase (PERK), and p-PERK (Tyr 980) were obtained from Abcam (Cambridge, MA, USA). Antibodies against β-actin and FLAG-tag were obtained from Sigma-Aldrich. Peroxidase-conjugated anti-mouse IgG, -goat IgG, and -rabbit IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA, USA).

Plasmid transfection. Cells (at 60-70% confluence in a 12-well plate) were transfected with the FLAG-wt ATM, FLAG-kd ATM, or *ATM* shRNA expression plasmid using Lipofectamine 2000. The expression of FLAG-wt ATM, FLAG-kd ATM, and ATM in transfected cells was assessed by western blotting using antibodies specific to FLAG and ATM.

Cell proliferation assay. Cell viability was assessed by fluorescence-activated cell sorting (FACS) analysis of cellular PI uptake (22). The cells were seeded at 3×10^4 cells/well in 24-well tissue culture plates. The cells were grown for overnight to about 60% confluence and treated with either methanol as the vehicle control or GA (300 μM) for 36 h. For the vehicle control, methanol was diluted in culture medium to the same final concentration of methanol [0.01% (vol/vol)] as that in the medium with GA. At the end of the incubation, treated cells were harvested and stained with 10 μg/ml of PI solution. The stained cells were analyzed using a FACSCount flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and the results were analyzed using CellQuest software (BD Biosciences).

Assays for the detection of caspase-3 activity. Caspase-3 activity was measured using the PhiPhiLux G1D2 kit (OncoImmunin, College Park, MD, USA) according to the manufacturer's protocols. The treated cells were incubated with the PhiPhiLux fluorogenic Caspase substrate at 37˚C for 1 h and were then analyzed using a FACSCount flow cytometer (23).

Measurement of cytosolic Ca^{++}. The Ca^{++} level was determined by measuring the retention of indo-1 acetomethoxy (Indo-1/AM) (Invitrogen, Carlsbad, CA, USA). Briefly, the treated cells were incubated with 3 μg/ml Indo-1/AM for 30 min at 37˚C. The cells were then pelleted by centrifugation at 160 × *g*. The pellets were resuspended and washed twice with PBS. The level of Ca^{++} was evaluated as previously described (23).

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential (ψm) was determined by measuring the retention of the dye 3,3'-dihexyloxacarbocyanine ($DiOC_6$). Briefly, treated cells were incubated with 40 nM $DiOC_6$ for 30 min at 37˚C. Cells were then pelleted by centrifugation at 160 × *g*. Pellets were resuspended and washed twice with PBS. The $\Delta\psi_m$ was determined with a FACSCount flow cytometer (24).

Detection of ROS. Briefly, treated cells were then resuspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (10 μM) and incubated for 30 min at 37˚C. The level of ROS was determined using a FACSCount flow cytometer (24).

Measurement of DNA fragmentation. Histone-associated DNA fragments were determined using the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science, Mannheim, Germany). Briefly, vehicle- or GA-treated cells were

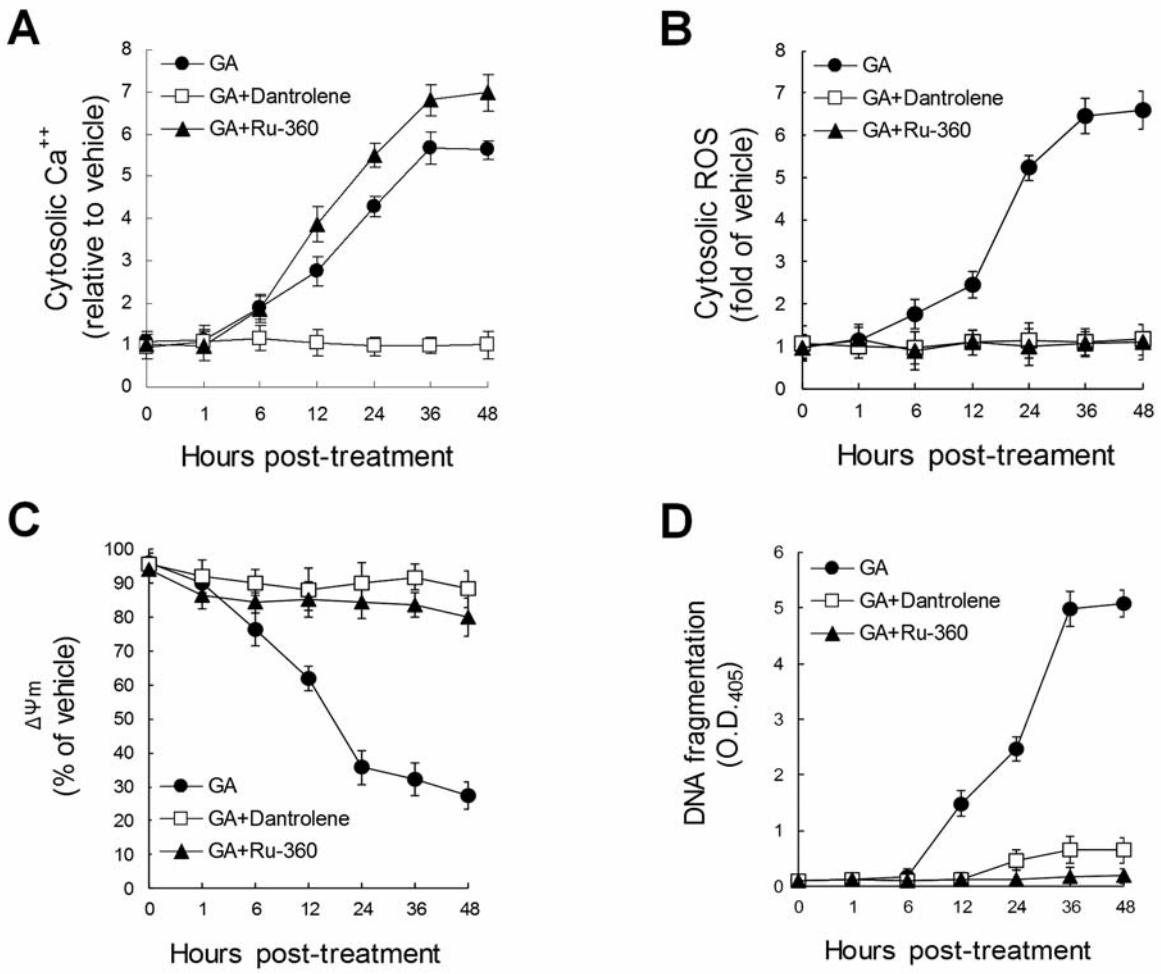

Figure 1. *Requirement of endoplasmic reticulum calcium (Ca^{++}) release for gallic acid (GA)-induced mitochondrial dysfunction, cytosolic reactive oxygen species (ROS) production, and apoptosis. Cells were treated with either vehicle, GA, GA plus dantrolene (25 μM), or GA plus Ru-360 (0.5 μM) for 36 h. The decrease in 3,3'-dihexyloxacarbocyanine fluorescence was measured by flow cytometry. The generation of cytosolic Ca^{++} and ROS was monitored by measuring increased fluorescence of Indo-1 and 2,7-dichlorodihydrofluorescein by flow cytometry. DNA fragmentation was determined using a Cell Death Detection ELISA kit. The values presented are the mean±standard errors from three independent experiments.*

incubated in a hypertonic buffer for 30 min at room temperature. After centrifugation, the cell lysates were transferred into an anti-histone-coated microplate to bind histone-associated DNA fragments. Plates were washed after 1.5 h of incubation, and nonspecific binding sites were saturated with blocking buffer. Plates were then incubated with peroxidase-conjugated anti-DNA for 1.5 h at room temperature. To determine the amount of retained peroxidase, 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) was added as a substrate, and a spectrophotometer (Thermo Labsystems Multiskan Spectrum, Frankin, MA, USA) was used to measure the absorbance at 405 nm (24).

Western blot analysis. Treated or transfected cells were lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1 μg/ml aprotinin, 100 mM Na_3VO_4, 50 mM NaF, 0.5% NP-40). Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA, USA). Proteins were separated by electrophoresis on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gel and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). Membranes were blocked overnight with phosphate-buffered saline (PBS) containing 3% skim milk and then incubated with primary antibody against ATM, p-ATM (Ser 1981), caspase-3, caspase-12, CHOP, COX2, cytochrome *c*, GRP78, JNK, or p-JNK. Proteins were detected with horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, or donkey anti-goat antibodies and Western Blotting Luminol Reagent. To confirm equal protein loading, β-actin was measured.

Detection of cytochrome c. Subcellular fractionation was performed as described previously (25). The treated cells were washed twice with ice-cold PBS and scraped into a 200 mM sucrose solution containing 25 mM HEPES (pH 7.5), 10 mM KCl, 15 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 μg/ml aprotinin. The cells were

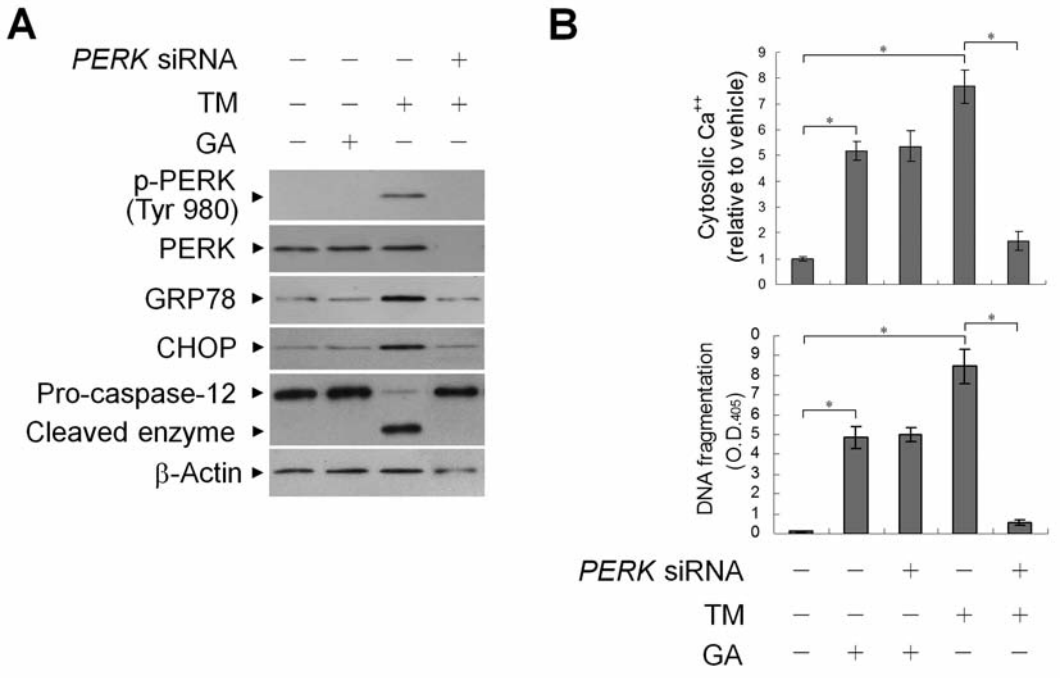

Figure 2. *Endoplasmic reticulum (ER) stress response is not responsible for gallic acid (GA)-induced calcium (Ca^{++}) efflux from the ER. At 12 h after transfection with control or protein kinase RNA-like endoplasmic reticulum kinase (PERK) siRNA, the cells were treated with either vehicle, GA, or tunicomycin (TM) (1.0 μM) for an additional 36 h. The levels of the indicated proteins in the total lysates were determined by western blot analysis using specific antibodies. β-Actin was used as an internal loading control. The generation of cytosolic Ca^{++} was monitored by measuring increased fluorescence of Indo-1M by flow cytometry. DNA fragmentation was determined using a Cell Death Detection ELISA kit. The values presented are the mean±standard errors from three independent experiments. *Significantly different at $p<0.05$. ATM, Ataxia telangiectasia mutated; JNK, c-Jun N-terminal kinase; COX2, Cytochrome c oxidase subunit II; Cyt c, cytochrome c.*

disrupted by passage through a 26-gauge hypodermic needle 30 times and then subjected to centrifugation for 10 min in an Eppendorf microcentrifuge (5804R) at 750 × *g* and 4˚C to remove unlysed cells and nuclei. The supernatant was collected and then subjected to centrifugation for 20 min at 10,000 × *g* and 4˚C to form a new supernatant and pellet. The resulting pellet was saved as the mitochondrial (Mt) fraction, and the supernatant was further centrifuged at 100,000 × *g* for 1 h at 4˚C. The new supernatant was saved as the cytosolic (Cs) fraction, and the pellet was reserved as the ER/microsomal (Ms) fraction. The resulting Mt fraction was lysed in RIPA buffer (1% sodium deoxycholine, 0.1% SDS, 1% Triton X-100, 10 mM Tris-HCl (pH 8.0), and 0.14 M NaCl) for western blot analysis. The purity of each subcellular fraction was confirmed by western blotting using specific antibodies against the mitochondrial marker COX2 and the Cs marker β-actin (26).

Statistical analysis of data. Statistical calculations of the data were performed using the unpaired Student's *t*-test and ANOVA analysis. A value of $p<0.05$ was considered statistically significant.

Results

Apoptosis induced by GA is dependent on ER Ca^{++} depletion. Previous reports have shown that GA is capable of inducing the production of ROS and activating the mitochondria-death signal pathway (27, 28). Weng *et al*. showed that the half-maximal inhibitory concentration (IC_{50}) of GA against the human OC SCC-4 cell line was 300 μM (20). To investigate whether GA-induced ROS release and mitochondrial cell death are ER Ca^{++}-sensitive, 300 μM GA were used to treat this OC cell line in all subsequent experiments. The levels of Ca^{++} in the cytosol, which were determined by flow cytometry, increased in cells after treatment with GA. ER Ca^{++} channel inhibitor dantrolene did inhibit GA-induced loss of Ψm and increase in cytosolic Ca^{++} level (Figures 1A and C). As a consequence, dantrolene blocked cellular ROS production and DNA fragmentation induced by GA (Figure 1B and D). Co-treatment of cells with Ru360, a specific inhibitor of mitochondrial calcium uptake, completely inhibited the induction of cytosolic ROS production and DNA fragmentation and the alteration of Ψm that were caused by GA (Figure 1B-D). Ru-360 treatment did not affect the level of cytosolic Ca^{++} induced by GA, whereas the treatment was able to increase cytosolic Ca^{++} levels significantly as compared with GA-treated cells (Figure 1A). These findings, therefore, indicate that cytosolic ROS production, loss of Ψ_m, and apoptosis is dependent on

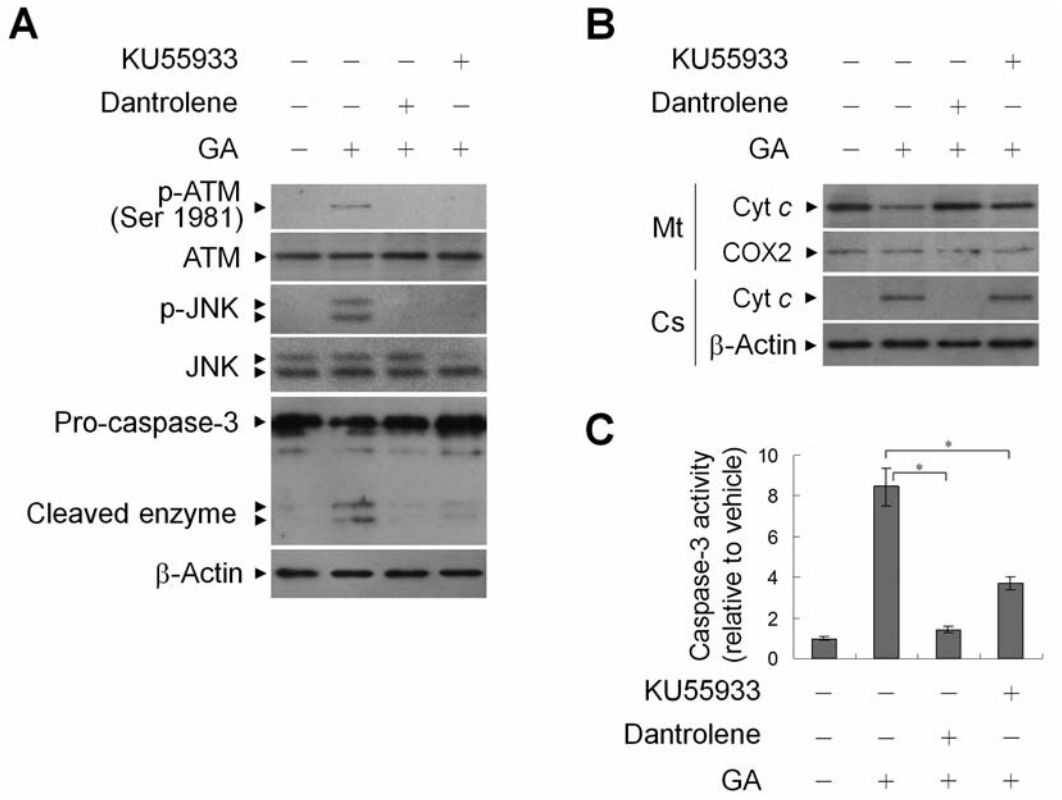

Figure 3. *Gallic acid (GA)-induced endoplasmic reticulum calcium (Ca^{++}) release is required for ataxia telangiectasia mutated (ATM) activation. A-C: Cells were treated with either vehicle, GA, GA plus dantrolene (25 μM), or GA plus KU55933 (5.0 μM) for 36 h. The levels of the indicated proteins in the total lysates were determined by western blot analysis using specific antibodies. β-Actin was used as an internal loading control. Caspase-3 activity was measured using flow cytometry. The level of cytochrome c (Cyt c) in the cytosol and mitochondria was determined by western blotting using specific antibodies. Cytochrome c oxidase subunit II (COX2) and β-actin were used as internal controls for the mitochondria and cytosol, respectively. JNK, c-Jun N-terminal kinase. The values presented are the mean±standard errors from three independent experiments. *Significantly different at p<0.05.*

the release of Ca^{++} from the ER to the cytosol induced by GA. The consequence of increased mitochondrial Ca^{++} uptake is associated with mitochondrial dysfunction, ROS generation, and apoptosis induced by GA.

ER stress response is not involved in GA-induced Ca^{++} efflux from the ER. It has been documented that ER stress can induce ER Ca^{++} release, promoting mitochondrial dysfunction and subsequent apoptosis (29). To determine whether GA-induced Ca^{++} release from ER store requires the ER stress response, we examined the expression levels of ER stress-related genes. Cells treated with tunicamycin, a glucosamine-containing antibiotic that specifically inhibits the asparagine-linked *N*-glycosylation of proteins, resulting in induction of unfolded protein response (UPR) regulator GRP78 and UPR-activated transcriptional factor CHOP expression, phosphorylation of ER stress sensor protein kinase RNA-like endoplasmic reticulum kinase (PERK), and pro-caspase-12 cleavage, and cytosolic Ca^{++} increase, which can be attenuated by *PERK* siRNA. These events were not observed in cells treated with GA (Figure 2A and B), although GA caused an increase in the cytosolic level. These data indicate that the ER stress response is not responsible for GA-induced Ca^{++} efflux from the ER.

GA-induced ER Ca^{++}-mediated ROS production contributes to activation of ATM-JNK signaling. To address whether GA-induced ER Ca^{++} release modulates ATM activation, cells were co-treated with dantrolene or a selective and competitive ATM kinase inhibitor KU-55933. Figure 3 shows that dantrolene treatment blocked GA-induced phosphorylation of ATM and JNK, proteolytic processing of pro-caspase-3, and the release of cytochrome *c* from mitochondria. The inhibition of ATM activity by KU55933 had a clear inhibitory effect on GA-induced phosphorylation of JNK, but there was no effect on mitochondrial cytochrome

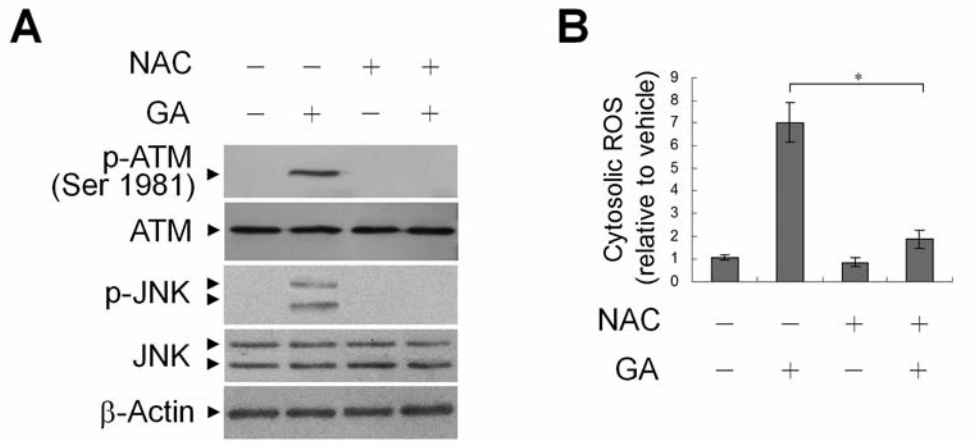

Figure 4. *Requirement of gallic acid (GA)-induced reactive oxygen species (ROS) for ataxia telangiectasia mutated (ATM) kinase phosphorylation. A-B: Cells were treated with either vehicle, GA, N-acetylcysteine (NAC) (100 μM), or GA plus NAC for 36 h. The levels of the indicated proteins in the total lysates were determined by western blot analysis using specific antibodies. β-Actin was used as an internal loading control. The generation of ROS was monitored by measuring increased fluorescence of 2,7-dichlorodihydrofluorescein by flow cytometry. The values presented are the mean±standard errors from three independent experiments. *Significantly different at p<0.05.*

c release. Moreover, KU55933 did not completely suppress GA-induced cleavage of pro-caspase-3 and induction of caspase-3 activity (Figures 3A and C). These observations suggest the involvement of ER Ca^{++} release in GA-induced activation of ATM-JNK signaling and caspase-3. Increased ATM activity contributes to GA-induced JNK phosphorylation and pro-caspase-3 cleavage but not to the induction of the release of cytochrome *c* from mitochondria.

To verify that production of ROS by GA is important for ATM phosphorylation, cells were co-treated with the ROS scavenger NAC. As shown in Figure 4, NAC co-treatment not only suppressed the increase of ROS but also caused the inhibition of GA-induced ATM and JNK phosphorylation. Therefore, ATM activation requires GA-induced mediated ROS production.

ATM activation is essential for the enhancement of GA-induced apoptosis but not for ROS production. To further confirm the role of ATM in GA-induced apoptotic cell death, FLAG-wt ATM, FLAG-kd ATM, and ATM shRNA were ectopically expressed in GA-treated cells. Compared with GA-treated control vector-transfected cells, overexpression of FLAG-wt ATM in GA-treated cells resulted in an increase in the levels of JNK phosphorylation and caspase-3 activity, which simultaneously increased apoptosis, as shown by an increase of DNA fragmentation. Although ectopic expression of FLAG-kd ATM or ATM shRNA overcame the induction of JNK phosphorylation induced by GA, this did not completely inhibit GA-induced induction of caspase-3 activity and apoptosis (Figure 5A and B). Expression of GA, FLAG-kd ATM or ATM shRNA in GA-treated cells had no effect on the level of ROS production (Figure 5B). These results indicate that ATM activation is not required for cytosolic ROS production but is essential for apoptosis induced by GA.

Discussion

In the present study, our findings indicate that the induction of apoptosis by GA appears to be due to the activation of ROS-mediated mitochondrial and ATM-JNK death signaling pathways by promoting the release of Ca^{++} from the ER, because dantrolene was capable of blocking GA-induced $\Delta\Psi_m$, ROS generation, mitochondrial cytochrome *c* release, caspase-3 activation, and ATM-JNK signaling activation. Dantrolene blocks ER Ca^{++} release at the ryanodine receptor, that is located in the ER membrane and is responsible for the release of Ca^{++} from intracellular stores (30). ER stress was implicated in the induction of Ca^{++} release from the ER. We did not observe an increase in the levels of GRP78, CHOP, and PERK phosphorylation in GA-treated cells. Silencing of PERK expression by siRNA in OC cells efficiently blocked the tunicomycin-induced induction of GRP78, CHOP, pro-caspase-12 cleavage, and cytosolic Ca^{++} elevation; however, *PERK* siRNA transfection had no inhibitory effect on the GA-induced cytosolic Ca^{++} level. Therefore, it is logical to suggest that the ER stress response is not involved in GA-induced release of ER Ca^{++}. There has been accumulating evidence demonstrating that the increased expression of B-cell lymphoma 2 (BCL2)-associated x protein (BAX) or BCL-2 antagonist killer 1 (BAK) promotes depletion of ER Ca^{++} and subsequent accumulation of Ca^{++} within the

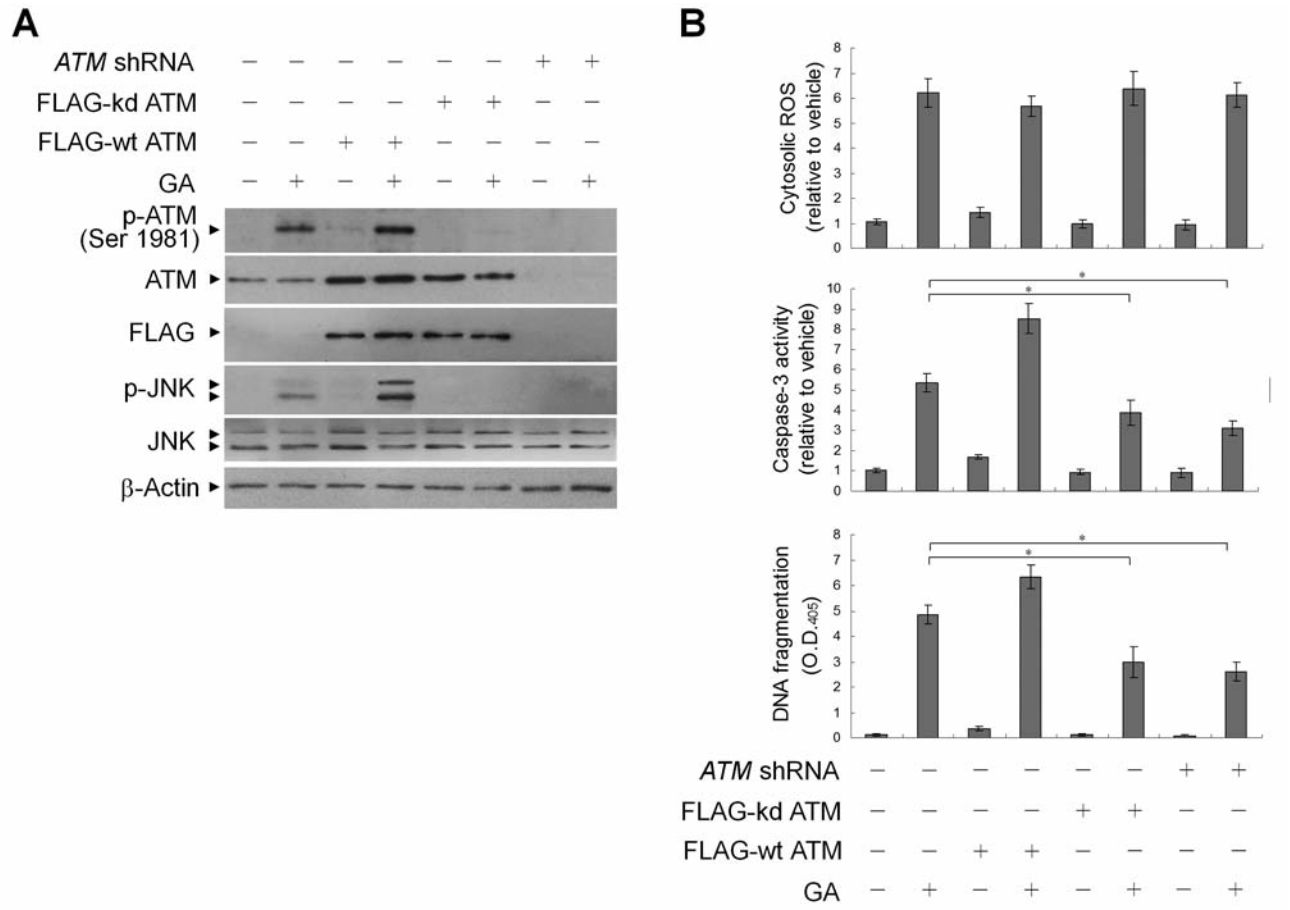

Figure 5. *Involvement of the activation of ataxia telangiectasia mutated (ATM)-c-Jun N-terminal kinase (JNK) signal pathway in gallic acid (GA)-induced apoptosis. A-B: At 12 h after transfection with vector alone, pcDNA3.1(+) FLAG-wild ATM (FLAG-wt ATM), pcDNA3.1(+) FLAG-kinase-dead ATM (FLAG-kd ATM), and pATM short hairpin RNA (ATM shRNA), cells were treated with either vehicle or GA for 36 h. The levels of the indicated proteins in the total lysates were determined by western blot analysis using specific antibodies. β-Actin was used as an internal loading control. The generation of cytosolic Ca^{++} and ROS was monitored by measuring increased fluorescence of Indo-1M and 2,7-dichlorodihydrofluorescein by flow cytometry. DNA fragmentation was determined using a Cell Death Detection ELISA kit. The values presented are the mean±standard errors from three independent experiments. *Significantly different at $p<0.05$.*

mitochondria (31-33). Overexpression of BCL2 did inhibit the effects of BAX or BAK on ER Ca^{++}-dependent release of mitochondrial cytochrome *c* or apoptosis (33). Localization of BAX and BAK to the ER membrane was shown to be required for ER stress-induced pro-caspase-12 cleavage and subsequent activation of caspase-3 for apoptosis initiation (34, 35). The BCL2 family of proteins can modulate ER-mitochondrial Ca^{++} trafficking and thereby control cell survival (36, 37). GA treatment did not affect the level of BCL-X_L, whereas the treatment increased BAX and BAK levels. Consistent with previous findings, GA caused p53 phosphorylation at Ser 15 (data not shown) (20). p53 Ser 15 is the primary target of the DNA-damage response, and can be phosphorylated by ATM kinase (38). Ser15 phosphorylation is required for transcriptional activation of p53-targeted genes (*p21*, *BAX*, and *BAK*) and contributes to the pro-apoptotic function of p53 (38). Previous work shows that p-p53 (Ser 15)-mediated expression of the BAX/BAK protein in response to DNA damage plays an important role in the control of ER Ca^{++} homeostasis. A direct interaction of GA with double-stranded DNA has been suggested to promote DNA damage (39). Whether or not GA-enhanced p-p53-mediated transcriptional activation of BAX and BAK is required for the induction of ER Ca^{++} release in OC cells remains to be further investigated.

Our finding shows that ER Ca^{++}-dependent ROS-mediated ATM-JNK signaling contributes to GA-induced apoptosis. Although apoptosis induction of genotoxic drug-resistant cancer cells is caused by high levels of ROS *via* ATM-JNK signaling (6), GA-induced ROS-dependent

apoptosis of OC cells does not rely completely on the activation of ATM-JNK signaling because the inhibition of ATM-JNK signaling by KU55933 or ectopic expression of FLAG-kd ATM or ATM shRNA partially suppresses GA-induced caspase-3 activation and apoptosis. Both activation of mitochondrial and ATM-JNK apoptotic signal pathways were shown to be required for apoptosis of OC cells in response to GA. A pharmacological approach provided evidence that suppression of ATM activity is required for the pro-apoptotic action of p53 in mitochondria through independent of changes expression of p53-targeted genes. It was also found that loss of ATM activity results in an increased level of ROS (40). Although we did not directly identify the p53 apoptotic activity involved in mitochondrial apoptotic priming, overexpression of FLAG-kd ATM similar to that of attenuation of by *ATM* shRNA did not suppress GA-induced increase in cellular ROS production. These results suggest that the ATM-JNK signal pathway acts downstream of ER Ca^{++}-mediated ROS and that their activation was responsible for strengthening of ROS-induced apoptosis.

In summary, this study shows that GA-induced apoptosis of human OC SSC-4 cells is dependent on the ER Ca^{++}-dependent ROS-mediated activation of mitochondrial apoptotic and ATM-JNK signal pathways. The characterization of this mechanism in human OC cells may provide a theoretical basis for utilizing GA to treat cancer.

Conflicts of Interest

The Authors disclose that there are no financial or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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