Leukocyte Elastase Induces Lung Epithelial Apoptosis via a PAR-1-, NF- κ B-, and p53-Dependent Pathway

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Leukocyte elastase induces apoptosis of lung epithelial cells via alterations in mitochondrial permeability, but the signaling pathways regulating this response remain uncertain. Here we investigated the involvement of proteinase-activated receptor-1 (PAR-1), the transcription factor NF- κ B, and the protooncogene p53 in this pathway. Elastase-induced apoptosis of lung epithelial cells correlated temporally with activation of NF- κ B, phosphorylation, and nuclear translocation of p53, increased p53 up-regulated modulator of apoptosis (PUMA) expression, and mitochondrial translocation of Bax resulting in enhanced permeability. Elastase-induced apoptosis was also prevented by pharmacologic inhibitors of NF- κ B and p53 and by short interfering RNA knockdown of PAR-1, p53, or PUMA. These inhibitors prevented elastase-induced PUMA expression, mitochondrial translocation of Bax, increased mitochondrial permeability, and attenuated apoptosis. NF- κ B inhibitors also reduced p53 phosphorylation. We conclude that elastase-induced apoptosis of lung epithelial cells is mediated by a PAR-1-triggered pathway involving activation of NF- κ B and p53, and a PUMA- and Bax-dependent increase in mitochondrial permeability leading to activation of distal caspases. Further, p53 contributes to elastase-induced apoptosis by both transcriptional and post-transcriptional mechanisms.

Keywords: inflammation; lung injury; neutrophils; proteinase; mitochondria

Unregulated activation of neutrophils with release of cytotoxic compounds including reactive oxygen species (ROS) and proteinases such as leukocyte elastase (LE) is thought to contribute to the pathogenesis of inflammatory injury to the kidneys (1), heart (2), GI tract (3, 4), and lungs (5–9). In these organs, cellular injury, if sufficiently severe, may proceed to death via necrosis or apoptosis. Apoptosis is a physiologic process that is pivotal during development and in tissue remodeling during repair after injury, but if excessive or unregulated, can contribute to organ dysfunction (10, 11). In the lung, evidence of extensive alveolar epithelial cell apoptosis is observed in murine models of lipopolysaccharide-induced acute lung injury (12) and in humans with the acute respiratory distress syndrome (ARDS) (13, 14). Similarly, lungs from patients with chronic obstructive pulmonary disease (COPD) exhibit large numbers of apoptotic alveolar and bron-

CLINICAL RELEVANCE

These data provide insights into the mechanisms by which leukocyte elastase induces apoptosis of lung epithelia. This has important implications for our understanding of the acute respiratory distress syndrome and emphysema, and provides novel targets for therapeutic intervention.

chial epithelial cells and endothelial cells, and this excessive and unregulated apoptosis is believed to contribute to the pathogenesis of the disease (9).

Current concepts of apoptosis suggest that two major signaling pathways, the extrinsic and intrinsic pathways, regulate this process (15). The former is mediated via activation of “death” receptors, whereas the latter is dependent on alterations in mitochondrial permeability. We have recently reported that LE induces lung epithelial apoptosis via proteinase-activated receptor (PAR)-1, leading to the activation of the intrinsic apoptotic pathway (16). However, the mechanisms of apoptosis of lung epithelial cells induced by LE remain incompletely understood.

NF- κ B is a ubiquitously expressed transcription factor that plays a critical role in immune and inflammatory responses and in apoptosis (17–20). The role of NF- κ B in apoptosis is complex, with both pro- and anti-apoptotic effects documented (21, 22). Although NF- κ B is most commonly considered to suppress apoptosis via expression of anti-apoptotic genes, it can also promote apoptosis in response to specific signals (23).

The pro-apoptotic effects of p53, the archetypical tumor-suppressor gene, are key in tumor inhibition (24). However, the molecular mechanisms underlying these effects remain incompletely understood (25). There is evidence that p53 activates NF- κ B during oxidant-induced apoptosis (26). p53 is known to be a direct transcriptional activator of several genes in the apoptotic pathway including Bax (27, 28), Noxa, and p53 up-regulated modulator of apoptosis (PUMA) (29, 30). In addition, recent studies suggest that p53 can regulate apoptosis by post-transcriptional mechanisms involving direct interactions with Bcl-2 family members and modulation of mitochondrial membrane permeability (31).

Herein, we investigate the role of PAR-1 leading to activation of NF- κ B, p53, PUMA, and Bax in apoptosis of lung epithelial cells induced by LE.

MATERIALS AND METHODS

Cell Culture

Primary human small airway epithelial (HSAE) cells (Cambrex, Walkersville, MD) were grown in small airway cell basal medium supplemented with growth factors and antibiotics according to the manufacturer's instructions. BEAS-2B, a human bronchial epithelial

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Figure 1. Leukocyte elastase (LE) induces lung epithelial apoptosis in a time-dependent manner. Cell death detection assay (A, C) and cleaved caspase-3 staining (B, D), were used for the detection of apoptosis. Two different lung epithelial cell types, BEAS-2B cells (A and B), and HSAE cells (C and D) were used. In experiments with HSAE cells, cells in the supernatant were sedimented using a cytocentrifuge and then stained as above. The graphs (A, C) represent the absorbance values relative to control (buffer). The solid bars represent 0.1 U/ml of LE, and the hatched bars represent 0.3 U/ml of human LE. Values are mean $\pm$ SD; * P < 0.05 and ** P < 0.01 compared with control, and +, # P < 0.05 compared with each concentration of LE treatment. (A) n = 8. (C) n = 4. For cleaved caspase-3 staining (B, D), apoptotic cells are shown in green. Representative data from 1 of 4 experiments are shown.

cell line, was provided by Dr. Reen Wu (University of California, Davis). Cells were grown in 1:1 mix of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 Nutrient Mix (DMEM/F12) (GIBCO/BRL, Grand Island, NY) supplemented with 10 μ g/ml of human recombinant insulin, 25 ng/ml of recombinant human epidermal growth factor, 5 μ g/ml of transferrin, 2% (vol/vol) penicillin-streptomycin (all from GIBCO/BRL), and 0.1 μ M of hydrocortisone (Sigma, St. Louis, MO). Both cell types were grown at 37°C in 5% CO₂. Epithelial cells were seeded onto 6- or 12-well tissue culture plastic plates coated with human type VI collagen (Sigma), and after confluence, used for experiments. When using 6-well plates, 0.5 $\times$ 10⁶ cells in 2 ml were seeded and in 12-well plates, 0.2 $\times$ 10⁶ cells were seeded in 1.5 ml media.

Reagents

Human LE isolated from sputum was from EPC (Owensville, MO). IL-1 β and TNF- α were obtained from BioSource International, Inc. (Camarillo, CA). Bay 11-7082, an inhibitor of I κ B- α phosphorylation, I κ B kinase inactive control peptide, I κ B kinase inhibitor peptide, and Pifithrin (PFT)- α were obtained from Calbiochem (San Diego, CA). PAR-1-activating peptide (PAR-1AP: Thr-Phe-Leu-Leu-Arg, TFLLR-NH₂) and PAR-1 control peptide (Arg-Leu-Leu-Phe-Thr, RLLFT-NH₂) were obtained from the Alberta Peptide Institute (Edmonton, AB, Canada).

Antibodies

Primary antibodies included: anti-phospho Ser-32 I κ B- α (rabbit polyclonal); anti-I κ B- α (rabbit polyclonal); anti-phospho Ser-536 NF- κ B p65 (rabbit polyclonal); anti-NF- κ B p65 (rabbit polyclonal); anti-phospho Ser-15 p53 (rabbit polyclonal); anti-phospho Ser-46 p53 (rabbit polyclonal); anti-cleaved caspase-3 (rabbit polyclonal); anti-caspase-3 (rabbit polyclonal); anti-cleaved caspase-3 (rabbit polyclonal) (fluorescein conjugated); anti-mitochondria COX IV (rabbit monoclonal); anti-GAPDH (rabbit monoclonal) (all from Cell Signaling Technology, Beverly, MA); anti-Bcl-x_L (murine monoclonal); anti-Bcl-x_{S/L} (rabbit polyclonal); anti-Bax (rabbit polyclonal); anti-PUMA (rabbit polyclonal), and anti Thrombin R (H-111) antibody (Santa Cruz Biotechnology, Santa Cruz, CA); anti-p53 (murine monoclonal); anti-cytochrome c (murine monoclonal) (BD Biosciences, San Jose, CA); anti- β -actin (murine monoclonal) (ICN, Aurora, OH); anti-H2B (rabbit polyclonal) (Millipore, Temecula, CA).

Apoptosis Analysis

Human lung epithelial cell apoptosis was quantified using the Cell Death Detection ELISA kit (Roche, Mannheim, Germany) that detects the histone region of mono- and oligonucleosomes released during apoptosis. Absorbance at 405 nm in a 96-well plate was

Figure 2. LE induces NF- κ B DNA binding activity during lung epithelial apoptosis. (A) Electrophoretic mobility shift assay (EMSA) analysis of NF- κ B DNA binding activity. The arrow indicates the NF- κ B DNA binding complex. The positive control represents cytomix (10 ng/ml of TNF- α and IL-1 β) treatment of lung epithelial cells. A sample of the positive control was incubated with unlabeled consensus (nNF- κ B), or mutant oligonucleotide (mNF- κ B), respectively. Representative data from 1 of 3 experiments are shown. (B) Western blot analysis of I κ B- α and NF- κ B. The first two panels illustrate I κ B- α phosphorylation at serine 32, and total cellular levels of I κ B- α , respectively. The third and fourth panels illustrate NF- κ B p65 phosphorylation. The fifth panel illustrates α -actin levels as a control for protein loading. Representative data each from 1 of 5 experiments is shown. The first two panels are from one gel and the third through fifth panels are from another gel run in parallel. (C) EMSA analysis of NF- κ B DNA binding activity after treatment of cells with the PAR-1 activating peptide (PAR-1AP) or control peptide. The arrow indicates the NF- κ B DNA binding complex. Representative data from 1 of 3 experiments are shown. (D) Western analysis of phosphor- and total I κ B- α in cells treated with the PAR-1AP or control peptide. β -actin is used as a control for protein loading. (E) Supershift EMSA for p65, p50, p52, Rel B, and c-Rel illustrating DNA binding complexes 4 hours after treatment with LE. The arrow indicates the super-shifted complexes. Representative data each from 1 of 3 experiments are shown. (F) Treatment of BEAS-2B cells with PAR-1 but not control siRNA results in significant diminution of PAR-1 protein levels as determined by Western analysis. The graph represents the mean $\pm$ SD of $n = 3$ experiments. (G) Inhibition of PAR-1 in BEAS-2B lung epithelial cells using siRNA prevents elastase-induced phosphorylation of I κ B- α (upper bands) at serine 32 and degradation of total cellular levels of I κ B- α (middle bands) as determined by Western blot analysis. Levels of β -actin are illustrated as a control for protein loading. (H, I) Densitometric analysis of Western blots of phosphor-I κ B- α (H) and total cellular levels of I κ B- α (I) normalized to β -actin from the experiment depicted in G. These data are representative of the results from 3 separate experiments conducted on different days with different passages of the epithelial cells. (J) Inhibition of PAR-1 using blocking antibodies prevents elastase-induced activation of NF- κ B as determined using an NF- κ B-luciferase reporter plasmid. Co-transfection with a Renilla luciferase plasmid was done as a control for transfection efficiency. These data are representative of the results from 3 separate experiments conducted on different days with different passages of the BEAS-2B epithelial cells.

Figure 2. (continued).

measured using a microplate reader (THERMO max; Molecular Devices, Sunnyvale, CA). Apoptosis was measured in duplicate from 1×10^5 lung epithelial cells from each treatment group and expressed as the absorbance ratio relative to control (32).

Electrophoretic Mobility Shift Assay

Dishes were gently scraped, and cells were collected by centrifugation at $300 \times g$ for 5 minutes. Cells were lysed for 15 minutes at 4°C in a solution containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 0.5% Nonidet P-40. Nuclei were collected by centrifugation at $1,500 \times g$ for 30 seconds and then suspended in a solution of 20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. The mixture was shaken vigorously for 15 minutes at 4°C , and the supernatant was collected after centrifugation for 5 minutes at $10,000 \times g$. Binding reactions were performed with $2 \mu\text{g}$ of nuclear protein in 20 mM HEPES (pH 7.9), 100 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA, 20% glycerol, $2 \mu\text{g}$ of salmon sperm DNA, 2 mM MgCl_2 , and 10,000 cpm of ^{32}P -labeled oligonucleotide. DNA complexes were separated on a 4% polyacrylamide gel in Tris-borate-EDTA. The consensus oligonucleotide for the NF- κ B-binding site (sequence: 5'-AGT TGA GGG GAC TTT CCC AGG C-3') and the mutant oligonucleotide for the NF- κ B-binding site (sequence: 5'-AGT TGA GGC GAC TTT CCC AGG C-3') were labeled by standard procedures. For the gel supershift assays, anti-NF- κ B p65, anti-NF- κ B p50, anti-NF- κ B p52, Rel B, and c-Rel (Santa Cruz Biotechnology) were used.

SDS-PAGE and Immunoblotting

For SDS-PAGE of whole cell extracts, cells were collected with boiling lysis buffer (2% SDS, 10% glycerol, 65 mM Tris/HCl, pH 6.8, 50 mM dithiothreitol) supplemented with a protease inhibitor cocktail (Roche). Subcellular fractions were also collected with the same lysis buffer described above. SDS-PAGE and immunoblotting were performed as described previously in detail (3). The data were analyzed by densitometry using NIH Image.

siRNA Transfection

Short interfering (si)RNA against p53, PUMA, and PAR-1 were provided from Qiagen (Mississauga, ON, Canada). Fifty and 100 nM of p53 siRNA and 100 nM control siRNA, and 10 nM and 50 nM of PUMA siRNA, were transfected into BEAS-2B cells (30–50% confluence) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. After 72 hours of incubation, the degree of apoptosis was assessed and the protein levels were assessed by Western analysis. PAR-1 siRNAs were designed as described (16). Control or PAR-1 siRNA (50 nM) was transfected into BEAS-2B cells with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions for "reverse transfection." After 48 hours of incubation, cells were stimulated with 0.1 U/ml LE for 1 or 4 hours, solubilized in lysis buffer and the cell lysates subjected to SDS-PAGE and immunoblot analysis for PAR-1 (anti-thrombin receptor antibody; Santa Cruz Biotechnology), p-IκB- α , and IκB- α (both antibodies from Cell Signaling Technology, Beverly, MA). The Western blots were analyzed by densitometry using NIH Image J.

NFκB Luciferase Reporter Assay

BEAS-2B cells were co-transfected with 500 ng NF- κ B firefly luciferase plasmid (provided by Dr. David Riches, National Jewish Health, Denver, CO) and 200 ng Renilla luciferase (pGL4; Promega, Madison, WI) plasmid using Lipofectamine 2000, according to the manufacturer's instructions. After 48 hours, in selected experiments, cells were pretreated with $20 \mu\text{g}/\text{ml}$ anti-thrombin receptor (H-111) antibody (Santa Cruz Biotechnology) or normal rabbit IgG for 1 hour before stimulation with 0.1 U/ml LE for 1 hour. Luciferase activity was assessed according to manufacturer's instructions (Promega) using a luminometer. The firefly luciferase activity (reflecting NF- κ B activation) was normalized to that of Renilla luciferase activity.

Cell Fractionation

Lung epithelial cells cultured in 60-mm plastic culture dishes were incubated with LE 0.1 U/ml for 0, 4, 12, and 24 hours. Cells were

Figure 3. NF- κ B inhibitors reduce lung epithelial apoptosis induced by LE. (A) Lung epithelial cells were pretreated with 5 μ M of Bay11-7082 1 hour before exposure to LE for an additional 24 hours. (B) The cells were treated with 50 μ g/ml of I κ B kinase inactive control peptide (I κ BK CP) or the I κ B kinase inhibitory peptide (I κ BK IP) 2 hours before exposure to LE for an additional 24 hours. The graphs represent apoptosis relative to LE treatment samples. Values are mean $\pm$ SD; * P < 0.05, ** P < 0.01 compared with the control samples; ++ P < 0.01 compared with the samples of LE treatment without Bay11-7082 or with I κ B kinase control peptide (n = 8).

washed once with PBS, scraped into PBS, sedimented at $1,000 \times g$ for 5 minutes, and resuspended in hypotonic buffer (10 mM NaCl, 5 mM MgCl₂, 10 mM Tris-HCl [pH 7.5], 100 μ M PMSF). Cells were allowed to swell on ice for 10 minutes and homogenized with a tight pestle using a 21-G needle (10 strokes) before lysis by additional of isotonic homogenizing buffer (2.5 $\times$ MS buffer, 525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl [pH 7.5], and 2.5 mM EDTA [pH 7.5]). After mixing, cell fragments were sedimented at $1,300 \times g$. Supernatants were collected and centrifuged at $17,000 \times g$ for 15 minutes. After centrifugation, pellets were resuspended in 1 $\times$ MS buffer and used as the heavy membrane fraction containing mitochondria. The supernatants were further centrifuged at $100,000 \times g$ for 1 hour, and resulting supernatants were used as the cytosol fraction. These fractions were used for Western analysis.

Immunoprecipitation

Cells were fractionated according to published methods (33, 34). Cells were lysed by homogenization in lysis buffer (10 mM HEPES [pH 7.4], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitors). Before centrifugation, NP-40 and NaCl were added to 0.5% and 200 mM. Ammonium sulfate (15–20%) was added to precipitate

proteins, and the concentration increased to 20 to 40% to concentrate cytoplasmic extracts to detect PUMA and p53. Proteins from both cytoplasmic and nuclear fractions were cleared of nonspecific protein/IgG interactions with normal mouse IgG before immunoprecipitation using anti-Bcl-x_L (mouse monoclonal) antibody. Protein A/G plus agarose (Santa Cruz Biotechnology) was used at each stage to sediment the immune complexes. All precipitates were washed extensively with the lysis buffer and precipitated proteins were eluted using Bcl-x_L (H-5) peptide in HE buffer (10 mM HEPES [pH 7.4], 1 mM EDTA). The proteins were released by boiling for 5 minutes in Laemmli sample buffer, and separated by SDS-PAGE as described (16).

Cleaved Caspase-3 Staining

Lung epithelial cells were cultured on glass chamber slides (Lab-Tek, Naperville, IL) and incubated with PBS (as a negative control), LE for 18 hours, with or without preincubation of I κ B kinase inhibitor peptide, I κ B kinase inactive control peptide, or PFT- α . Cells were labeled with fluorescein-conjugated anti-cleaved caspase-3 antibody according to the manufacturer's instructions. After labeling, cells were observed using fluorescence microscopy (LEICA DM-IRB) and Open lab (Improvision Inc., Lexington, MA) was used for data acquisition and analysis.

Statistical Analysis

Parametric data were compared by using t tests for mean values or ANOVA with correction for multiple comparisons (Fisher's PLSD test) when more than two variables were analyzed using STATView software.

RESULTS

LE Induces Lung Epithelial Apoptosis

To characterize the mechanisms by which LE induces apoptosis of lung epithelial cells, we quantified elastase-induced apoptosis by monitoring the presence of histone-associated mono- and oligonucleosomes (Figures 1A and 1C) and by assessment of cleavage of caspase 3 (Figures 1B and 1D). We used the airway epithelial cell line, BEAS-2B (Figures 1A and 1B), as well as primary cultures of human small airway epithelial cells (SAEC) (Figures 1C and 1D). As illustrated, human LE induced apoptosis in a time- and dose-dependent manner peaking between 12 and 24 hours (Figures 1A and 1C). With more prolonged exposure (> 48 h), many epithelial cells became necrotic, especially at the highest concentration (0.3 U/ml) of elastase. These observations were confirmed independently by immunofluorescence assessment of cleavage of caspase-3 using an anti-cleaved caspase-3 antibody (Figures 1B and 1D). These observations confirm that treatment of lung epithelial cells with LE induces apoptosis at physiologic concentrations and in a dose- and time-dependent manner.

NF- κ B DNA Binding Activity of Lung Epithelial Cells Is Induced by LE

To ascertain the potential role of NF- κ B in elastase-induced apoptosis, we assessed NF- κ B DNA binding activity in nuclear extracts of BEAS-2B cells and human SAEC using an electrophoretic mobility shift assay (EMSA). Treatment of cells with elastase resulted in NF- κ B activation in a time-dependent manner peaking at 4 hours that returned to baseline levels by 24 hours (Figure 2A). The NF- κ B-binding site mutant oligonucleotide was used as a competitor to demonstrate the specificity of the assay. We also used Western analysis to assess the total cellular levels and phosphorylation status of I κ B- α . Figure 2B (panels 1 and 2) illustrates that phosphorylation of I κ B- α was enhanced after 4 hours of the treatment and further, that I κ B- α was degraded in response to elastase treatment.

Figure 4. LE induces phosphorylation of p53. The *first panel* illustrates phosphorylation of p53 at serine 15, the *second panel* illustrates phosphorylation of p53 at serine 46, the *third* shows total p53, and the *last panel* represents α -actin (control) expression from BEAS-2B cells after treatment with LE using Western analysis. The *graphs* illustrate densitometry units normalized to the level of α -actin. Values represent mean $\pm$ SD. Representative data each from 1 of 5 experiments are shown.

We have previously reported that the pro-apoptotic effects of LE are mediated in part via activation of PAR-1 (16). To determine if activation of NF- κ B was mediated via PAR-1 under these conditions, epithelial cells were treated with the PAR-1AP or control peptide. Figure 2C illustrates that the PAR-1AP but not the control peptide resulted in NF- κ B activation. In addition, the PAR-1AP but not the control peptide resulted in phosphorylation of I κ B- α on serine 32, peaking at 4 hours (Figure 2D). This was accompanied by a decrease in levels of total I κ B- α (Figure 2D), compatible with its known proteasome-mediated degradation.

To identify the individual subunits of the NF- κ B complex involved in the elastase-induced NF- κ B activation, we used a “super shift” assay with antibodies to specific components of the NF- κ B complex. Figure 2E illustrates that LE induced NF- κ B complexes containing heterodimers of p50 and p65 but not p52, Rel-B, or c-Rel. In addition, Western analysis revealed that the p65 subunit of NF- κ B was phosphorylated 4 hours after treatment with elastase (Figure 2B, *panels 3 and 4*), providing independent confirmation of the involvement of p65 in this pathway.

To provide more direct evidence for the role of PAR-1 in LE-mediated activation of NF- κ B, two independent approaches were taken. First, we used siRNA to inhibit PAR-1 expression as previously reported (16). Under these conditions, in which PAR-1 expression was significantly diminished (Figure 2F), there was a corresponding diminution of elastase-induced phosphorylation of I κ B (Figure 2G, *upper band*). Further, PAR-1 inhibition prevented elastase-induced degradation of total I κ B (Figure 2G, *middle band*). Figures 2H and I represent the densitometric analysis of the corresponding Western blots. As a second independent method to inhibit PAR-1, we used blocking antibodies. Inhibition of PAR-1 by a blocking (but not

a control) antibody prevented NF- κ B activation as determined using an NF- κ B-luciferase reporter system (Figure 2J).

NF- κ B Inhibitors Reduce LE-Induced Apoptosis

To investigate the physiologic consequences of NF- κ B activation under these conditions and whether it served as a pro- or anti-apoptotic factor, we assessed the effects of two pharmacologic inhibitors of NF- κ B—Bay 11-7082, an I κ B- α inhibitor, and the I κ B kinase inhibitory peptide—on elastase-induced apoptosis. Both Bay 11-7082 and the I κ B kinase inhibitory peptide, but not the control peptide, largely prevented apoptosis of lung epithelial cells (Figures 3A and 3B). These data indicate that NF- κ B acts primarily as a pro-apoptotic factor under these conditions.

LE Activates p53

To investigate the pathways downstream of NF- κ B that mediate epithelial apoptosis, we focused on p53 based on previous reports in other cell types (35). p53 plays a major role in the cellular response to DNA damage and other genomic aberrations and activation of p53 can lead to either cell cycle arrest and DNA repair or to apoptosis (36). With respect to pro-apoptotic pathways, phosphorylation of p53 on serine 46 enhances its ability to induce apoptosis (37). As illustrated in Figure 4, p53 was phosphorylated on both serine 15 and serine 46 in response to elastase treatment. Phosphorylation of p53 on serine 15 was detectable after 4 hours and preceded phosphorylation of serine 46, the latter peaking 24 hours after elastase treatment. The total cellular levels of p53 were not altered under these conditions. Additional studies revealed no change in phosphorylation of p53 at ser20, ser37, or ser392 under these conditions (not illustrated). We conclude that p53 is phosphorylated and (by inference) activated in response to LE.

Figure 5. Role of p53 and NF- κ B in elastase-induced lung epithelial apoptosis. (A) Cells were pre-treated with 50 μ M of the p53 inhibitor PFT- α for 2 hours and then exposed to LE for an additional 24 hours. The graph represents apoptosis relative to LE-treated samples. Values are mean $\pm$ SD; * P < 0.05, ** P < 0.01 compared with control samples without PFT- α ; ++ P < 0.01 compared with leukocyte elastase treated samples (n = 7). (B) BEAS-2B cells were transfected with 50 nM p53 or control siRNA followed by exposure to LE for an additional 24 hours. The upper panel illustrates p53 expression and the lower panel α -actin protein expression assessed by Western analysis. Representative data from 1 of 4 experiments are shown. The graph shows densitometry units normalized to levels of α -actin. The values are mean $\pm$ SD. (C) BEAS-2B cells were treated with LE 0.1 U/ml or buffer 24 hours after transfection with p53 or control siRNA. Values are mean $\pm$ SD; ** P < 0.01 compared with control siRNA transfection and buffer only. ++ P < 0.01 compared with control siRNA transfection followed by LE treatment (n = 6). (D) Treatment of cells with an NF- κ B inhibitor reduces phosphorylation of p53 at serine 46. Cells were pretreated with 50 μ g/ml of either IkBK CP or IkBK IP and then exposed to LE. Total cellular protein was collected for Western analysis. The left upper panel represents phosphorylation of p53 at serine 46 in the presence or absence of the IkBK CP. The upper right panel shows cells pretreated with the IkBK IP. α -actin levels are illustrated as a control. Representative data each from 1 of 3 experiments are shown. (E) An NF- κ B inhibitor blocks elastase-induced increases in nuclear and cytosolic p53. Nuclear and cytosolic proteins were collected from cells pretreated with Bay11-7082 at 0, 4, 12, and 24 hours after treatment with 0.1 U/ml LE followed by Western analysis using anti-p53 antibody. Histone H2B was used as a control for the nuclear fraction and GAPDH was used as a control for the cytosolic fraction. Representative data from 1 of 3 experiments are shown. The graphs depict densitometric analysis normalized to the level of the protein markers of each fraction. The solid bars represent samples treated with 0.1 U/ml of leukocyte elastase, and the hatched bars represent samples pretreated 5 μ M of Bay11-7082 before exposure to LE. Values are mean $\pm$ SD.

Figure 6. LE induces PUMA expression by lung epithelial cells. (A) Western analysis of PUMA, Noxa, Bax, and Bcl-x_L. Total cellular protein was collected 0, 4, 12, and 24 hours after treatment with 0.1 U/ml LE. The panels illustrate PUMA, Noxa, Bax, and Bcl-x_L, respectively, and the last panel illustrates α -actin as a control. Representative data each from 1 of 5 experiments are shown. (B) Immunoprecipitation (IP) analysis of the PUMA-Bcl-x_L and p53-Bcl-x_L complexes. Cytoplasmic proteins were collected after 0, 4, 12, and 24 hours of treatment with LE (0.1 U/ml). After IP, proteins in the immune complexes were solubilized and analyzed by SDS-PAGE and Western analysis. The first panel illustrates p53, and the second panel PUMA co-immunoprecipitation with Bcl-x_L. The last panel represents Bcl-x_L as a control for loading. Representative data each from $n = 3$ experiments are shown. The graph shows densitometry units normalized to the level of Bcl-x_L. Values are mean $\pm$ SD.

Blockade of p53 Reduces LE-Induced Lung Epithelial Cells Apoptosis

Previous studies have documented that p53 exerts potent pro-apoptotic effects (24, 38, 39). To determine more definitively the role of p53 in elastase-induced apoptosis in our experimental system, we used two independent approaches to block p53, and examined the effects on elastase-induced apoptosis. As an initial approach, we used pifithrin- α (PFT- α), a pharmacologic inhibitor of p53. A quantity of 50 μ M PFT- α largely prevented apoptosis of lung epithelial cells induced by LE (Figure 5A). As an alternative approach, we used RNA interference. As illustrated in Figure 5B, siRNA directed against p53 substantially reduced p53 protein levels as assessed by Western analysis. Maximum inhibition of

p53 expression without apparent cellular toxicity was achieved at 50 nM of p53 siRNA. Under these conditions, elastase-induced epithelial apoptosis was substantially reduced (Figure 5C). Together, these observations provide direct evidence that LE induces lung epithelial apoptosis via p53 activation.

NF- κ B Inhibitor Blocks Elastase-Induced Phosphorylation of p53 and Nuclear and Cytosolic p53

To investigate the relationship between NF- κ B and p53 in elastase-induced apoptosis, we assessed the effects of an NF- κ B inhibitor on phosphorylation of p53 on serine 46. Figure 5D illustrates that the I κ B kinase inhibitory peptide, but not the control peptide, blocked elastase-induced p53 phosphorylation under these conditions. To characterize the mechanism of this response, we examined the effect of the NF- κ B inhibitor on the subcellular localization of p53. LE also induced an increase in nuclear p53 that was prevented by the NF- κ B inhibitor, Bay 11-7082. Similarly, the delayed (12–24 h) increase in cytosolic p53 was also prevented by the inhibitor. Together, these data indicate that p53 is downstream of NF- κ B in the pro-apoptotic pathway triggered by LE and that phosphorylation and translocation of p53 is regulated by NF- κ B.

LE Induces Expression of PUMA

To explore the pathways downstream of p53 mediating elastase-induced apoptosis, we focused on potential target genes of p53. These include Bax, a member of the pro-apoptotic Bcl-2 family, and Noxa and PUMA, BH3 only domain-containing proteins that are transcriptionally regulated by p53 (29, 30). Conversely, Bcl-x_L, also a member of the Bcl-2 family, functions to suppress apoptosis. We assessed whether expression of these apoptosis-modulating proteins was altered after treatment with elastase using Western analysis. These studies revealed that expression of PUMA was induced in a time-dependent manner by LE (Figure 6A). In contrast, expression of Bax, Noxa, and Bcl-x_L was not altered under these conditions.

In addition to transcriptional regulation, p53 can exert post-transcriptional effects on these proteins. For example, cytoplasmic p53 binds to Bcl-x_L protein and in turn, PUMA can bind to Bcl-x_L displacing p53 (34). To determine if elastase induced alterations in binding of PUMA, p53, and Bcl-x_L, we used immunoprecipitation. As illustrated in Figure 6B, elastase treatment induced increased binding of PUMA to Bcl-x_L, whereas binding of p53 to Bcl-x_L was diminished. These observations are consistent with the notion that elastase increases PUMA expression that is followed by binding of PUMA to Bcl-x_L and displacement of p53.

LE Alters p53 Localization

Recent studies have provided evidence that cytosolic and mitochondrial p53 regulate mitochondrial permeability through activation of Bax, an example of a transcription-independent regulation of apoptosis (31, 40–42). To characterize further the post-transcriptional effects of p53 in elastase-induced apoptosis, we assessed alterations in the subcellular distribution of p53 using cell fractionation followed by Western analysis of cytosolic, mitochondrial, and nuclear fractions. As illustrated in Figure 7A, treatment of epithelial cells with LE induced complex alterations in the subcellular distribution of p53. There was an increase in mitochondrial levels of p53 detectable at 4 hours and sustained to 24 hours. There was also a slightly delayed increase in nuclear p53 reaching a maximum between 12 and 24 hours. Cytosolic levels of p53 were diminished after 4 hours, compatible with mitochondrial and nuclear translocation. The cytosolic levels of p53 subsequently increased to baseline levels by 12 hours.

Figure 7. (A) LE induces alterations in the subcellular distribution of p53. Nuclear, mitochondrial, and cytosolic cell fractions were collected at 0, 4, 12, and 24 hours after treatment of cells with 0.1 U/ml of LE. The upper panels illustrate levels of nuclear p53, mitochondrial p53, and cytosolic p53. Histone H2B, COX IV, and GAPDH are used as markers for each of these fractions. Representative data each from 1 of 3 experiments are shown. The graphs depict densitometric analysis normalized to the level of the protein markers of each fraction. Values are mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$. (B) LE induces Bax translocation from cytosol to mitochondria, and release of mitochondrial cytochrome c to the cytosol. Cells fractions were collected after 0, 4, 12, and 24 hours of incubation with 0.1 U/ml of leukocyte elastase. COX IV is used as a marker for the mitochondrial fraction and GAPDH as a marker for the cytosolic fraction. Representative data from 1 of 5 experiments are shown.

These observations are compatible with both transcriptional (i.e., nuclear translocation) and post-translational (i.e., mitochondrial effects) effects of p53 in elastase-induced apoptosis.

Mitochondrial Permeability Is Increased via Bax Translocation

In other systems, enhanced expression and binding of PUMA to Bcl-x_L results in displacement of p53 and accumulation of free cytosolic p53 that can in turn activate Bax (34, 42). Accordingly, we sought to determine if elastase induced Bax translocation to the mitochondria, resulting in cytosolic release of cytochrome c, using Western analysis of mitochondrial and cytosolic fractions. Figure 7B illustrates that elastase treatment of epithelial cells induced mitochondrial translocation of Bax after 12 hours. This correlated with release of cytochrome c to the cytosol with reciprocal reduction in the mitochondrial levels of the enzyme. COX IV was used here as a mitochondrial marker and GAPDH was used as a cytosolic marker. These observations are consistent with the notion that leukocyte elastase induces the translocation of Bax to the mitochondria, increasing mitochondrial membrane permeability and inducing release of cytochrome c to the cytosol, conditions that promote apoptosis.

Inhibition of NF- κ B and p53 Reduces PUMA Expression

To characterize the downstream consequences of NF- κ B-dependent p53 modulation in elastase-induced apoptosis, we used inhibitors of p53 and NF- κ B and monitored expression of PUMA. Figure 8A illustrates that both NF- κ B and p53 inhibitors blocked elastase-induced PUMA expression. Further,

inhibition of NF- κ B or p53 prevented elastase-induced formation of PUMA-Bcl-x_L complexes (Figure 8B), indicating that NF- κ B and p53 mediate elastase-induced apoptosis via enhanced expression of PUMA and binding to Bcl-x_L.

Blockade of PUMA Attenuates Elastase-Induced Apoptosis of Lung Epithelial Cells

To determine more directly the role of PUMA in elastase-induced apoptosis in our experimental system, we used RNA interference. As illustrated in Figure 8C, siRNA against PUMA substantially reduced the levels PUMA protein as assessed by Western analysis. The maximum inhibition of p53 protein expression without apparent cellular toxicity was achieved at 50 nM of PUMA siRNA. Under these conditions, inhibition of PUMA significantly reduced epithelial apoptosis induced by LE (Figure 8D). These observations provide direct evidence that PUMA is pro-apoptotic under these conditions.

Inhibition of NF- κ B and p53 Reduces Elastase-Induced Mitochondrial Permeability

To provide more direct evidence for the role of NF- κ B and p53 in alterations of mitochondrial permeability, we inhibited p53 and NF- κ B, and monitored mitochondrial translocation of Bax and release of cytochrome c to the cytosol using Western analysis. As illustrated in Figure 9A, pretreatment of cells with the NF- κ B inhibitor Bay 11-7082 or the p53 inhibitor PFT- α substantially attenuated elastase-induced mitochondrial translocation of Bax. In addition, elastase-induced translocation of cytochrome c was

Figure 8. The role of NF- κ B in elastase-induced PUMA expression. (A) Inhibition of NF- κ B or p53 reduces PUMA expression. Cells were pretreated with 50 μ g/ml of IkBK CP, IkBK IP, 5 μ M of Bay11-7082, and 50 μ M of PFT- α , respectively. Total cellular proteins were collected 0, 4, 12, and 24 hours after treatment with LE. The *upper panels* illustrate PUMA expression by Western analysis. α -actin was used as a control for protein loading. Representative data from 1 of 3 experiments are shown. (B) Inhibition of NF- κ B and p53 reduces the PUMA-Bcl-x_L complex. Cells were pretreated with Bay11-7082 or PFT- α . Cytoplasmic proteins were collected 24 hours after treatment with LE. After immunoprecipitation of Bcl-x_L, the immune complexes were solubilized and analyzed by SDS-PAGE, followed by Western blotting. The *upper panel* represents co-immunoprecipitation of PUMA and Bcl-x_L. The *lower panel* illustrates Bcl-x_L levels as a control. Representative data each from 1 of 3 experiments are shown. The *graph* depicts densitometry units normalized to the level of Bcl-x_L. Values are mean $\pm$ SD. (C) siRNA knockdown of PUMA substantially reduces the levels PUMA protein as assessed by Western analysis. (D) siRNA knockdown of PUMA substantially reduces elastase-induced of lung epithelial cell apoptosis with maximum inhibition observed with 50 nM siRNA.

also reduced under these conditions. These observations indicate that LE-induced epithelial apoptosis is directly related to activation of NF- κ B and p53 through mitochondrial translocation of Bax and release of cytochrome *c* into the cytosol.

Inhibition of NF- κ B and p53 Reduces Activation of Caspase-3

To determine the role of NF- κ B and p53 in the terminal events involved in apoptosis, we assessed cleavage of caspase-3 using Western analysis and immunofluorescence microscopy. Figure 9B illustrates that treatment of cells with the IkB kinase inhibitory peptide but not the control peptide reduced cleavage of caspase-3 after elastase treatment. Similarly, treatment of cells

with PFT- α also diminished LE-induced cleavage of caspase-3. These results were confirmed using immunofluorescence microscopy to detect cleaved caspase-3 (Figure 9C, *panels b* and *c*). Similar effects were observed in lung epithelial cells pretreated with PFT- α (Figure 9C, *panel d*). These observations provide strong evidence for the role of NF- κ B and p53 in the distal stages of apoptosis induced by elastase as reflected by caspase-3 cleavage.

DISCUSSION

In this study, we provide evidence that apoptosis of lung epithelial cells induced by leukocyte elastase is mediated by

Figure 9. (A) Inhibition of p53 and NF- κ B reduces mitochondrial Bax and cytosolic cytochrome c. Cells were pretreated with 5 μ M of Bay11-7082 or 50 μ M of PFT- α . After pretreatment, cells were incubated with LE for 24 hours followed by cellular fractionation. The first panel represents mitochondrial Bax protein expression and the second panel shows COX IV used here as a marker for the mitochondrial fraction. The third panel shows cytosolic cytochrome c levels, and the fourth panel illustrates GAPDH as a marker for the cytosolic fraction. Representative data from 1 of 4 experiments are shown. The graphs depict densitometry units normalized to the levels of each fraction marker. Values are mean $\pm$ SD. (B) The upper panel illustrates cleaved caspase-3 levels, and the lower panel shows α -actin levels by Western analysis. BEAS-2B cells were pretreated with I κ B kinase inactive control peptide or I κ B kinase inhibitory peptide for 1 hour, or with 50 μ M of PFT- α for 2 hours. Representative data from 1 of 4 experiments are shown. (C) BEAS-2B cells were pretreated as described above and exposed to LE for an additional 18 hours. The upper panels represent anti-cleaved caspase-3-labeled epithelium (green fluorescence). The lower panels represent transmitted light images. Representative data from 1 of 3 experiments are shown.

PAR-1-dependent activation of NF- κ B leading to p53 activation, induction of PUMA expression, mitochondrial translocation of Bax with release of mitochondrial cytochrome c, and activation of effector caspases (Figure 10). These data provide a comprehensive molecular mechanism by which leukocyte elastase induces apoptosis of lung epithelial cells via that has pathophysiologic relevance for diverse pulmonary diseases.

Our observations build on an emerging literature indicating that proteolytic enzymes such as elastase can trigger both physiologic and pathologic cellular responses by nondegradative mechanisms. For example, treatment of Kupffer cells with pancreatic elastase induced NF- κ B activation in a rat model of pancreatitis (43). The aspartic proteinase cathepsin D can directly cleave caspase 8, thus initiating neutrophil apoptosis (44). LE induces NF- κ B activation in A549 cells, leading to IL-8 gene transcription (45). Our studies confirm and extend these observations; we provide evidence that LE induces NF- κ B activation in lung epithelial cells, leading to apoptosis. However, there are apparently contradictory reports as to whether NF- κ B exerts pro-apoptotic or anti-apoptotic effects (21–23). In the current study, NF- κ B appears to act predominantly as a pro-apoptotic factor, inasmuch as both NF- κ B inhibitors, Bay 11-7082 and the I κ B kinase inhibitory peptide, blocked apoptosis induced by LE. A recent publication has underscored that both pro- and anti-apoptotic effects of NF- κ B are possible and that the predominating effect is dependent on the experimental circumstances (46).

It is known that NF- κ B can transcriptionally up-regulate p53 (47–49), and this response has been implicated as a primary

mechanism in camptothecin-induced apoptosis in cortical neurons (50). Our data indicate that LE induces NF- κ B-dependent activation of p53 via phosphorylation of serine 46 that in turn leads to enhanced expression of PUMA. Further, NF- κ B inhibition prevented translocation and phosphorylation of p53, indicating that p53 is downstream of NF- κ B in this pathway.

Our data provide evidence for a key role for p53 in elastase-induced epithelial apoptosis. We used two independent approaches to inhibit p53: pharmacologic inhibition with PFT- α and siRNA gene knockdown. The concentration of PFT- α was chosen based on previous reports in the literature using cardiac (51) and neural cells (52). Both pharmacologic inhibition and siRNA-mediated gene silencing of p53 yielded similar results.

As mentioned above, PUMA, a BH3 only protein and pro-apoptotic member of the Bcl-2 family, is an essential component of a highly conserved pathway leading to apoptosis (25). Members of the BH3 only family, via binding to the anti-apoptotic protein Bcl-2 or its close relatives, initiate an apoptotic program that proceeds through Bax-like family members, leading to activation of the effector caspases (53, 54). Transcription of these genes, including Bax, PUMA, and Noxa (another BH3 only subfamily member), is known to be directly activated by p53-binding sites in the regulatory region of the gene (29, 30, 55). In the current study, PUMA was the only member of this subfamily that was induced by elastase.

Our data also provide evidence that LE induces translocation of Bax from the cytosol to the mitochondria (Figure 7), thus linking Bax to elastase-induced apoptosis. There is evidence in the literature that Bax is a key pro-apoptotic factor in lung cells

Figure 10. Model of the proposed pathways involved in apoptosis of lung epithelial cells induced by LE. PAR-1 is triggered in response to LE treatment, leading to activation of NF- κ B and p53 and enhanced expression PUMA (transcriptional effect). Increased levels of PUMA protein displace p53 from Bcl- x_L , liberating free cytosolic p53 that in turn activates Bax that translocates to the mitochondria activating the intrinsic apoptotic pathway (post-translational effect).

in response to hyperoxia in mice (56). The p53 apoptosis-inducing protein 1 (p53AIP1) is also known to be directly activated by p53 (37). This protein is located in mitochondria, and when overexpressed, induces apoptosis of cells via phosphorylation of p53 at serine 46. Our preliminary studies failed to demonstrate any alterations in p53AIP1 expression response to LE (data not shown).

Recent studies have defined several distinct roles for p53 in induction of apoptosis, including both transcriptional and post-transcriptional mechanisms (31). Alterations in the subcellular localization of p53 directly alter mitochondrial permeability and induce apoptosis via a series of complex interactions with Bcl-2 family members (34, 42, 57). In response to cellular stress induced by DNA damage, the anti-apoptotic protein Bcl- x_L sequesters cytoplasmic p53 while nuclear p53 induces transcription of PUMA. PUMA then binds Bcl- x_L , displacing p53, which in turn activates Bax to induce mitochondrial permeability (Figure 10). Bax is predominantly cytosolic, but in response to pro-apoptotic stimuli it is translocated to mitochondria, where it inserts into outer mitochondrial membrane and undergoes conformational changes that lead to increased permeability of the membrane (58). Accumulation of cytosolic p53 directly induces activation of Bax (42). Further, in response to genotoxic stress, there is p53-dependent enhanced transcription of PUMA that in turn binds Bcl- x_L displacing p53, thereby allowing p53-dependent activation of Bax (34, 57). Our data support this model and provide strong evidence that NF- κ B signals elastase-induced apoptosis via transcriptional and post-transcriptional consequences of p53 activation.

Although our current study clearly demonstrates that LE induces lung epithelial apoptosis via a p53-dependent pathway, there are other potential mechanisms. As illustrated in Figure

5C, p53 siRNA diminished elastase-induced apoptosis by around 50%, despite the nearly complete knock-down of p53 expression, suggesting the involvement of other pathways. Examples of other potentially relevant pathways include the PI3 kinase-Akt/PKB pathway and the MAP kinase pathway (59–61).

Our studies indicate that PAR-1 is upstream of NF- κ B in the pathway involved in elastase-induced apoptosis. However, it is possible that other surface receptors are also activated by elastase. Of particular interest is the family of death receptors. Fas-mediated cell death through NF- κ B activation was observed in cells treated with TNF- α and IFN- γ (62). Also, NF- κ B differentially regulates TNF-related apoptosis-inducing ligand (TRAIL) receptor-like DR5 expression involving histone deacetylase 1 (63). Previous publications have suggested that the role of the Fas-R/Fas-L system in neutrophil-induced epithelial apoptosis of GI epithelial cells may be minor (64). However, recent studies have provided evidence that NF- κ B mediates apoptosis of Kupffer cells in response to pancreatic elastase through transcriptional activation of Fas/FasL (43). Thus, death receptors may contribute to pro-apoptotic signaling in epithelial cells in response to neutrophil elastase.

Our observations have relevance to diverse lung diseases characterized by inflammatory injury to lung parenchymal cells, such as acute lung injury (ALI)/ARDS and COPD. Epithelial apoptosis in the setting of increased levels of leukocyte elastase is observed in ALI/ARDS (13, 14). It is also recognized that LE plays a pivotal role in the pathogenesis of COPD by mechanisms involving apoptosis of lung epithelial cells (65). In this regard, mitigation of inappropriate apoptosis may be a viable therapeutic strategy for treatment of ALI/ARDS and COPD. Thus, a better understanding of the mechanisms by which LE induces epithelial apoptosis may help inform treatment of lung diseases that are consequent to unregulated inflammation.

The relevance of observations in our *in vitro* model system to events *in vivo* in intact animal models and in humans merits consideration. In this regard, intratracheal administration of elastase into murine lungs induces apoptosis of lung epithelial cells (66, 67), supporting the relevance of *in vitro* observations to events in intact animals. Second, inhibition of neutrophil elastase attenuates lung injury in several animal models (68–71) and in patients with ALI after cardio-pulmonary bypass (72). However, treatment with a neutrophil elastase inhibitor was not effective in improving clinical outcomes in a multinational study of 492 patients with ALI (73). It is noteworthy that the concentrations of elastase used in our *in vitro* studies are within the range of levels recorded in intact animals and humans. For example, elevated levels of neutrophil elastase (up to 400 ng/ml) were noted in BAL fluid from horses exposed to moldy hay (74). Further, levels of active neutrophil elastase ranging from 0.15 to 86 μ M have been recorded in the epithelial lining fluid recovered by BAL from patients with cystic fibrosis (75, 76). Thus, the concentrations of elastase in our *in vitro* model system are well within the range of those present in intact lungs in animals and humans.

In summary, we provide evidence that LE induces apoptosis of lung epithelial cells via PAR-1-, NF- κ B-, and p53-dependent pathways involving up-regulation of PUMA leading to enhanced mitochondrial permeability consequent to Bax translocation. Under these circumstances, NF- κ B functions as a pro-apoptotic factor. Activated NF- κ B signals p53 phosphorylation, PUMA induction, and Bax translocation from cytosol to mitochondria, leading to increased permeability of mitochondria, cleavage of caspase-3, and the completion of the apoptotic process.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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