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Gallic Acid Induces Apoptosis of Lung Fibroblasts via a Reactive Oxygen Species-Dependent Ataxia Telangiectasia Mutated-p53 Activation Pathway

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Idiopathic pulmonary fibrosis (IPF) is a progressive chronic disorder characterized by the activation of fibroblasts and the overproduction of extracellular matrix. Fibroblast resistance to apoptosis leads to increased fibrosis. Targeting fibroblasts with apoptotic agents represents a major therapeutic intervention for debilitating IPF. Gallic acid (3,4,5-trihydroxybenzoic acid), a naturally occurring plant phenol, has been reported to induce apoptosis in tumor cell lines and renal fibroblasts. However, the effects of gallic acid on lung fibroblasts have not been investigated. The aim of the present study is to determine the effects of gallic acid on primary cultured mouse fibroblasts. Our results showed that gallic acid induces the apoptotic death of fibroblasts via both intrinsic and extrinsic apoptotic pathways by the elevation of PUMA, Fas, and FasL protein levels. Moreover, intracellular reactive oxygen species (ROS) generation and 8-hydroxy-2′-deoxyguanosine production were observed in gallic acid-stimulated fibroblasts. Mechanistic studies showed that gallic acid induces early phosphorylation of $p53^{Ser18}$ and histone $2AX^{Ser139}$ (H2AX) via ataxia telangiectasia mutated (ATM) activation in response to ROS-provoked DNA damage. When mouse lung fibroblasts were treated with caffeine, an ATM kinase inhibitor, the levels of p53, phosphorylated $p53^{Ser18}$, and cell death induced by gallic acid were significantly attenuated. Additionally, pretreatment with antioxidants drastically inhibited the gallic acid-induced 8-hydroxy-2′-deoxyguanosine (8-OH-dG) formation and phosphorylation of $p53^{Ser18}$ and $ATM^{Ser1981}$, as well as apoptosis. Our results provide the first evidence of the activation of ROS-dependent ATM/p53 signaling as a critical mechanism of gallic acid-induced cell death in primary cultured mouse lung fibroblasts.

KEYWORDS: Idiopathic pulmonary fibrosis; gallic acid; apoptosis; reactive oxygen species; p53; ataxia telangiectasia mutated

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive, lethal lung disorder with poorly understood pathogenetic mechanisms. It affects about 5 million persons worldwide with median survival of about 3 to 6 years from the time of diagnosis (*1*). IPF is characterized by the loss of lung epithelium and the formation of scar tissue within the lungs with accumulation of fibroblasts and myofibroblasts that deposit excessive extracellular matrix including collagen. Risk factors associated with pulmonary fibrosis include smoking, environmental exposure (e.g., to asbestos or silica), gastro-esophageal reflux disease, infectious agents, and genetic factors (*2*). The current treatments for IPF typically target the inflammatory response; however, they have little effect. This may be explained by increasing evidence that the abnormal wound repair process in response to alveolar epithelial injury, rather than inflammation, is responsible for IPF (*3*).

During efficient wound repair, the activated fibroblasts differentiate to α-smooth muscle actin (α-SMA)-expressing myofibroblasts that promote wound contraction followed by regeneration of epithelial and/or endothelial cells (*4*). These myofibroblasts

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along with endothelial cells and macrophages undergo apoptosis to exit the wound healing process (*5*). It has been speculated that during impaired epithelial wound healing, abnormal apoptotic activities occur in fibroblasts and epithelial cells, with reduced apoptosis causing accumulation of abnormal fibroblasts and myofibroblasts, and abnormal fibroblasts inducing alveolar epithelial cell death (*6*). Thus, pathways that activate pro-survival/antiapoptosis in fibroblasts/myofibroblasts may contribute to fibrosis. Since fibroblasts and myofibroblasts are one of the key players in wound healing, much effort has been expended to develop new therapeutic strategies that reduce persistent activation of these cells by targeting apoptosis, epithelial/endothelial mesenchymal transitions, telomerase activity, and stem cells, etc. (*7*).

Gallic acid (3,4,5-trihydroxybenzoic acid), a natural plant phenol, is abundant in green tea, red wine, and grapes, etc. The antioxidant activity of gallic acid protects human lymphocytes from hydrogen peroxide-induced lipid peroxidation and DNA fragmentation (*8*). Gallic acid has also been reported to act as a skin-whitening agent by inhibition of tyrosinase activity and melanin biosynthesis in B16 melanoma cells (*9*). In animal models, gallic acid reduces oxidative stress and enhances the levels of glutathione (GSH), GSH peroxidase, GSH reductase, and GSH *S*-transferase in hepatic tissue, as well as catalase in serum (*10*). In addition, gallic acid inhibits the saturation of fatty acid (*11*) and has antiangiogenesis activities (*12*). It also possesses antibacterial and antiviral activities (*13*), as well as anti-inflammatory activities (*14*). In the cardiovascular system, gallic acid inhibits the vasorelaxant response via the inhibition of NO production (*15*) to provide a cardioprotective effect by binding to *P*-selectin (*16*). Gallic acid also prevents amyloid β protein-induced apoptosis in neuron cells (*17*) and inhibits chemical-induced liver and kidney damage in rats (*18*), as well as possesses antimutagenic activity (*19*). Notably, gallic acid has been shown to induce apoptosis in tumor cells with higher sensitivity than that of normal cells (*20*, *21*). Hsu et al. reported that gallic acid induces apoptosis in preadipocyte cells (*22*). Since gallic acid has the ability to induce apoptosis in cancers and preadipocytes, we attempted to explore whether gallic acid affects the survival of lung fibroblasts. This is the first study to address gallic acid-induced apoptotic cell death and the underlying mechanisms involved in gallic acid-induced apoptosis in primary cultured mouse lung fibroblasts.

MATERIALS AND METHODS

Reagents. Gallic acid, ascorbic acid, *N*-acetylcysteine (NAC), pifithrin (PFT), and caffeine were purchased from Sigma (St. Louis, MO, USA). Anti-Fas, anti-Fas-ligand, and anticytochrome *c* antibodies were purchased from BD Biosciences PharMingen. Anti-p53 and antiphospho-p53^{Ser18} were obtained from Cell Signaling Technology, Inc. Anti-DR4, anti-DR, and antiphospho ataxia telangiectasia-mutated (ATM^{Ser1981}) kinase antibodies were procured from Abcam plc (Cambridge, UK). Anti-α-SMA, anti-Bcl-2, anti-Bax, and anti-Bcl-xL were from Santa Cruz Biotechnology, Inc. Caspase activity assay kit was obtained from R&D Systems (Minneapolis, MN, USA). Terminal deoxynucleotidyl transferase-mediated dUTP-fluorescin nick end-labeling (TUNEL) assay kit was purchased from Roche Diagnostics (Mannheim, Germany). Caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK), and caspase-9 inhibitor (Z-LEHD-FMK) were purchased from Kamiya (Thousand Oaks, CA, USA). Anti-Fas ligand antibody was procured from Transduction Laboratory. Antiactive Bax antibody was obtained from Calbiochem, EMD Biosciences (San Diego, CA, USA). Anti-γH2AX^{Ser139} antibody was purchased from Upstate (Temecula, CA, USA).

Lung Fibroblast Isolation and Culture. ICR mice aged 8−10 weeks were dissected under asphyxia. The lungs and upper airway were removed and placed into 10 cm Petri-dishes containing HSBB buffer. After rinsing twice with HBSS buffer, the tissues were minced into 1−3 mm pieces and incubated with trypsin, collagenase, and DNase. The samples were filtered, and equivalent medium was added to discontinue decomposition. After centrifugation, cells were harvested and cultured in DMEM−10% FBS supplemented with 1% L-glutamine, 1% nonessential amino acid, penicillin (100 units/mL), and streptomycin (100 μg/mL). Isolated murine pulmonary fibroblasts were characterized by detecting their immunoreactivity to α-smooth muscle actin (α-SMA), a structural protein of activated fibroblasts (**Figure 1**). The viability of isolated lung fibroblasts was approximately 75%. All experiments were performed with primary mouse lung fibroblasts at 3 to 15 passages. Average plating efficiency was >95%.

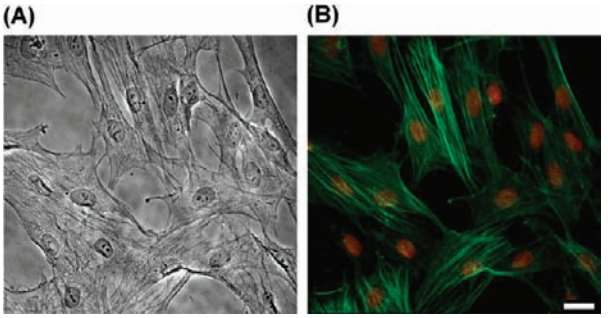

Figure 1. Identification of mouse lung fibroblasts (MLF) by α-SMA. MLFs were immuno-stained with α-smooth muscle actin antibody (green, FITC) and propidium iodide (DNA, red). Magnification 400×; scale bar, 10 μm.

Western Blot Analysis. Cells were lysed at 4 °C in RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.25% Sodium deoxycholate, 5 mM EDTA (pH 8.0), and 1 mM EGTA and supplemented with protease and phosphatase inhibitors. After 20 min of lysis on ice, cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined by the Bradford method. Equal amounts of proteins were separated onto SDS−polyacrylamide gels and then electrophoretically transferred from the gel onto a PVDF membrane (Millipore, Bedford, MA). After blocking, the membrane was reacted with specific primary antibodies overnight at 4 °C and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. The blots were visualized using the ECL-Plus detection kit (PerkinElmer Life Sciences, Inc. Boston, MA, USA).

Flow Cytometry Analysis. Cells (1×10^6) were treated with or without varying doses of gallic acid for 24 h and then harvested and centrifuged at 1200 rpm for 5 min.The cell pellets were fixed with 75% ethanol for 30 min at 4 °C. After centrifugation at 1200 rpm for 5 min, the cell pellets were resuspended in 1 mL of solution containing 0.1 mg/mL RNase A and 40 μg/mL propidium iodide, and incubated for 30 min at 37 °C. Cells were analyzed using FACS-Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Clonogenic Assay. Clonogenic assay was performed by seeding 200 cells per well onto 6-well plates. After 24 h of incubation, cells were treated with various concentrations (0, 2.5, 5, 10, 20, 30, and 40 μg/mL) of gallic acid for 48 h and then washed twice with PBS to remove any remaining gallic acid. Fresh medium was added, and the cells were incubated for another 5 days. The colonies were stained using crystal violet. Colonies comprising at least 50 cells were counted manually, and growth inhibitory curves were derived from a minimum of three independent experiments, each performed in triplicate.

Apoptotic Cell Determination. After treatment, cells were fixed with 2% paraformaldehyde for 20 min and washed twice with PBS. Then, the cells were permeabilized with 0.1% Trion-X100 for 30 min and incubated in TUNEL reaction buffer for 2 h. TUNEL assay protocol was carried out according to the manufacturer's instructions. After reaction with TUNEL buffer, cells were incubated with DAPI for 10 min, and the images were visualized using a fluorescence microscope. TUNEL-positive cells were counted as apoptotic cells by flow cytometry.

Caspase Activity Assay. Caspase activity was determined by a caspase fluorometric kit (R&D Systems, Mineapolis, MN, USA), according to the manufacturer's instructions. Briefly, cell lysates were incubated with caspase-3, -8, and -9 substrates. Relative caspase activity was measured by spectrophotometric analysis when the cleavage products released fluorochrome that emitted fluorescence at 505 nm after excitation

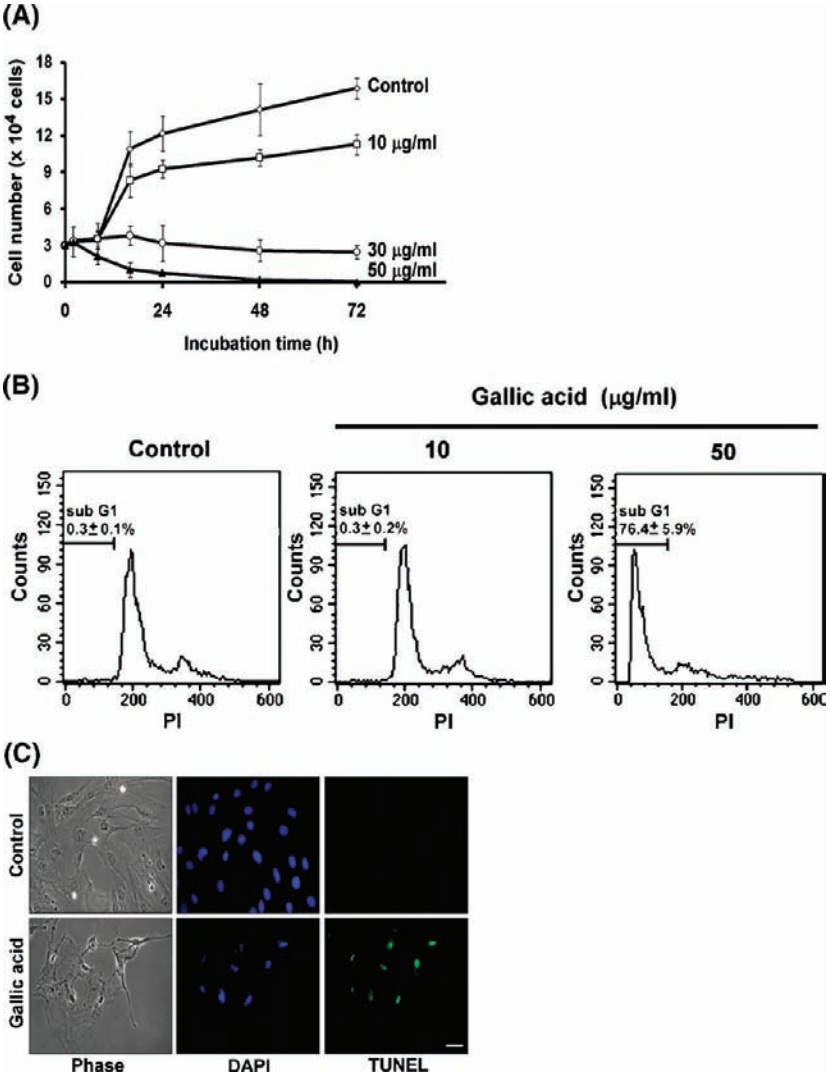

Figure 2. Gallic acid induces apoptotic cell death in murine pulmonary fibroblasts. (**A**) Antiproliferation effect. MLFs were treated with varying concentrations (0, 10, 30, and 50 μg/mL) of gallic acid for 2, 8, 16, 24, 48, and 72 h. After treatment, cell number was calculated by the Trypan blue dye exclusion method. (**B**) Flow cytometry analysis. MLFs were treated with or without varying concentrations of gallic acid (0, 10, and 50 μg/mL) for 24 h, and then cells were fixed and incubated with propidium iodide. After incubation, cells were harvested and analyzed by flow cytometry. (**C**) DAPI and TUNEL assay. MLFs were treated with 50 μg/mL gallic acid for 24 h. TUNEL assay was then performed to detect apoptotic cells (green), and the nuclear DNA was stained with DAPI (blue). The stained cells were examined using fluorescence microscopy. Magnification 400×; scale bar, 10 μm.

by light at 400 nm wavelength. The level of caspase activity in the cell lysate was directly proportional to the fluorescence signal detected with a fluorescent microplate reader.

Measurement of Reactive Oxygen Species (ROS) Generation. Dihydroethidine is a specific superoxide tracing dye, and DCF-DA has been used frequently to monitor H_2O_2 and hydroxyl radical levels in cells. To detect the levels of intracellular ROS production, cells were incubated for the indicated times in the absence or presence of gallic acid and then treated with 5 μM dihydroethidine or 5 μM H_2DCF-DA (Molecular Probes, Eugene, OR, USA) for 30 min prior to harvesting. After rinsing twice with PBS, cells were detached, and fluorescence was measured with a FACSCalibur flow cytometer using Cell Quest software.

Determination of 8-Hydroxy-2′-deoxyguanosine (8-OH-dG). Fibroblast DNA was isolated using Genimic DNA Mini Kit (Geneaid Biotech Ltd., Tao-Yuan, Taiwan), according to the manufacturer's instructions. DNA concentration was determined by the absorbance at 260 nm (A260 1.0: 50 μg DNA). Samples containing 100 μg of DNA were resuspended in 50 μL of reaction mixture, containing 100 mM sodium acetate (pH 5.0) and 5 mM $MgCl_2$, and digested with DNase I (Sigma-Aldrich) for 60 min at room temperature. The samples were incubated at 37 °C for 1 h after 2 units of alkaline phosphatase and 1 mM Tris-HCl (pH 7.4) were added. The amount of 8-OH-dG was determined using an enzyme-linked immunosorbent assay according to the manufacturer's instructions (ELISA, JaICa, Shizuoka, Japan). The 8-OH-dG amount was presented as the 8-OH-dG pg/μg DNA ratio.

Statistical Analysis. All data are presented as the mean ± SD of nine replicates from three independent experiments. Figures are representative of three separate experiments with similar patterns. Statistical differences were evaluated using the Student's *t*-test and considered significant at the $p < 0.05$, $p < 0.01$, or $p < 0.001$ level.

RESULTS

Induction of Apoptosis by Gallic Acid in Murine Lung Fibroblasts. To explore whether gallic acid has a beneficial effect on the treatment of lung fibrosis by blocking fibroblast accumulation, murine lung fibroblasts were treated with various concentrations of gallic acid for the indicated time periods. As shown in **Figure 2A**, gallic acid inhibited cell growth and was cytotoxic to fibroblast cells. The growth of lung fibroblasts was significantly inhibited by gallic acid at concentrations higher than 10 μg/mL and was almost completely inhibited at concentrations higher than 30 μg/mL (**Figure 2A**). Following incubation with 50 μg/mL gallic acid for 48 h, nearly 100% of fibroblasts were killed

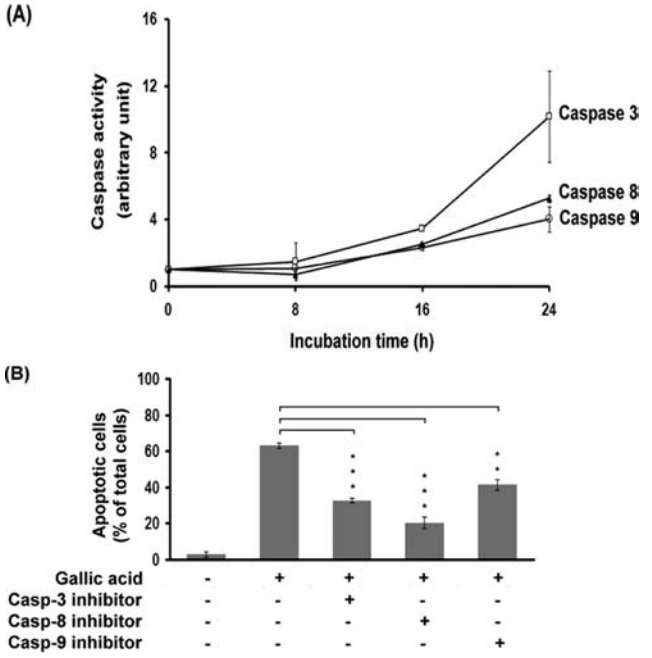

Figure 3. Gallic acid induces caspase-dependent apoptosis. (**A**) Activation of caspases. MLFs were incubated with 50 μg/mL gallic acid for the indicated time periods. The cell lysates were then extracted, and the caspase activity was determined. Data are presented as the mean ± SD of nine replicates from three independent experiments. (**B**) Caspase inhibitors blocked gallic acid-triggered apoptosis. MLFs were pretreated with 50 μM caspase inhibitors for 1 h and then incubated with 50 μg/mL gallic acid for another 24 h. Apoptotic cells were determined by TUNEL assay. Data are expressed as the mean ± SD from three independent experiments.

(**Figure 2A**). These results indicate that low concentration of gallic acid exhibits a cytostatic effect, while high concentration of gallic acid causes cytotoxicity. Clonogenic assay was used to confirm the growth inhibitory effect of gallic acid. The results showed that the half maximal inhibitory concentration of gallic acid is 16.8 μg/mL for 48 h of treatment.

Additionally, treatment of fibroblasts with 50 μg/mL gallic acid caused shrinkage and blebbing of cell morphology, as well as detachment of cells from culture dishes (data not shown). To examine if gallic acid induces apoptotic cell death, flow cytometry analysis was performed. As shown in **Figure 2B**, approximately 76% sub-G1 population was observed after 50 μg/mL gallic acid treatment for 24 h, indicating that gallic acid induces apoptosis. DAPI staining and TUNEL assay further confirmed that gallic acid induces apoptosis in pulmonary fibroblasts (**Figure 2C**).

Activation of caspases is one of the most widely recognized features of apoptosis; thus, the effect of gallic acid on caspase activity was examined. The results showed that the activities of caspase-3, -8, and -9 increase with increasing incubation time in gallic acid-treated fibroblasts (**Figure 3A**). Pretreatment with inhibitors of caspase-3, -8, and -9 significantly attenuated the gallic acid-elicited apoptosis (**Figure 3B**).

Gallic Acid Induces Both Intrinsic and Extrinsic Apoptosis. On the basis of the above results, caspase-3, -8, and -9 activities increase in gallic acid-treated fibroblasts indicating that both extrinsic and intrinsic apoptotic pathways are activated. Proteins of the Bcl-2 family are involved in intrinsic mitochondria-mediated apoptosis. Thus, we examined the expressions of pro-survival proteins, Bcl-2 and Bcl-X_L, and pro-apoptotic proteins, Bax and PUMA. Expression of PUMA protein drastically changed following gallic acid treatment (**Figure 4A**), while the levels of Bcl-2, Bcl-X_L, and Bax were not affected. Bax plays a key role in apoptotic death. It normally resides in the cytosol but

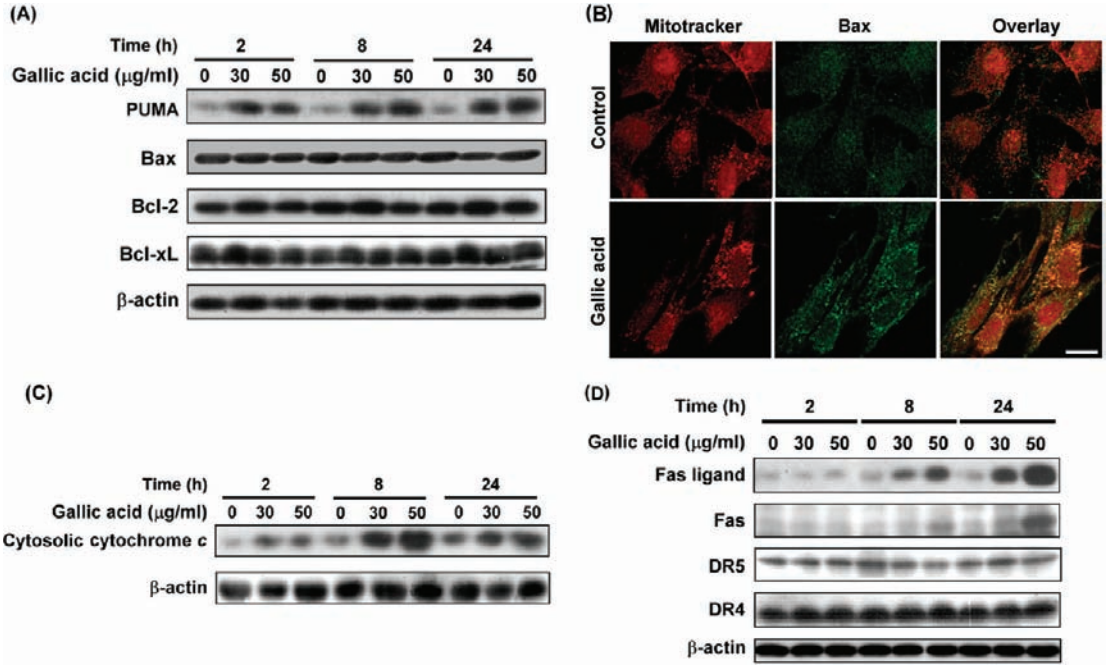

Figure 4. Regulation of apoptosis-related molecules by gallic acid. MLF cells were treated with the indicated concentrations of gallic acid. (**A**) The expressions of Bcl-2 family members (PUMA, Bax, Bcl-X_L, and Bcl-2) were detected after 2, 8, and 24 h treatment by Western blot analysis. (**B**) Bax mitochondrial translocation was observed after 8 h with 50 μg/mL gallic acid incubation. Immunostaining with antibody against active Bax (green color) and costaining with Mitotracker (red color) were performed to localize cellular distribution of Bax. Magnification 630×; scale bar, 20 μm. (**C**) Cytochrome *c* release. Cell lysates were isolated from tested cells, and Western blot was performed with antibody against cytochrome *c*. (**D**) Regulation of death-receptor proteins. After treatment, MLF cell lysates were analyzed by Western blot with antibodies against Fas ligand, Fas, DR4, and DR5. β-Actin was used as an internal loading control.

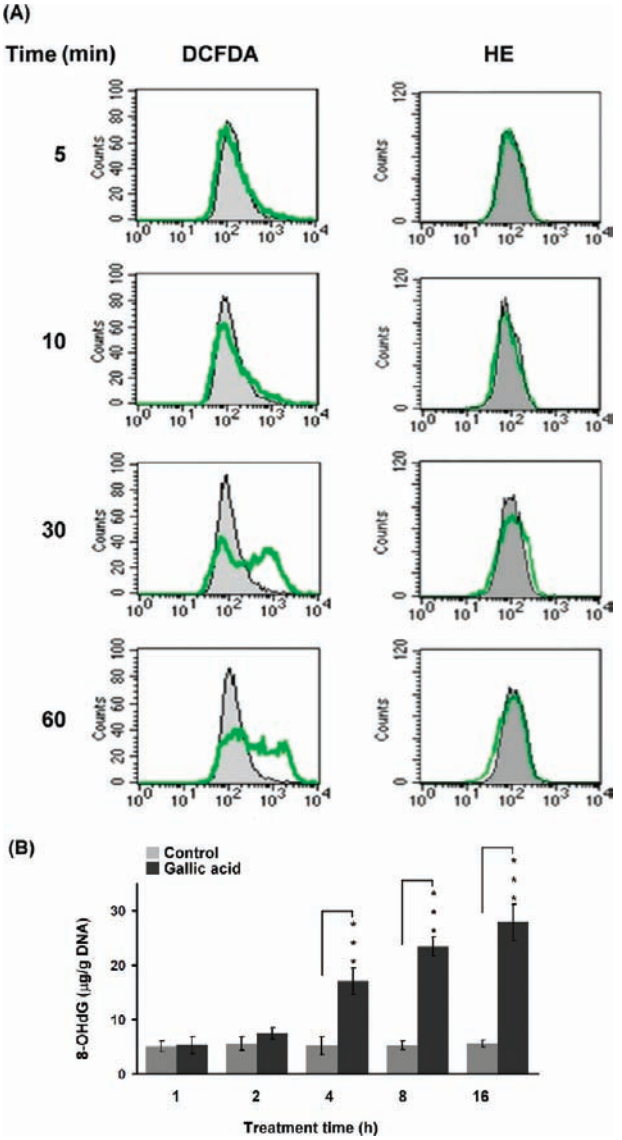

Figure 5. Gallic acid increases ROS production and disrupts mitochondrial membrane potential. MLFs were treated with or without 50 μg/mL gallic acid (**A**), and the ROS levels were measured by FACS analysis after incubation with gallic acid for 5, 10, 30, and 60 min. (**B**) 8-OH-dG levels were measured by ELISA assay after 1, 2, 4, 8, and 16 h of gallic acid treatment.

translocates into the mitochondria, driving mitochondrial cytochrome *c* release at the onset of apoptotic stresses. Therefore, the cellular distributions of Bax and cytochrome *c* were investigated. As depicted in **Figure 4B**, using antibody against active Bax to trace the cellular distribution of Bax, treatment with gallic acid caused mitochondrial translocation of Bax. Moreover, gallic acid induced cytochrome *c* release to cytosol, which peaked at 8 h (**Figure 4C**). Next, to determine if gallic acid triggers an extrinsic apoptotic process, the levels of death receptors were analyzed. As revealed in **Figure 4D**, levels of both Fas ligand and Fas were elevated in a time- and dosage-dependent manner in gallic acid-treated cells. The DR4 and DR5 levels did not change in response to gallic acid administration.

Gallic Acid Promotes ROS Production and 8-OH-dG Formation. Studies of the signaling pathways involved in apoptosis have indicated that intracellular ROS induced by gallic acid plays an important role in eliciting an early signal in apoptosis (*23*). To examine the effect of gallic acid on ROS production and

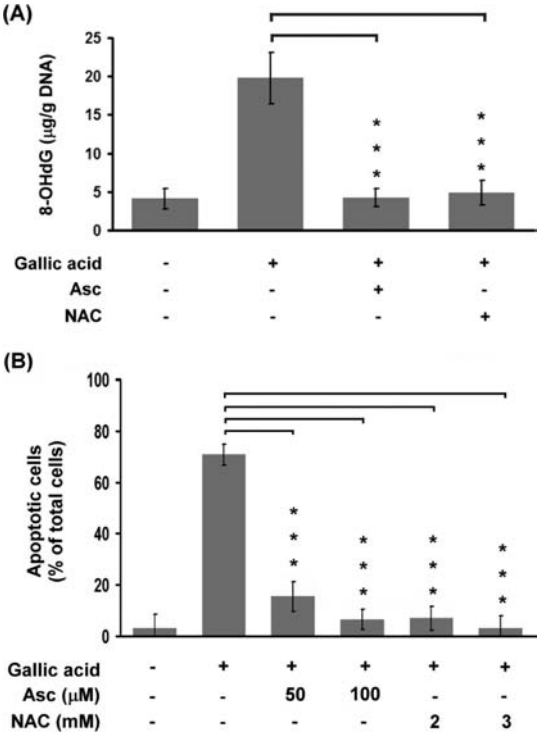

Figure 6. Antioxidants block gallic acid-mediated biological effects. MLFs were preincubated with ascorbic acid (100 μM) or *N*-acetylcysteine (2 mM) for 30 min and then treated with 50 μg/mL gallic acid. (**A**) The levels of 8-OH-dG were measured by ELISA assay following 8 h of gallic acid exposure. (**B**) The apoptotic cells were determined by TUNEL assay after 24 h of treatment. Data were expressed as the mean ± SD from 3 independent experiments. Asc, ascorbic acid; NAC, *N*-acetylcysteine.

mitochondrial membrane potential in pulmonary fibroblasts, specific fluorescence probes, H_2DCFDA and dihydroethidine (HE), were used to monitor the levels of hydrogen peroxide and superoxide radical, respectively. In **Figure 5A**, the level of intracellular hydrogen peroxide detected by oxidation of DCF-DA increased after stimulation with 50 μg/mL gallic acid when compared with that of untreated cells. The relative level of hydrogen peroxide was elevated at 30 min after gallic acid treatment. The superoxide content was not affected by gallic acid treatment as there was no change in HE fluorescence intensity (**Figure 5A**). 8-OH-dG is widely used as a biomarker for oxidative gene damage in cells. To examine whether gallic acid-induced hydrogen peroxide production causes DNA damage in lung fibroblasts, the amount of 8-OH-dG was measured. As shown in **Figure 5B**, the level of 8-OH-dG increased in a time-dependent manner in cells exposed to gallic acid.

ROS Generation Plays an Important Role in Eliciting DNA Damage and Apoptosis Induced by Gallic Acid. To address the role of ROS in gallic acid-elicited apoptosis, lung fibroblasts were pretreated with antioxidants (including NAC and ascorbic acid) 30 min prior to gallic acid addition. Results showed that treatment of fibroblasts with ascorbic acid or NAC eliminates 8-OH-dG production (**Figure 6A**) and inhibits apoptotic cell death (**Figure 6B**) induced by gallic acid. These results imply that ROS are generated by gallic acid and play a key role in apoptosis induction.

Involvement of ATM and p53 Activation in Gallic Acid-Mediated Apoptosis. It has been reported that the toxicity associated with some phenolic compounds is mediated by their oxidative activity, which can accelerate oxidative damage in vitro, to DNA or to proteins and carbohydrates (*24*). p53 is one of the essential players

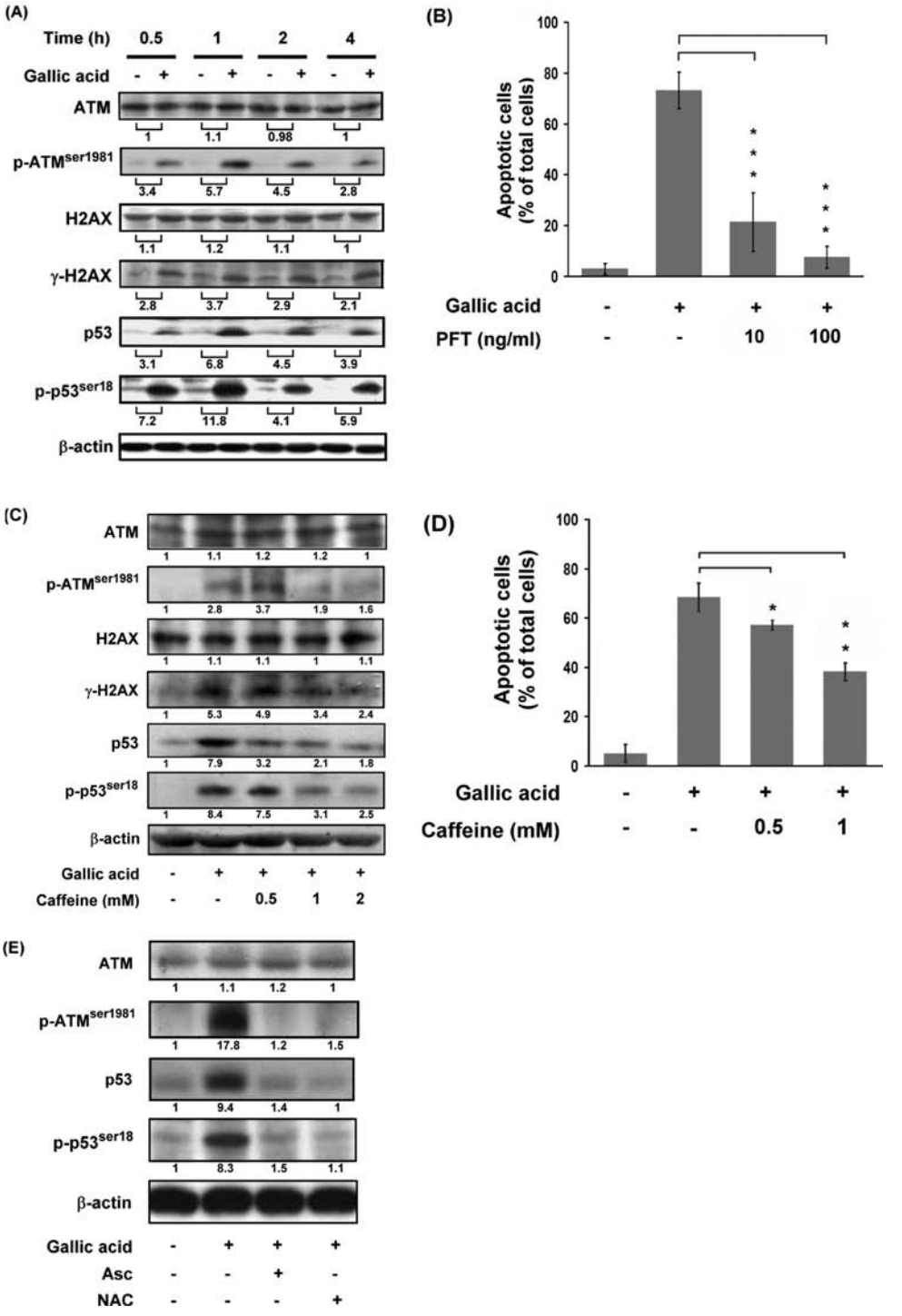

Figure 7. Involvement of ATM and p53 in gallic acid-induced apoptotic process. (**A**) Activation of ATM and p53. MLFs were treated with or without 50 μg/mL gallic acid for 0.5, 1, 2, and 4 h. After treatment, cell extracts were isolated and analyzed by Western blot analysis with specific antibodies against ATM and phosphorylated-ATM, -H2AX, and -p53. (**B**) Attenuation of gallic acid-triggered apoptosis by p53 inhibitor. MLFs were pretreated with PFT (an inhibitor of p53) for 1 h and then incubated with 50 μg/mL gallic acid for another 24 h. Then, apoptotic cells were determined by TUNEL assay. (**C**) Effects of ATM inhibitor caffeine on p53 phosphorylation and apoptosis induced by gallic acid. MLFs were pretreated with caffeine for 1 h and then incubated with 50 μg/mL gallic acid for another 1 h. Cell lysates were harvested, and Western blot analysis was performed to examine the levels of phosphorylated-ATM, -H2AX, and -p53. (**D**) The apoptotic cells were determined by TUNEL assay after 24 h of treatment. (**E**) Antioxidants inhibit ATM and p53 phosphorylation. MLFs were pretreated with 100 μM ascorbic acid or 2 mM *N*-acetylcysteine for 30 min and then incubated with gallic acid for another 1 h. The levels of phosphorylated-ATM and -p53 were determined by Western blot. Quantitation was by densitometry, and fold of expression levels was calculated relative to that found in control cultures. The values are presented as means from three independent experiments.

in the oxidative DNA damage-induced apoptotic process and is activated through phosphorylation at the Serine-18 residue by activated ATM kinase in mice. Apoptosis-related molecules, including Fas and PUMA, are the targets of p53. To investigate if p53 and ATM are involved in gallic acid-induced apoptosis, the levels of total p53, phosphorylated $p53^{Ser18}$, and activated $ATM^{Ser1981}$ were measured in cells treated with or without gallic acid. As shown in **Figure 7A**, gallic acid treatment resulted in

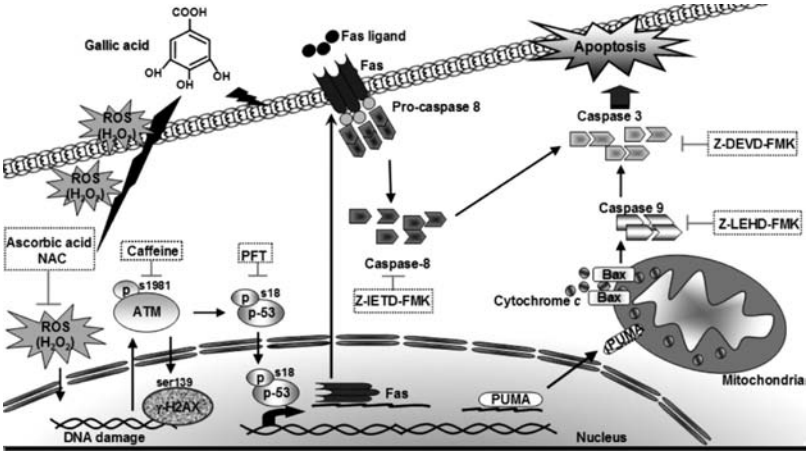

Figure 8. Schematic model of gallic acid-induced apoptosis pathway in primary cultured murine lung fibroblasts. Incubation of fibroblasts with gallic acid activates ROS-mediated DNA damage signaling pathway by triggering ATM-dependent activation of p53. The transcriptional activation of p53 upregulates the proapoptotic molecules, such as PUMA and Fas, and provokes caspase activation via both intrinsic and extrinsic pathways, consequently leading to apoptotic cell death.

strong phosphorylation of ATM at Ser-1981 and also caused a marked increase in the levels of phosphorylated forms of $p53^{Ser18}$ and γ-$H2AX^{Ser139}$ (substrates of ATM). Addition of p53 inhibitor PFT 1 h prior to gallic acid treatment significantly suppressed cell death (**Figure 7B**), implying that p53 is an important mediator of gallic acid-induced cell death. Moreover, pretreatment with caffeine, an inhibitor of ATM, markedly attenuated gallic acid-induced p53 and H2AX phosphorylation (**Figure 7C**), as well as cell death (**Figure 7D**), implying that ATM is an upstream regulator of p53 activation. To address whether gallic acid-induced ROS production is important to p53 phosphorylation activation, lung fibroblasts were exposed to antioxidants and/or gallic acid, and then analyzed by Western blot. As expected, antioxidants (both ascorbic acid and NAC) significantly suppressed the gallic acid-induced $ATM^{Ser1981}$ and $p53^{Ser18}$ phosphorylation (**Figure 7E**), suggesting that ROS induced by gallic acid plays a crucial role in ATM and p53 phosphorylation in lung fibroblasts.

DISCUSSION

A wide range of naturally occurring substances have been shown to protect against experimental pulmonary inflammation and fibrosis, and numerous studies have suggested that phytochemicals possess strong anti-inflammation and antifibrosis properties. Inducing apoptosis of overgrowing fibroblasts is considered to be one of the possible mechanisms of these natural compounds in combating fibrosis. Gallic acid has been reported to induce apoptosis in various tumor cells and adipocytes (*20*−*25*). In this study, gallic acid induced apoptosis in mouse lung fibroblasts via both intrinsic and extrinsic death pathways to activate caspase-8, -9, and -3 activities, and ROS-mediated ATM and p53 activation played key roles in gallic acid-induced apoptosis.

It is well known that gallic acid is a strong antioxidant. However, its role in apoptosis is achieved by pro-oxidant activity (*26*). It has been shown that Cu(II) oxidizes propyl gallate and other flavonoids upon complexation and generates hydrogen peroxide and Cu(I) (*27*). In this study, we found that treatment of lung fibroblasts with gallic acid increases hydrogen peroxide accumulation (**Figure 5A**). Moreover, pretreatment with antioxidants, ascorbic acid and NAC, completely inhibited gallic acid-triggered apoptotic death, suggesting that hydrogen peroxide production plays a crucial role in gallic acid-elicited cytotoxicity of lung fibroblasts. This finding was similar to that in rat hepatoma dRLH-84 and HeLa cells, human hepatoma PLC/PRF/5 cells (*28*), and human leukemia HL-60RG cells (*29*) in which gallic acid-induced apoptosis is mediated by hydrogen peroxide.

Yoshino et al. reported that gallic acid and related compounds cause copper-dependent DNA strand breaks and formation of 8-hydroxy-2′-deoxy guanosine (8-OHdG) (*30*). Gallic acid has been shown to induce DNA damage and ataxia-telangiectasia-mutated kinase (ATM) activation, which leads to increases in the phosphorylation of histone 2AX (H2AX) that triggers cell cycle arrest and apoptosis in human prostate carcinoma DU145 cells (*31*). ATM kinase plays an essential role in maintaining genome integrity by coordinating cell cycle arrest, DNA damage repair, and apoptosis (*32*). DNA damage triggers the phosphorylation of ATM at serine 1981, and this activation of ATM results in the subsequent phosphorylation of γH2AX and p53 (*32*). The ATM-mediated phosphorylation of p53 inhibits Mdm2 (murine double minute) binding and leads to the accumulation and increased transcriptional activation capacity of p53 (*33*). Our results showed that gallic acid-elicited hydrogen peroxide generation is accompanied by the formation of 8-OH-dG (**Figure 5B**) and the elevation of phosphorylated-ATM, -γH2AX, and -p53 levels (**Figure 7A**), which implies ROS-induced DNA damage. Pharmacological inhibition of p53 significantly reduced gallic acid-induced apoptosis (**Figure 7B**), suggesting that p53 is one of the main players in gallic acid-triggered apoptosis in lung fibroblasts. Moreover, an ATM inhibitor, caffeine, significantly attenuated gallic acid-elicited p53 phosphorylation and apoptotic cell death (**Figure 7C** and **D**), suggesting that ATM is an upstream molecule of the p53 signaling pathway.

Accumulating evidence demonstrates that p53 regulates many genes involved in apoptosis, including Fas and PUMA (*33*). A previous study demonstrated that gallic acid-induced apoptosis is accompanied by an increase in p53, Fas, and FasL protein levels, as well as by modulation of Bcl-2 family molecules, in 3T3-L1 preadipocytes (*22*). Consistent with those findings, the expressions of intrinsic apoptosis pathway-related molecule PUMA and extrinsic apoptosis pathway-associated molecules Fas ligand and Fas increased in gallic acid-treated lung fibroblasts (**Figure 4A** and **D**). Moreover, gallic acid provoked the activation of caspase-3, -8, and -9, and preincubation with caspase inhibitors effectively attenuated gallic acid-induced apoptosis (**Figure 3**). These results

indicated that treatment of pulmonary fibroblasts with gallic acid causes ROS-mediated DNA damage that activates ATM-p53 signaling and subsequently provokes both Fas- and PUMA-mediated apoptosis pathways. PUMA, a BH3-only protein, plays an important role in p53-mediated apoptosis. Several studies have shown that PUMA is responsible for the mitochondrial alterations in many tumor cell lines during p53-dependent apoptotic process. These include the release of cytochrome *c* from mitochondria and reduction in mitochondrial membrane potential (*34*). It has been reported that the apoptosis induced by PUMA is completely dependent on the presence of the intact Bax (*34*). PUMA activates Bax by inducing Bax mitochondrial translocation and multimerization in colorectal cancer cells (*35*). Similarly, our findings showed that the kinetics of apoptosis do not correlate with the Bax protein level (**Figure 4A**). Instead, the p53-induced PUMA protein level and Bax mitochondrial translocation, as well as cytochrome *c* release (**Figure 4A**, **B**, and **C**), were kinetically compatible with gallic acid-induced cell death. These results suggest that Fas level, PUMA content, and Bax mitochondrial translocation contribute to both the extrinsic and intrinsic apoptotic death of gallic acid-treated lung fibroblasts.

In conclusion, our observations indicate that gallic acid induces intracellular ROS and elicits DNA damage. This subsequently induces ATM-mediated p53 transcriptional activation to upregulate the expressions of PUMA, Fas, and FasL. Upregulation of Fas and FasL provokes the extrinsic apoptotic pathway by activating caspase-8 and -3, while upregulated PUMA and mitochondria translocated Bax initiate the intrinsic apoptotic pathway by the release of cytochome *c* and activation of caspase-9 and -3 leading to apoptosis of murine lung fibroblasts (**Figure 8**). Our study clearly establishes a mechanistic role of gallic acid in limiting lung fibroblast growth in vitro and provides a basis for further studies of this bioactive agent in vivo to address its preventive and therapeutic potential for idiopathic pulmonary fibrosis.

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