Bleomycin induces the extrinsic apoptotic pathway in pulmonary endothelial cells

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Mungunsukh O, Griffin AJ, Lee YH, Day RM. Bleomycin induces the extrinsic apoptotic pathway in pulmonary endothelial cells. Am J Physiol Lung Cell Mol Physiol 298: L696–L703, 2010. First published February 12, 2010; doi:10.1152/ajplung.00322.2009.—Bleomycin, a chemotherapeutic agent, can cause pulmonary fibrosis in humans and is commonly used to induce experimental pulmonary fibrosis in rodents. In cell culture, bleomycin causes single- and double-stranded DNA breaks and produces reactive oxidative species, both of which require iron (Fe^{2+}) and O_2. The mechanism of bleomycin-induced apoptosis is controversial due to its complexity. We investigated bleomycin apoptotic signaling events in primary pulmonary endothelial cells. Time course experiments revealed that bleomycin induced apoptosis within 4 h. Caspase-8, the initiator caspase for the extrinsic pathway, was activated within 2 h and preceded activation of the effector caspses-3 and -6 (4 h). Caspase-9, the initiator of the intrinsic pathway and release of cytochrome c from the mitochondria were not detected at these time points. Bleomycin induced the expression of Bcl-2 and Bcl-x_L, Bcl-2 family member proteins that protect cells from the mitochondria-dependent intrinsic apoptosis. Real-time quantitative RT-PCR results demonstrated that, at 4–8 h, bleomycin induced expression of TNF and TNF receptor family genes known to induce the extrinsic apoptotic pathway. Silencing of the death receptor adaptor protein Fas-associated death domain by short interfering RNA significantly reduced bleomycin-induced apoptosis. Apoptosis was also abrogated by caspase-8 inhibition, but only slightly reduced by caspase-3 inhibition. Together, these data suggest that bleomycin initiates apoptosis via the extrinsic pathway.

PULMONARY FIBROSIS IS CHARACTERIZED by the remodeling of the alveolar architecture, notably the loss of normal alveolar epithelial and endothelial cells and their replacement by activated fibroblasts that induce alterations in the extracellular matrix. This process ultimately results in the irreversible loss of the lung’s ability to transfer oxygen into the bloodstream. The chemotherapeutic agent bleomycin can induce pulmonary fibrosis in humans (1, 5, 7) and has become widely used as a fibrogenic agent in animal models of experimentally induced pulmonary fibrosis (2, 26). Bleomycin catalyses single- and double-stranded DNA breaks through direct binding to DNA in the presence of iron (Fe^{2+}) and O_2 (6, 25). Bleomycin also produces reactive oxidative species, including superoxide, H_2O_2, and/or organoperoxides, which participate in its cytotoxicity and in triggering apoptosis (25).

Pulmonary epithelial and endothelial cell death by apoptosis is considered as a key event in the initiation and progression of pulmonary fibrosis (2, 11). Two pathways of apoptosis are known in mammals, the mitochondria-dependent, or intrinsic, pathway, and the death-receptor-mediated, or extrinsic, pathway. The intrinsic apoptotic pathway is triggered in response to DNA damage and other types of severe cell stress and involves dysregulation of pro- and antiapoptotic proteins of the Bcl-2 superfamily. Normally, the antiapoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-x_L, prevent the permeabilization of the mitochondrial outer membrane by inhibiting the formation of membrane pores by the proapoptotic Bcl-2 proteins, Bax and Bak. Under proapoptotic conditions, mitochondrial pores formed by Bax and Bak allow the release of cytochrome c, leading to the activation of the intrinsic apoptotic initiator caspase, caspase-9. The complex of cytochrome c/apoptotic protease activating factor-1/caspase-9, or the apoptosome, triggers activation of the common effector caspases-3, -6 and -7, and leads to DNA breakdown and the final dissolution of the cell (10, 20). In contrast, the extrinsic apoptotic pathway is triggered by ligand stimulation of death receptors (DRs) of the TNF receptor superfamily, resulting in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and the extrinsic pathway initiator caspase, caspase-8. Upon recruitment to this complex, caspase-8 is activated and initiates apoptosis by direct cleavage of the downstream common effector caspases (10).

In vivo, excessive apoptosis of pulmonary cells has been correlated with bleomycin-induced upregulation of Fas and Fas ligand (FasL) in murine models of pulmonary fibrosis (12, 16). However, cell culture experiments using primary rat lung epithelial cells and transformed mouse lung epithelial (MLE) cells provided evidence for activation of the intrinsic pathway of apoptosis by bleomycin in a c-Jun NH2-terminal kinase (JNK) dependent manner (17). In a separate report, also using MLE, bleomycin was shown to activate caspase-8, suggesting the involvement of the extrinsic pathway of apoptosis (28).

Here, we investigated bleomycin-induced apoptosis in pulmonary artery endothelial cells (PAEC). The time course of proteolytic activation of caspases, mitochondrial dysfunction, and apoptosis-related DNA fragmentation suggests that, in this cell type, activation of the extrinsic apoptotic pathway precedes intrinsic apoptotic events.

MATERIALS AND METHODS

Reagents. Fetal bovine serum (FBS; 100–106) was purchased from Gemini Bio-Products (Woodland, CA). RPMI-1640 medium, fungizone, penicillin/streptomycin, and Dulbecco’s PBS were purchased from Invitrogen (Carlsbad, CA). Bleomycin was purchased from Hospira (Lake Forest, IL). Caspase inhibitors were obtained from R&D Systems (Minneapolis, MN), and all other inhibitors were purchased from EMD Chemicals (Gibbstown, NJ). Dominant negative (DN) caspase-8 expression vector was a gift of Dr. Fukuoka Takahiko Horiuchi, Department of Medicine and Biosystemic Sci-
ence, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

**Cell culture.** Human bronchial epithelial cells (BEnPc) were purchased from Cell Applications and cultured in the prepared B/TePc (tracheal epithelial cell) growth medium (Cell Applications, San Diego, CA). Bovine PAEC were purchased from ATCC (Manassas, VA). Passage 2–8 cells were used for all experiments. PAEC were cultured in RPMI 1640 with 10% FBS, 1% penicillin/streptomycin, and 0.5% fungizone. Cells were grown in 5% CO₂ at 37°C in a humidified atmosphere cell culture incubator. PAEC were treated at 80–90% confluence. Concentrations of drugs and inhibitors for culture were as follows: bleomycin (4.5 μM/ml), Z-IETD-FMK (caspase-8-inhibitor, 10 μM), Z-DEVD-FMK (caspase-3-inhibitor, 10 μM). Control cells were treated for the same time, either with medium or with the same amount of DMSO used to prepare drug/inhibitor solutions.

**Neutral comet assay.** The neutral comet assay was used to measure double-stranded DNA breaks as an indication of apoptosis. Confluent PAEC were treated with 4.5 μM/ml bleomycin for 16 h. Cells were embedded in 1% low-melting point agarose and placed on a comet slide, according to the manufacturer’s protocol (Trevigen, Gaithersburg, MD). Slides were then placed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% sodium-lauryl sarcosinate, 1% Triton X-100, and pH 9.9) for 20 min, washed by immersion in 1× TBE buffer (0.089 M Tris, 0.089 M boric acid, and 0.002 M EDTA, pH 8.0). The nuclei underwent electrophoresis for 10 min at 6 mA in a horizontal mini-electrophoresis apparatus (Bio-Rad, Hercules, CA) with 1× TBE buffer. Then cells were fixed with 75% ethanol for 10 min and air-dried overnight. Cells were stained with 1× Sybe Green (Molecular Probes, Eugene, OR) and visualized with an Olympus BX61 fluorescence microscope (Olympus, Center Valley, PA) using a 10 magnification at 478-nm excitation and 507-nm emission wavelengths. Approximately 100 cells were randomly selected and microscopically scored, according to tail length. Comets were defined as apoptotic cells, as described by Krown et al. (14).

**DNA laddering assay.** PAEC were grown to confluency on 100-mm dishes and treated with bleomycin (4.5 μM/ml) for the indicated times. Cells were washed once with cold PBS, collected by centrifugation, and resuspended in 300-μl lysis buffer [Tris EDTA (TE) buffer containing 0.2% Triton X-100]. Then cells were incubated on ice for 10 min (mixed vigorously by vortexing every 2 min). Cell debris and intact nuclei were removed by centrifugation at 15,000 g for 15 min at 4°C. RNA was removed by RNase A (0.06 mg/ml) incubation for 30 min at 37°C. Then 0.5% of SDS and 0.15 mg/ml of proteinase K were added to the lysate and incubated overnight at 50°C. DNA fragments were precipitated by the addition of 0.1 volume of 5 M NaCl and 1 volume of isopropanol and subsequent incubation on ice for 10 min. After centrifugation for 15 min at 15,000 g, DNA fragments were dissolved in 30 μl TE buffer. Six microliters of loading buffer was added to this solution and incubated for 10 min at 50°C and then analyzed by 2% agarose gel electrophoresis.

**Lactate dehydrogenase assay.** Lactate dehydrogenase (LDH) assay was used to measure necrotic death of PAEC and performed using the LDH Cytotoxicity Detection Kit (Clontech, Mountain View, CA), according to the manufacturer’s instruction. Cell death is expressed as the percentage of the LDH measured in the medium divided the total LDH release after treatment with 1% Triton X-100.

**DePsipher assay.** Bleomycin-induced mitochondrial membrane dysfunction was measured in PAEC using the DePsipher Kit (Trevigen, Gaithersburg, MD). Cells were allowed to reach confluency and treated with bleomycin (4.5 μM/ml) for 8, 24, and 48 h; hydrogen peroxide (1 mM) was included as a positive control. Slides were prepared, and fields from each treatment were selected at random and microscopically scored according to cell color. Results are expressed in terms of percent mitochondrial membrane disturbance out of total cell number.

**Caspase assays.** Caspase-6 and -8 activities were measured using the Caspase-Glo assay (Promega, Madison, WI). PAEC grown to confluency on a 60-mm dish were treated with bleomycin (4.5 μM/ml) for the indicated times. After being washed once with 1× PBS, cells were incubated in lysis buffer (100 μl; 30 mM Tris, pH 7.5; 150 mM NaCl; 10% glycerol; 1% Triton X-100; and protease inhibitor, Roche, Mannheim, Germany) for 10 min on ice. Then cells were scraped and transferred into a fresh tube and sonicated three times for 10 s at 50% output control using an ultrasound (Heat Systems-Ultrasonics, Plainview, NY). Cell debris were removed by centrifugation for 15 min (15,000 g). Cell lysates were diluted with sterile water (1:1 vol/vol) and incubated with Caspase-Glo assay reagent (1:1 vol/vol) for 30 min at room temperature, and luminescent signals were collected using a Dynex MLX Microtiter Plate Luminometer (Dynex Technologies, Chantilly, VA). Caspase-9 activities were measured using the Caspase-Glo assay, according to the manufacturer’s cell-based assay protocol (Promega, Madison, WI). Cells grown in a 96-well white-walled cell culture plate (30,000–50,000 cells/well) were treated for the indicated times. To collect luminescent signals, 0.1 ml of Caspase-Glo assay reagent, including the MG-132 inhibitor, was added to each well containing 0.1 ml cell culture and incubated for 2 h at room temperature on a rotating shaker. Caspase-3 activity was measured using the caspase colorimetric substrate/inhibitor QuantiPak kit (BIOMOL International, Plymouth Meeting, PA), according to the manufacturer’s instruction, with the following modifications: cells were incubated in lysis buffer for 10 min on ice; the assay mixture contained 35-μl reaction buffer, 35-μl cell lysate, and 5-μl substrate; and caspase activities were measured after 16-h incubation at 37°C. Caspase-3 substrate (Ac-DEVD-pNA) was purchased from A.G. Scientific (San Diego, CA).

**Quantitative real-time reverse transcription polymerase chain reaction.** Total RNA was isolated from cells using an RNaseasy kit (Qiagen, Valencia, CA). Genomic DNA was removed using the RNase-free DNase set (Qiagen, Valencia, CA). RNA was quantitated spectroscopically (ND-1000 Spectrophotometer, NanoDrop, Wilmington, DE), and integrity was assessed by capillary electrophoresis (Experion, Bio-Rad, Hercules, CA). RNA (1.0 μg) was subjected to reverse transcription with GeneAmp RNA PCR kit, according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). cDNA was diluted 10-fold with water, and then 2 μl of cDNA were used for 20-μl qPCR (quantitative real-time reverse transcription polymerase chain reaction). PCR was performed in triplicates using 6 μM of each primer and 10 μl of SybrGreen PCR master mix (Applied Biosystems, Foster City, CA). Primers for qR-PCR were designed using the ProbeFinder software, version 2.35 (Roche Applied Sciences, Indianapolis, IN). The following primer pairs were used to analyze bovine gene expressions: caspase-3, 5'-CTC TGT GTG GTG TGG TCA G and 5'-TCT ACT GCC GGT TCT TTT TC; FAS, 5'-CCC ATT CCA TGA ATT AGT CGA T and 5'-AGT GGA GCT GGG GAT GTA AT; FADD, 5'-GGG ACC TCG GAG TAT CTG AC and 5'-GAG TCC GGG GGT ACT TCT C; TNF-α, 5'-TCC CCT TCC TCC TGG TTG C and 5'-CGG CCA GAA ACT CAC TTC T; tumor necrosis factor receptor superfamily, member 12a (TNFRSF12a), 5'-GCC GAC GTA GAC TGC AT and 5'-GAA GTC GCT GTG CGG TCT; TNFRSF9, 5'-AAC AGC CCA AGA AGA TG and 5'-TTC TCA AGA GAG TCC CAA CAC A; Bcl-2, 5'-GGG CAA CAA ATA TGC AGA AG and 5'-TGG TGC ATC AGC AAC AAT G; Bcl-xL, 5'-GGT ATT GGT GAG TCG GAT CG and 5'-TCC AAG GCT GTA GGT GA. As internal control, mRNA levels of α-tubulin were determined. For quantification, the comparative threshold cycle method was used to assess relative changes in mRNA levels between the untreated (control) and the drug-treated samples.

**Gene expression profiling.** Gene expression profiles were created using the RT²-Profilier PCR array, apoptotic pathway gene array (SuperArray, Rockville, MD). Total RNA was isolated from cells, and 1-μg RNA was converted to cDNA, as described above. This cDNA was used for a 96-well plate RT²-Profilier PCR array (SuperArray). The expression of each gene tested was determined relative to a set of
multiple internal controls for normalization. The comparative threshold-cycle method was used to assess fold changes in mRNA levels between the untreated (control) and bleomycin-treated samples.

**Western blots.** To prepare whole cell lysates, confluent cells grown on 35-mm dishes were washed once with cold PBS and resuspended in 100 μl of 1× SDS buffer (62.5 mM Tris-HCl, pH 6.8; 2% wt/vol SDS; 10% glycerol; 50 mM DTT; and 0.01% bromophenol blue). Following cell lysis, DNA was sheared using an ultrasonic sonicator (Heat Systems-Ultrasonics, Plainview, NY) at 50% output control for 15 s at 4°C. Samples were heated for 5 min at 95°C. Mitochondria-free extract was obtained using the mitochondria isolation kit for cultured cells, according to the manufacturer’s protocol (Pierce, Rockford, IL). Whole cell lysates or mitochondria-free cytosolic extracts (10–20 μg protein/sample) were subjected to a SDS polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membrane (0.2-μm pore size). Membrane was blocked with 3% blocking reagent (Roche, Indianapolis, IN). Membranes were cut into two slices: one containing the higher (>32 kDa), and another one containing the lower molecular mass (<32 kDa) peptides. Slices containing lower molecular mass peptides were incubated with the primary antibodies: rabbit polyclonal anti-cytochrome c (1:250, no. sc-7159) or anti-Bcl-x (1:500, no. sc-634, Santa Cruz, Santa Cruz, CA); or rabbit monoclonal anti-active caspase-3 (1:1000, no. 9664) or anti Bcl-2 (1:1000, no. 2870, Cell Signaling Technology, Danvers, MA). To normalize the results, slices containing higher molecular mass peptides of the same membranes were blotted with β-actin. Proteins were detected with horseradish peroxidase-linked secondary antibodies (1:5,000) and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). WCIF ImageJ software (http://www.uhnresearch.ca/facilities/wcif/index.htm) was used for densitometry analysis.

**Silencing of FADD expression.** BEpC were grown in B/TEpC growth medium (catalog no. 511–500, Cell Applications, San Diego, CA) in a 35-mm cell culture dish to 30% confluence, medium was changed to an antibiotic-free basal medium (1.5 ml per dish, cat. no. 510–500, Cell Applications). After 24 h, cells were transfected with 40 pmol/dish FADD-short interfering RNA (siRNA)-1 or -2 (cat. no. 4392420; ID: s16706 and s16707, respectively; Ambion, Austin, TX) using the Lipofectamine RNAiMAX Reagent, according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Silencer GAPDH siRNA (75 pmol/dish, cat. no. AM-4624, Ambion) and negative control 1 (75 pmol/dish, cat. no. 4611G, Ambion) were used as controls. Twenty-four hours posttransfection, cells were transfected again with the same amount of siRNA as described before. The next day, medium was changed by regular growth medium, after 24-h cells were used for downstream assay.

**Cloning of enhanced green fluorescent protein-tagged histone H2B.** Total mRNA was prepared from cultured primary human BePC (Cell Applications) and used for reverse transcription, as described above. PCR was performed with 2 μl cDNA using the GC-Rich System (Roche Applied Science, Indianapolis, IN), according to the manufacturer’s instructions. Primers used were as follows: 5’-cgc gga ttc TTA GGC TCG ATG TAC TTG GTG AC-3’ (forward) and primer 5’-ccc gg agg tac cgC CAC CAT GCC AGA GCC AGC GAA GTC TGC T3’ (reverse), with upper case bases corresponding to the human histone H2B (GenBank: AF531286.1) sequence, and lower case bases indicating 5' extensions with restriction enzyme sites (bold) for BamHI I and Kpn I, respectively. PCR cycles were as follows: 1× 94°C 3 min, 5× 94°C 30 s, 55°C 30 s, 72°C 2 min; 22× 95°C 30 s, 55°C 30 s, 72°C 1 min; and 1× 72°C 7 min. PCR product was analyzed by 1.5% agarose gel electrophoresis, purified from the gel, and cloned into the BamHI I and Kpn I restriction sites of the pEGFP-N1 vector (BD Biosciences, San Jose, CA).

**Plasmid transfection with DN caspase-8 and H2B-enhanced green fluorescent protein.** One day before transfection, cells were plated at 1.4 × 10⁶ cells/well in a 12-well plate. One microgram of DNA per well was transfected using the FuGENE 6 Transfection Reagent (Roche Applied Science), according to the manufacturer’s instructions, in serum-free, antibiotic-free medium. When the H2B-enhanced green fluorescent protein (EGFP) reporter was cotransfected with the DN caspase-8 vector (or empty vector), the ratio of H2B-EGFP-to-DN expression vector was 1:2, with a total concentration of DNA equal to 1 μg. Cells were transfected for 6 h, and then medium was replaced with 0.01% FBS medium containing antibiotics. The next day, cells were treated with bleomycin for the times indicated for each experiment.

**Comet assay with DN caspase-8.** PAEC were transfected with DN caspase-8, as described above. An EGFP-tagged histone (H2B-EGFP) expression vector was included to allow identification of transfected cells. Transfected cells were treated with bleomycin for 24 h, and apoptotic cells were identified microscopically. Untreated cells were used as control. Cells were stained with propidium iodide and green fluorescent cells (cells positive for H2B-EGFP expression) forming red comet tails were defined as apoptotic cells.

**Statistics.** All results are expressed as means ± SE, with P < 0.05 considered statistically significant. Two-tailed Student’s t-test was used to analyze differences between two groups.

**RESULTS**

The extrinsic pathway of apoptosis is induced by bleomycin.

To elucidate the mechanism of bleomycin-induced apoptosis, we first determined the concentration of bleomycin required to induce apoptosis. Pulmonary artery endothelial cells (PAEC) grown in serum-free, antibiotic-free medium were exposed to various concentrations of bleomycin for 24 h. As shown in Fig. 1A, bleomycin induced a dose-dependent decrease in cell viability, as measured by lactate dehydrogenase release into the culture media.

**Western Blot Analysis.** To confirm that bleomycin induced apoptosis, we performed Western blot analysis on whole cell lysates. As shown in Fig. 1B, bleomycin treatment resulted in a decrease in the expression of the anti-apoptotic protein Bcl-2, and an increase in the expression of the pro-apoptotic protein caspase-3.

**LDH Assay.** To further confirm the induction of apoptosis, we performed a lactate dehydrogenase (LDH) assay. As shown in Fig. 1C, bleomycin treatment resulted in a significant increase in LDH release into the culture media, indicating cell death.

**Comet Assay.** To confirm the occurrence of DNA fragmentation, we performed a comet assay. As shown in Fig. 1D, bleomycin treatment resulted in a significant increase in the percentage of cells with fragmented DNA, indicating apoptosis.

**CONCLUSION**

Bleomycin-induced apoptosis in PAEC is mediated by the extrinsic pathway of apoptosis, involving the activation of caspase-3 and the release of lactate dehydrogenase. Further studies are needed to elucidate the molecular mechanisms underlying bleomycin-induced apoptosis.
induce at least 80% apoptosis in primary pulmonary endothelial cells. Treatment of cells with 4.5 mU/ml bleomycin for 16 h induced 80–100% apoptosis, as shown by the neutral comet assay. This concentration of bleomycin is similar to the concentration that would be used for chemotherapeutic use in humans (~3 mU/ml blood; prescribing information dosage and administration; Hospira, Lake Forest, IL).

Next, a time course of bleomycin treatment to induce apoptosis was performed. Neutral comet assays detected appearance of DNA fragmentation at 4–16 h posttreatment of bleomycin (Fig. 1A). To confirm that this fragmentation was due to apoptosis, DNA laddering assay was performed. As shown in Fig. 1B, bleomycin induced DNA laddering within 4 h of treatment (Fig. 1B). Release of LDH from cells was not detected until 20 h of bleomycin treatment (LDH assay; Fig. 1C), indicating that necrotic cell death was not present before this time point. Overall, these data show that bleomycin (4.5 mU/ml) initiates apoptosis within 4 h in primary PAEC.

We next focused on identifying the pathways leading to apoptosis by bleomycin. Apoptotic events, including caspase activation, loss of mitochondrial outer membrane potential, and mitochondrial release of cytochrome c, were individually determined. Enzymatic assays revealed that activation of caspase-8, the initiator caspase of the extrinsic pathway, occurred within 2 h of bleomycin treatment (Fig. 2A). In contrast, activation of caspase-9, the initiator caspase of the intrinsic pathway, was not active at this time point, and significant activation was only detected after 8 h (Fig. 2B). The effector caspase, caspase-6, was activated within 4 h of bleomycin treatment (Fig. 2C). An assay to detect enzymatic activation of the effector caspases-3/7 showed that these caspases were active within 8 h of bleomycin treatment (Fig. 2D). Because of the overlAPPoing substrate specificity of caspases-3 and -7, Western blots with anti-active caspase-3 were performed to determine the time course of caspase-3 activation. As shown in Fig. 2E, caspase-3 was proteolytically activated at 4 h posttreatment. Altogether, these data suggest that caspase-8 acts as an initiator caspase of bleomycin-induced apoptosis in PAEC. In agreement with this, loss of mitochondrial membrane potential, as determined by the DePsipher assay, was not observed until ~24 h following bleomycin treatment (Fig. 3A). Besides, significant release of cytochrome c from the mitochondria, a key event of the intrinsic pathway, was not detected until ~16 h (Fig. 3B).

Regulation of apoptotic gene expression by bleomycin. To explore the genes involved in bleomycin-induced apoptosis, we first conducted pathway-focused PCR array using human BEpC treated with bleomycin for 4, 8, and 12 h (Supplemental Table 1; the online version of this article contains supplemental data), as focused arrays are not currently available for bovine sequences. Genes from the TNF and TNF receptor families, as well as caspase family, were activated in BEpC in response to bleomycin (supplemental data). Therefore, we designed primers for the bovine orthologs and performed qPCR analysis. As expected, several proapoptotic genes for the extrinsic apoptotic pathway were activated in bovine PAEC after bleomycin treatment. Significant activations were detected for Fadd, Tnf, Fas, Casp3, Tnfrsf9, and Tnfrsf12a at 4 or 8 h postbleomycin (Fig. 4). Interestingly, the PCR array data with human BEpC also showed that bleomycin activated the expression of a number of antiapoptotic genes for the intrinsic apoptotic pathway (Supplemental Table 1). Therefore, we analyzed antiapoptotic Bcl-2 and Bcl-xL gene expressions in PAEC using qPCR (Fig. 5, A and B) and their protein expression by Western blotting (Fig. 5C). As expected, we observed both gene activation and increased protein expression in response to bleomycin at 4–8 h posttreatment (Fig. 5).

Fig. 2. Time course of bleomycin-induced caspase activation. PAEC were grown to confluence, treated with 4.5 mU/ml bleomycin for the indicated times, and used for caspase assays. Untreated cells were used as control. A–C: caspase-8, caspase-9, and caspase-6 enzymatic assay using the Caspase-Glo 8, 9, and 6 substrate, respectively. D: caspase-3/7 activity was determined using the Caspase-3 Colorimetric Substrate/Inhibitor QuantiPak kit. Values are means ± SE. *P < 0.05, statistical significant from control. RLU, relative light units. E: representative Western blot with anti-active caspase-3 antibody. Experiment was reproduced 3 times.
Caspase-8, but not caspase-3, inhibition blocks bleomycin-induced apoptosis. Time course studies showed caspase-8 and caspase-3 activation before caspase-9. Pharmacological inhibitors were used to further elucidate the roles of these individual caspases in bleomycin-induced apoptosis. In PAEC, the caspase-8 inhibitor Z-IETD-FMK (10 M) almost completely abrogated bleomycin-induced apoptosis, as determined by neutral comet assays (Fig. 6A). This concentration of Z-IETD-FMK has been shown to inhibit caspase-8 completely and cross react only weakly with caspase-3 and caspase-9 (4). In contrast, the caspase-3/7 inhibitor Z-DEVD-FMK reduced apoptosis only by 23% (Fig. 6B). Next, PAEC were transfected with a DN caspase-8 expression vector and subjected to neutral comet assay, with or without bleomycin treatment. The histone-EGFP expression vector was included in cotransfections to enable differentiation between transfected and nontransfected cells. Analysis of comets in transfected cells demonstrated that bleomycin-treated, DN caspase-8-expressing cells exhibited ~50% less apoptosis compared with bleomycin-treated cells transfected with EGFP alone (Fig. 6C). To determine whether caspase-8 was required for caspase-3 activation, Western blots for anti-active caspase-3 were performed in cells treated with the caspase-8 inhibitor before the addition of bleomycin. The caspase-8 inhibitor suppressed caspase-3 activation almost completely (Fig. 6D). These results suggest that caspase-8 activation occurs upstream of other apoptotic events.

Silencing of FADD prevents bleomycin-induced apoptosis. Time course results and inhibitor data suggested a critical role of caspase-8 in bleomycin-induced apoptosis in pulmonary endothelial cells. Caspase-8 is known to be activated upon DR activation as a critical part of the extrinsic apoptotic pathway (10). Furthermore, our PCR array data indicated that bleomycin induced expression of multiple DRs and ligands, including Fas, DR3, and DR4/5 in BEpC (Supplemental Table 1).
sequences from human was included in controls. The data was determined by neutral comet assay. Negative control s16707 and treated with bleomycin for 24 h, and apoptosis silenced it by 

silenced FADD expression by mRNA level was measured by qPCR. FADD-siRNA s16706 isolated from transfected cells (24-h posttransfection), and the MATERIALS AND METHODS section. Total RNA was then

BEpC were transfected with FADD-siRNA, as described in MATERIALS AND METHODS. Nonhomologous siRNA transfection was included in controls. Cells were transfected with dominant-negative caspase-8 (DN-Casp8). An enhanced green fluorescent protein (GFP)-tagged histone H2B expression vector was included to allow identification of transfected cells. Transfected cells were treated with bleomycin for 24 h. Untreated cells were used as control. Cells were stained with propidium iodide, and green fluorescent cells forming red comet tails were defined as apoptotic cells. Data show means ± SE from 3 independent experiments. Statistical significance from *untreated cells and ‡cells treated with bleomycin for 24 h, P < 0.05. D: representative Western blot of whole cell extracts using anti-active caspase-3. Cells were treated with either caspase-8 inhibitor or DMSO for 30 min before bleomycin exposure (6 h). Experiment was repeated at least 3 times.

fore, we focused on gene knockdown of the FADD adaptor, which is common to all of the DRs (13, 15). Two different FADD-siRNA were used, s16706 and s16707 in human BEpC, as siRNAs for bovine gene was not available. At first we performed comet assays with human BEpC to confirm that apoptosis in this cell type is modulated by bleomycin, similarly to PAEC. As shown in Fig. 7A, bleomycin induced DNA fragmentation in BEpC, as in PAEC, within 4 h. Next, BEpC were transfected with FADD-siRNA, as described in the MATERIALS AND METHODS section. Total RNA was then isolated from transfected cells (24-h posttransfection), and mRNA level was measured by qPCR. FADD-siRNA s16706 silenced FADD expression by ~75%, whereas s16707 silenced it by ~80% (Fig. 7B). Cells were transfected with s16707 and treated with bleomycin for 24 h, and apoptosis was determined by neutral comet assay. Negative control siRNA that has no significant homology to any known gene sequences from human was included in controls. The data indicate that bleomycin-induced apoptosis was inhibited by 50% by FADD-siRNA (Fig. 7C).

DISCUSSION

The chemotherapeutic agent bleomycin induces pulmonary fibrosis in humans and is one of the most commonly used agents to induce pulmonary fibrosis in animal models (1, 2). Apoptosis of pulmonary endothelial and epithelial cell plays an important role in the initiation and progression of pulmonary fibrosis (2, 9, 11, 32), and the mechanism of bleomycin-induced apoptosis in lung cells has been a critical topic for pulmonary fibrosis research (17, 18, 28, 30). The major finding of this study is that, in primary pulmonary endothelial cells, bleomycin induces early activation of the extrinsic apoptotic pathway, but not of the intrinsic apoptotic pathway. Time course studies with bleomycin showed that proteolytic activation of caspase-8 is upstream of other apoptotic events, such as activation of effector caspases-3/7 and -6, and apoptosis-related DNA fragmentation. Because caspase-8 is a well-known initiator caspase of the extrinsic apoptotic pathway (10), it seems very likely that bleomycin triggers apoptosis by the extrinsic apoptotic pathway. This finding is greatly supported by our results from both caspase-8 inhibitor and DN-negative caspase-8 expression studies, showing significant inhibition of bleomycin-induced apoptosis. Caspase-8 is believed to become activated by autoproteolytic cleavage after recruitment to the death-inducing signaling complex that is formed on activation of DRs by their ligand (23). In BEpC and pulmonary endothelial cells, we found that bleomycin increased the ex-
pression of several TNF ligand and TNF receptor family genes. Expression of DRs, such as FAS, DR4, and DR5, has been shown to be activated by p53 in response to DNA damage (8), and bleomycin has been shown to regulate expression of DNA damage-inducible proteins, including p53, that are associated with acute injury in the lung (21). The exact mechanism by which bleomycin regulates TNF-receptor expression, that triggers apoptosis, is currently under investigation in our laboratory.

Our study demonstrates that apoptosis-related DNA fragmentation is upstream of the loss of mitochondrial outer membrane potential and activation of caspase-9. Since loss of mitochondrial outer membrane potential is a key event of the intrinsic apoptotic pathway, and caspase-9 is known to be the initiator caspase of this pathway (10, 20), bleomycin clearly initiates apoptosis by the extrinsic and not by the intrinsic pathway. This finding is also supported by the time course study showing that caspase-9 is downstream of the activation of effector caspases-3/7 and -6. In addition, increased expression of the antiapoptotic proteins Bcl-2 and Bcl-xL was detected in response to bleomycin. These proteins are known to inhibit the intrinsic apoptotic pathway by preventing loss of mitochondrial outer membrane potential (24, 27). We hypothesize that upregulation of Bcl-2 and Bcl-xL could play a role in the delay of activation of the intrinsic pathway in PAEC.

In PAEC, inhibition of caspase-8, by either overexpression of DN caspase-8 or by the Z-IETD-FMK inhibitor, blocked bleomycin-induced apoptosis. However, inhibition of caspase-3/7 by the Z-DEVD-FMK inhibitor suppressed apoptosis only by 25%. Enzymatic assays showed that another executioner, caspase-6, which is regulated directly by p53 (19), was activated in response to bleomycin. Therefore, it is possible that inhibition of the caspase-3/7 alone was not sufficient to block apoptosis. The role of caspase-7 in bleomycin-induced apoptosis is yet not clear because of the overlapping substrate specificity with caspase-3 (29) and needs to be further elucidated.

Two recent studies investigated the mechanism of bleomycin-induced apoptosis in alveolar epithelial cell and obtained contradictory findings. Lee et al. (17) demonstrated in MLE and primary rat alveolar type II cells that bleomycin induced cell death through the intrinsic apoptotic pathway, through JNK-dependent activation of Bax. In a separate study, Wallach-Dayan et al. (28) found that, in MLE, bleomycin caused an increase of reactive oxidative species, resulting in upregulation of Fas/FasL expression, as well as increased activation of caspase-8, caspase-9, and mitochondrial dysfunction and apoptosis. Both studies in MLE cells utilized over 20-fold higher bleomycin dose and twice the incubation time (often ~48 h) to obtain 100% apoptosis (17, 28) compared with our study in primary PAEC and BEpC. MLE is a transformed cell line, and its apoptotic machinery may behave differently from that of primary cells (31), but it is also possible that differences between our study result from the use of different cell types.

Several studies have suggested that the extrinsic apoptotic pathway is essential in bleomycin model for lung injury and fibrosis (11, 16). Bleomycin induces the expression of TNF receptors in mice (22). In an in vivo study, Fas- and FasL-deficient C3H mice (lpr/lpr and gld/gld) displayed reduced susceptibility to bleomycin-induced pulmonary fibrosis (16). However, in a subsequent study, Aoshiba et al. (3) used lpr/lpr and gld/gld in the C57BL/6 murine strain and found no change in bleomycin-induced pulmonary fibrosis. The differences between susceptibility to bleomycin-induced lung injury may be partially explained by the use of different murine strains for the two studies, but our data also indicate that Fas/Fasl is not the only DR/ligand pair regulated by bleomycin. In agreement with our findings, bleomycin increased expression of Fas/Fasl and activated caspase-8 in MLE; however, a Fas/Fasl antagonist failed to inhibit apoptosis (28). Our gene expression studies with PAEC and BEpC indicated that bleomycin activates expression of other DRs, including DR4 and DR5. Thus targeting Fas/Fasl interaction alone may not have been sufficient to inhibit extrinsic apoptosis induced by bleomycin. A possible target could be FADD, a common adaptor molecule for all apoptosis-signaling DRs (13, 15). We achieved 80% silencing of FADD expression in BEpC by siRNA and obtained 50% inhibition of bleomycin-induced apoptosis. Complete silencing of FADD could have increased its effect, but this result, nevertheless, supports our findings of bleomycin-induced extrinsic apoptotic pathway.

In summary, our findings suggest a principal role for the extrinsic pathway in bleomycin-induced apoptosis in primary pulmonary endothelial cells. Because of the importance of endothelial cell apoptosis in the development of pulmonary fibrosis (9, 32), a complete understanding of the underlying molecular mechanism(s) will likely be important for the development of treatments for pulmonary fibrosis.

REFERENCES


DISCLOSURES

No conflicts of interest are declared by the author(s).


47. Kibrick JA, Aicher K, Dife...