p53 Mediates Cigarette Smoke–Induced Apoptosis of Pulmonary Endothelial Cells

Inhibitory Effects of Macrophage Migration Inhibitor Factor

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Exposure to cigarette smoke (CS) is the most common cause of emphysema, a debilitating pulmonary disease histopathologically characterized by the irreversible destruction of lung architecture. Mounting evidence links enhanced endothelial apoptosis causally to the development of emphysema. However, the molecular determinants of human endothelial cell apoptosis and survival in response to CS are not fully defined. Such determinants could represent clinically relevant targets for intervention. We show here that CS extract (CSE) triggers the death of human pulmonary macrovascular endothelial cells (HPAECs) through a caspase 9–dependent apoptotic pathway. Exposure to CSE results in the increased expression of p53 in HPAECs. Using the p53 inhibitor, pifithrin- α (PFT- α), and RNA interference (RNAi) directed at p53, we demonstrate that p53 function and expression are required for CSE-mediated apoptosis. The expression of macrophage migration inhibitory factor (MIF), an antiapoptotic cytokine produced by HPAECs, also increases in response to CSE exposure. The addition of recombinant human MIF prevents cell death from exposure to CSE. Further, the suppression of MIF or its receptor/binding partner, Jun activation domain–binding protein 1 (Jab-1), with RNAi enhances the sensitivity of human pulmonary endothelial cells to CSE via a p53-dependent (PFT- α -inhibitable) pathway. Finally, we demonstrate that MIF is a negative regulator of p53 expression in response to CSE, placing MIF upstream of p53 as an antagonist of CSE-induced apoptosis. We conclude that MIF can protect human vascular endothelium from the toxic effects of CSE via the antagonism of p53-mediated apoptosis.

Keywords: endothelial cells; p53; macrophage migration inhibitory factor; cigarette; apoptosis

Exposure to cigarette smoke is unquestionably the most frequent cause of chronic obstructive pulmonary disease (COPD), typified by emphysema. As the fifth leading cause of death worldwide, COPD is responsible for an estimated 2.5 million deaths annually (1). Accelerated endothelial cell apoptosis is now recognized as an important early determinant of lung destruction and resultant emphysema in COPD. Multiple investigators demonstrated that endothelial cell apoptosis is increased in the lung tissue of smokers and patients with COPD, compared with nonsmokers (2–8). Endothelial cell apoptosis is sufficient to induce emphysema in multiple animal models of COPD (4, 9, 10). The blockade of apoptosis prevents the

CLINICAL RELEVANCE

Exposure to cigarette smoke (CS) is the most common cause of emphysema. Mounting evidence links enhanced endothelial apoptosis causally to the development of emphysema. We conclude that MIF can protect human vascular endothelium from the toxic effects of CS via the antagonism of p53-mediated apoptosis.

development of disease in certain models, suggesting that apoptosis is necessary for the development of emphysematous changes in the lung (9–15). Oxidative stress, inflammation, and protease/antiprotease imbalance, all theorized to be mechanisms in the development of COPD, have the potential to promote endothelial apoptosis, emphasizing endothelial apoptosis as a potential nodal point in the pathogenesis of COPD. As such, apoptosis becomes an attractive target for therapeutic intervention. However, the molecular determinants of cigarette-induced endothelial cell apoptosis are incompletely defined. Cigarette smoke (CS) is known to contain numerous potential apoptogens, including reactive oxygen species (ROS) and the bacterial toxin LPS. Thus, the interplay between these cytotoxic agents within CS and the endogenous prosurvival and apoptotic cascades constitutes an area of active investigation where information on specific mechanisms could provide therapeutic targets to prevent endothelial cell apoptosis and ultimately the progression of COPD.

The tumor-suppressor protein, p53, is a well-recognized and well-characterized transcription factor and a key regulator of multiple critical cellular processes, including cell-cycle regulation, DNA repair, and apoptosis (16). Recently, p53 protein expression was demonstrated to be significantly higher in lung tissue from patients with emphysema secondary to smoking, compared with tissue from smokers without emphysema or nonsmokers, suggesting the potential for a direct relationship between the expression of p53 and the development of emphysema (17). Animal studies provide additional preclinical evidence that chronic exposure to CS is sufficient to increase p53 protein expression within the murine lung (18). Additional clinical data linking altered p53 expression with the risk of developing emphysema come from genetic studies that linked polymorphisms of p53 that increase protein expression with an enhanced risk of developing COPD (19). Despite the abundance of data implicating apoptosis causally with the development of emphysema, and data demonstrating changes in p53 expression during CS-induced emphysema, a causal link between p53 expression and CS-induced apoptosis is lacking.

Here, we provide evidence that cigarette smoke extract (CSE) kills human pulmonary endothelial cells (ECs) via a mitochondrial-dependent apoptotic pathway that requires the induction and function of p53. In addition, we identify potential negative regulators of p53 induction in response to

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CSE that may represent additional targets for intervention. Macrophage migration inhibitory factor (MIF), an antiapoptotic cytokine, was linked to tumorigenesis through its ability to antagonize p53 functionally (20). We previously demonstrated that MIF efficiently antagonizes the LPS-induced apoptosis of human pulmonary macrovascular endothelial cells (HPAECs) (21). Here, we provide additional evidence that MIF and its receptor (Jun activation domain-binding protein 1, or Jab-1) function to antagonize CSE-induced death, in part via suppression of the CSE-mediated expression of p53.

MATERIALS AND METHODS

Experimental Protocols

Reagents and cell lines. Primary HPAECs derived from five individual donors (CC-2530; Lonza, Walkersville, MD) were maintained according to the manufacturer's recommendations in endothelial growth media-2 (EGM-2) basal media supplemented with 2% FBS, hydrocortisone, human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), ascorbic acid, human epidermal growth factor (hEGF), heparin, and GA-1000, according to the manufacturer's recommendations. Cells were analyzed between passages 5–8, and were cultured, unless otherwise indicated, in complete media (Lonza). Cells were maintained in phenol red-free media for analysis in caspase activity assays. For each individual experiment, cells were matched for donor and passage number. Results from at least four different donors were pooled. Where indicated, cells were incubated with recombinant human MIF (100 ng/ml final concentration, catalogue number 298-MF; R&D Systems, Minneapolis, MN). Duplex RNAs encoding nontargeting negative control (ctrl) short interfering (si) RNA (On-Target Plus, Non-Targeting Pool), human MIF siRNA (sense strand, ACAGG GUCUACAUCAACUA), and Jab-1 siRNA (catalogue number J005814) were used for RNA interference (RNAi) and were manufactured by Dharmacon, Inc. (Lafayette, CO). A pool of four duplex siRNAs that target p53 was used to suppress total p53 expression (SiGENOME SMARTpool reagent; Dharmacon). The transfection of duplex RNA was performed using Geneporter B reagent (Genelantis, San Diego, CA), according to the manufacturer's recommendations. HPAECs were plated at a density of $1 \times 10^5/\text{cm}^2$, and transfected as previously described (21). The final concentration of RNA duplexes was 50 nM. The p53 inhibitor, cyclic pifithrin- α (PFT- α) (catalogue number P4236; Sigma-Aldrich, St. Louis, MO), was used at a final concentration of 10 μM , and the caspase 8 and 9 inhibitors, z-IEDT and z-LEHD (catalogue numbers FMK007 and FMK008, respectively; R&D Systems), were used at final concentrations of 1 μM . The pan-caspase inhibitor, z-VAD-fmk (R&D Systems), was used at a final concentration of 50 μM . The doses and timings of these treatments were based on the reported half-life of these agents, previous publications, and preliminary experiments demonstrating efficacy.

Generation of cigarette smoke extract. Kentucky 1R3F research-reference filtered cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY) were smoked using a peristaltic pump, as previously described (22), with several modifications. The filters were cut from the cigarettes before combustion. Each cigarette was smoked in 6 minutes, with less than 10 mm remaining. One cigarette was bubbled through 10 ml of endotoxin-free PBS, and this solution was defined as 0.1 cigarette/ml CSE (the gaseous phase of one tenth of a cigarette per milliliter of buffer), and was immediately adjusted to a pH of 7.4, and filtered through a 0.22- μm filter. PBS was treated in a similar fashion and used as vehicle control. CSE was diluted to a final concentration of 2×10^{-2} cigarettes/ml (i.e., one part CSE to four parts complete media) unless otherwise stated, and used fresh (within 15 minutes of preparation).

Apoptosis. Apoptotic cells were identified on the basis of altered nuclear morphology after staining with Hoechst dye 33342 (Invitrogen, Carlsbad, CA). Necrotic cell death was evaluated by the uptake of the normally cell-impermeable DNA dye, propidium iodide (Invitrogen). A minimum total of 500 cells per sample was evaluated using fluorescent microscopy. Sample identification was blinded both during image acquisition and analysis, to minimize the potential for bias.

Caspase 3/7 activity (Apo-One; Promega, Madison, WI) assays were performed 4 hours after treatment with CSE or vehicle according to the manufacturer's recommendations, and normalized to the protein concentration using a modified bicinchoninic acid assay compatible with reducing agents (Thermo Scientific, Rockford, IL).

Immunocytochemistry. For immunocytochemistry, cells were grown on glass-bottomed, 12-well dishes coated with 0.1% bovine gelatin. For cytochrome C analysis, cells were incubated with 100 nM Mitotracker Red CMX-ROS (Molecular Probes, Eugene, OR) in Opti-MEM (Invitrogen) for 45 minutes at 37°C and 5% CO₂. Mitotracker Red CMX-ROS stains mitochondrially, with an intact membrane potential. Media were replaced with fresh, complete EGM-2, and cells were treated with vehicle or CSE for an additional 4 hours before fixation and permeabilization with 4% paraformaldehyde, followed by three rinses with PBS and incubation with 0.05% goat serum/0.003% Triton-X 100. Samples were incubated with antibodies against cytochrome C (catalogue number 556432; B.D. Pharmagen, San Jose, CA.) at 1:2,000 in PBS/1% BSA/0.003% Triton-X 100 overnight. Detection was achieved using goat anti-mouse fluorescent-conjugated (488 nm, excitation wavelength) secondary antibodies (Molecular Probes). Samples were rinsed with PBS and finally stained with Hoechst 33342. Parallel staining without the addition of primary antibody revealed no detectable background staining. For Bax 6A7 staining, cells were grown on glass-bottomed, 12-well dishes coated with 0.1% bovine gelatin before exposure to vehicle or CSE in the presence or absence of PFT- α (10 μM) versus DMSO for 20 hours. Cultures were incubated with Mitotracker Red CMX-ROS (100 nM in Opti-MEM) for the final 45 minutes in culture. Cells were fixed in 4% paraformaldehyde and processed as described (23), with several modifications. After permeabilization, cells were incubated overnight with A67 (catalogue number 556467; B.D. Pharmagen), diluted 1:400 in PBS. Detection was achieved with goat anti-mouse fluorescent-conjugated (488 nm, excitation wavelength) secondary antibodies (Molecular Probes). Samples were rinsed with PBS and finally stained with Hoechst 33342. Images were captured, and evaluation was performed on a Nikon Eclipse TE2000-S fluorescent microscope equipped with Image Pro Plus 5.1 imaging software (Media Cybernetics, Bethesda, MD). Image acquisition was performed blinded to treatment arms. Images were pseudo-colored.

Western blotting. For Western blotting, cells were washed in cold PBS, and proteins were extracted in lysis buffer (Cell Signaling, Boston, MA) containing $1 \times$ protease inhibitor cocktail (catalogue number P8340; Sigma Chemical Co., St. Louis, MO), sonicated, and cleared by centrifugation. Proteins were separated by SDS-PAGE, and the gel was then electrophoretically transferred to a PVDF membrane (Millipore Corp., Billerica, MA). Specific proteins were detected with the antibodies anti-MIF (catalogue number MAB289; R&D Systems), anti-p53 (catalogue number 9292; Cell Signaling, Boston, MA), anti-Jab-1 (catalogue number sc-13157; Santa Cruz Biotechnology, Santa Cruz, CA), anti-caspase 9 (catalogue number 9508; Cell Signaling), and anti-actin (catalogue number csc-13157; Santa Cruz Biotechnology), as indicated. Horseradish peroxidase-conjugated secondary antibodies were then incubated with the blot, and antibody complexes were detected by chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical Evaluation of Data

All data are expressed as mean $\pm$ standard error. Unpaired or paired Student *t* tests were used for statistical comparisons when appropriate. Differences were considered significant at $P < 0.05$.

RESULTS

Cigarette Smoke Induces Caspase 9-Dependent Endothelial Cell Apoptosis

The sensitivity of HPAECs to CS was evaluated in primary cell culture. HPAECs were exposed to vehicle or CSE for 24 hours. Apoptotic cells were identified by nuclear condensation and fragmentation, after staining with the nuclear dye Hoechst 33342, as previously described (21) (Figures 1A and B). *Insets*

in Figures 1A and 1B demonstrate normal (*asterisks*) and fragmented (*arrowhead*) nuclear morphology in cells treated with vehicle (endotoxin-free PBS) or 2×10^{-2} cigarettes/ml for 24 hours. In culture, basal rates of HPAEC apoptosis varied slightly with passage number and donor, but were typically low (around $6.8\% \pm 0.3\%$), with no significant increase above basal levels resulting from exposure to the vehicle (PBS) for 24 hours (Figure 1C). Necrosis in untreated cells was minimal ($0.5\% \pm 0.5\%$), as assessed by staining with propidium iodide, and did not change with any of the treatments shown in Figure 1 (data not shown).

To define the biochemical requirements of the observed cell death, parallel studies were undertaken, using distinct peptide inhibitors of caspases, the enzymatic mediators of apoptotic cell death. Specifically, HPAECs were pretreated with the pan-caspase inhibitor, z-VAD-fmk, or the highly specific peptide inhibitor of the initiator caspases 8 or 9, z-IEDT-fmk or z-LEHD-fmk, respectively. Cells were subsequently challenged with CSE, analyzed for apoptotic morphology, and compared with cultures treated with the carrier, DMSO, as detailed in MATERIALS AND METHODS. Figure 2A shows that cell death in response to CSE was absolutely dependent on caspase activity, as demonstrated by its inhibition with z-VAD-fmk. Cell viability was maintained with the addition of z-LEHD-fmk but not z-IEDT-fmk, indicating that the activity of the initiator caspase 9 was required for CSE-mediated apoptosis, and not caspase 8.

Caspase 9 is an initiator caspase that functions to cleave and activate executioner caspases 3 and 7, which are the effector

enzymes of the apoptotic process. To evaluate the role of caspase 9 further in CSE-induced apoptosis, HPAECs were preincubated with z-LEHD-fmk or its carrier (DMSO), and challenged with CSE or vehicle. Total cell homogenates were analyzed for caspase 3/7 activity, as described in MATERIALS AND METHODS. Exposure to CSE increased caspase 3/7 activity, which was efficiently inhibited by pretreatment of cells with z-LEHD-fmk (Figure 2B). Thus, in addition to the morphologic changes characteristic of apoptosis, exposure to CSE was sufficient to activate executioner caspases 3 and 7 in HPAECs in a caspase 9-dependent manner.

To provide further evidence supporting the requirement for active caspase 9 during the CSE-induced apoptosis of HPAECs, we used a molecular approach. Cells were transduced with an adenovirus encoding dominant-negative caspase-9 (AdDN9) or an empty adenoviral vector (Adnull), as described in MATERIALS AND METHODS. Western blotting of total cell lysates demonstrated the ectopic expression of the transgene (Figure 2C, *inset*). Adnull or AdDN9 cells were exposed to vehicle or CSE for 24 hours, and assessed for apoptotic morphology. Cells transduced with AdDN9 were protected from CSE-induced apoptosis, compared with Adnull-transduced HPAECs (Figure 2C), providing additional evidence that caspase 9 activity is required for the efficient apoptosis of HPAECs exposed to CSE. These results indicate that CSE is sufficient to induce the apoptosis of HPAECs, via a caspase 9-dependent mechanism.

Caspase 9 is known to be activated in the context of apoptosome, a large tertiary complex of proteins composed of cytochrome C, procaspase 9, and apoptotic protease activating factor 1 (Apaf-1) (24). Various apoptogens, including cytochrome C, are sequestered in the mitochondria. Their activity is thus regulated by compartmentalization. After release into the cytosolic space, cytochrome C complexes with Apaf-1 and procaspase 9 to form apoptosome, which then promotes the cleavage of procaspase 9 into the active enzyme, caspase 9. Figure 2D demonstrates the representative immunocytochemistry of cytochrome C in HPAECs after exposure to CSE. Whereas cytochrome C typically exhibits a filamentous pattern colocalized with mitochondria (using Mitotracker Red) (Figure 2D, *solid arrowheads*), exposure to CS results in diffuse staining no longer associated with mitochondria (Figure 2D, *open arrowhead*), indicating release into the cytosol. To characterize the role of mitochondria further in the apoptotic response to CS, we evaluated more proximal molecular events upstream of mitochondrial outer membrane permeability (MOMP). Bax is a major regulator of mitochondrial function, and promotes cell death through its effects on MOMP (25). The activation of Bax is associated with a stereotypic, conformational change in the protein that leads to the exposure of a cryptic epitope in the N-terminus of the molecule. This epitope is recognized by conformation-specific antibodies, such as the monoclonal antibody 6A7 (26). Staining for active Bax (i.e., 6A7 immunoreactivity) was evident after exposure to CS ($14.3\% \pm 0.5\%$ versus $1.2\% \pm 1.1\%$ in vehicle-treated cells), and coincided with the nuclear fragmentation indicative of apoptosis (Figure 2E). These data indicate that the mitochondrial apoptotic pathway is activated in response to CS exposure *in vitro*.

Cigarette Smoke-Induced Death is p53-Dependent

After establishing a model to study the CSE-induced apoptosis of primary HPAECs, we specifically addressed the role of p53 in CSE-induced apoptosis. Chronic smoke exposure in patients and animal models was associated with increased p53 expression in the intact lung (17, 18). We found that acute exposure of HPAECs to CSE for 24 hours was sufficient to increase p53 protein expression greater than twofold (Figure 3A).

Figure 1. Cigarette smoke induces pulmonary endothelial cell apoptosis. Primary HPAECs were exposed to vehicle (PBS) versus cigarette-smoke extract (CSE) in complete media for 24 hours, and nuclei were stained with Hoechst dye. Fluorescent microscopy revealed increased apoptotic morphologic changes (i.e., nuclear condensation and fragmentation, *arrowheads*) in CSE-exposed cells (B) compared with vehicle-treated cells (A). (C) Quantification of apoptotic cell death in human pulmonary macrovascular endothelial cells (HPAECs) exposed to CSE for 24 hours demonstrated a significant increase in apoptosis at concentrations of 2×10^{-2} cigarette/ml. More than 500 events were analyzed per condition. Results are representative of at least eight independent studies. *P < 0.05 versus vehicle treatment.

Figure 2. Cigarette smoke induces endothelial cell apoptosis via the mitochondrial pathway. Primary HPAECs were exposed to vehicle (PBS) versus CSE in complete media for 24 hours, and nuclei were stained with Hoechst dye. (A) Preincubation with the pan-caspase inhibitor z-VAD-fmk (z-VAD, solid bar) or the caspase 9 inhibitor z-LEHD-fmk (z-LEDT, hatched bar) efficiently suppressed CSE-induced apoptosis compared with vehicle (DMSO, gray bar)-treated cells. The caspase 8 inhibitor (z-IEDT-fmk) had no demonstrable effect on apoptosis in response to CSE (z-IEDT, open bar). (B) The activity of executioner caspases 3 and 7 was increased in HPAECs exposed to CSE. (C) Preincubation with the caspase 9 inhibitor (z-LEDT-fmk) but not its carrier (DMSO) efficiently blocked the CSE-induced activity of caspases 3 and 7. HPAECs were infected with adenovirus encoding dominant-negative caspase 9 (DN9) versus empty vector (Adnull) before exposure to vehicle or CSE (2×10^{-3} cigarette/ml), and analyzed for apoptotic morphology. Cells expressing DN9 (gray bar) were protected from CSE-induced apoptosis compared with control (AdNull) lines (solid bars). Western blotting of total cell lysates demonstrated ectopic overexpression of the adenovirally encoded transgene (C, inset). (D) CSE induced mitochondrial cytochrome C (Cyto c) release. HPAECs were stained with Mitotracker Red (Mito Red, red, D ii) before exposure to CSE or vehicle for 4 hours, as described in MATERIALS AND METHODS. After fixation, cells were stained for cytochrome C (green, D i) and Hoechst (blue, D iii). Inset (D iii) shows

the merging of the three images, with the marked area digitally magnified. Solid arrowheads identify a cell with reticular cytochrome C staining within the mitochondria (higher magnification within inset), whereas open arrowheads indicate a cell with loss of mitochondrially localized cytochrome C staining. (E) CSE exposure induced activating conformational changes in Bax. HPAECs were exposed to vehicle or CSE before fixation and staining for conformationally active Bax (green), Mitotracker Red (red), and nucleic acid (blue), as described in MATERIALS AND METHODS. Top: Representative merged images from vehicle-treated (E i) and CSE-treated (E ii) cells. Exposure to CSE significantly increased the number of A67-reactive cells, indicative of conformational activation of Bax during CSE-induced death (E i versus E ii). Higher magnification of these cells revealed clusters of A67 immunoreactivity (solid arrowheads, E iii) in cells with fragmented nuclei (open arrowheads, E iii). More than 500 events were analyzed per condition. Results are representative of at least eight independent studies. * $P < 0.05$ versus vehicle treatment. ** $P < 0.05$ versus cells exposed to CSE.

To demonstrate that p53 contributed causally to CSE-induced apoptosis, HPAECs were preincubated with the pharmacologic inhibitor of p53, cyclic PFT- α or its carrier, DMSO,

before challenge with CSE. The pharmacologic inhibition of p53 efficiently suppressed apoptosis in the face of CSE, returning rates of death to baseline levels (Figure 3B). Numerous

studies demonstrated that PFT- α blocks the transcriptional effects of p53, as well as the apoptogenic translocation of p53 to the mitochondria, a cytosolic effect of p53 (27, 28). However, debate has arisen about the potential p53-independent effects of this inhibitor (29, 30). Therefore, a second distinct method was undertaken to confirm the requirement for p53 in CSE-mediated apoptosis. RNA interference in the form of duplex silencing RNA (Si RNA) directed against p53 was delivered via transient transfection, as detailed in MATERIALS AND METHODS. Previous studies demonstrated the efficient transduction of duplex RNA into HPAECs, with an efficiency of greater than 95% of the cells in culture (21). We were able to suppress the expression of p53 protein after exposure to CSE by more than 75% (Figure 3C). When p53-deficient cells (p53 Si transfectants) were compared with cells transfected with control siRNA (ctrl Si), p53-deficient cells were protected from CSE-induced cell death

(Figure 3D). Thus, using both a pharmacologic inhibitor and RNA interference, we provide evidence that p53 is necessary for the apoptogenic effects of CSE on HPAECs. Through multiple mechanisms that can interface upstream or downstream of the MOMP, p53 can contribute to apoptosis (16). To define the mechanism of p53-induced apoptosis further in response to CS exposure, we evaluated the effects of p53 inhibition with PFT- α on the activation of the mitochondrial pathway. More specifically, we evaluated the effects of PFT- α on a proximal event, the CSE-induced activation of Bax. The activation of Bax in response to CSE was efficiently antagonized by pretreatment with PFT- α (Figure 3E). Thus, the inhibition of p53 blocked the CS-induced activation of Bax, placing p53 function upstream of changes in Bax in response to CS.

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MIF Antagonizes CS-Induced Apoptosis

MIF is a cytokine produced by endothelial cells *in vivo* and *in vitro* (21, 31). Our previous work demonstrated that MIF is an endogenous inhibitor of apoptosis, functioning to suppress LPS-induced cell death via the stabilization of the endogenous caspase 8 inhibitor, Flice-like inhibitory protein short isomer (FLIPshort) (21). MIF protein expression was increased in response to CSE, as assessed by Western blotting of total cell lysates (Figure 4A). To determine if MIF functions to antagonize CSE-induced cell death, two complementary approaches were undertaken in primary HPAECs. First, cells were pretreated with recombinant human MIF (rMIF) or its carrier, and subsequently challenged with CSE, as described in MATERIALS AND METHODS. An analysis of apoptosis revealed that the addition of exogenous rMIF to cells in culture efficiently prevented CSE-induced apoptosis (Figure 4B). To understand the role of endogenous MIF, cells were transfected with siRNA directed at the MIF mRNA or control siRNA. Previous work demonstrated that this method efficiently suppresses MIF mRNA, as assessed by quantitative PCR (21). MIF siRNA efficiently suppressed MIF protein expression in response to CSE treatment, as demonstrated by Western blotting (Figure 4C). An analysis of parallel cultures revealed that a deficiency of MIF dramatically increased the sensitivity of HPAECs to the apoptogenic effects of CSE (Figure 4D). The dose-response curve shifted toward the left, as indicated

by the increased apoptosis of MIF Si transfectants at 2×10^{-3} cigarettes/ml, a dose insufficient to kill nontransfected cells (Figure 1C) or control Si transfectants (Figure 4D).

Importantly, the pretreatment of MIF Si transfectants with rMIF, to bypass the effects of RNAi on expression, resulted in levels of apoptotic death comparable to those seen in control Si transfectants, providing strong evidence for the specificity of the response. Thus, taken collectively, these experiments provide strong evidence that MIF functions to antagonize CSE apoptosis.

The MIF Binding Partner Jab-1 Protects against CS Toxicity

Jab-1 is the first identified binding partner of MIF (32). Jab-1 is involved in the proteasome-mediated protein degradation of several cell-cycle regulators and intracellular signaling interme-

diates (33). Alterations of Jab-1 function, mediated through its interactions with MIF, contribute to the differential turnover of certain proteins such as p27 (34). The role of Jab-1 in apoptosis is controversial, with conflicting evidence indicating that Jab-1 may promote or antagonize apoptosis in an array of cell types and contexts (35, 36). To address the contribution of Jab-1 to CSE-induced HPAEC death, cells were transfected with control Si or Si RNA directed against Jab-1 mRNA (Jab-1 Si). The analysis of total cell lysates by Western blotting demonstrated that Jab-1 Si was efficient in suppressing Jab-1 protein in culture (Figure 5). No differences in baseline apoptosis were detectable between ctrl Si and Jab-1 Si transfectants. An analysis of apoptosis after exposure to CSE indicated that Jab-1 deficiency, similar to MIF deficiency, resulted in enhanced sensitivity to CSE (Figure 5), suggesting that endogenous MIF and Jab-1 both act to antagonize CSE-mediated cytotoxicity.

The Death of MIF-Deficient and Jab-1-Deficient Cells Is p53-Dependent

Previous work indicates that MIF can promote tumorigenesis by inhibiting p53 functions, including apoptosis (20). After providing evidence that CS-induced death was p53-dependent and antagonized by MIF and Jab-1, we determined whether the enhanced cell death observed in MIF-deficient or Jab-1-deficient cells was mediated via p53. In these studies, RNAi directed against MIF or Jab-1 was coupled with the pharmacologic antagonism of p53, using PFT- α . As in previous experiments, the transfection of MIF Si (Figure 6A) or Jab-1 Si (Figure 6B) enhanced the sensitivity of HPAECs to CSE-induced apoptosis. Pretreatment with PFT- α was associated with the suppression of apoptosis in control Si, MIF Si, and Jab-1 Si transfectants after exposure to CSE, compared with cells pretreated with vehicle. The CSE-induced apoptosis of MIF Si and Jab-1 Si transfectants

Figure 4. Macrophage migration inhibitory factor (MIF) antagonizes CS-induced apoptosis. HPAECs were exposed to increasing concentrations of CSE for 24 hours, and total cell lysates were analyzed for MIF protein expression by Western blotting. (A) Exposure to 2×10^{-2} or 5×10^{-2} cigarettes/ml in complete media was associated with a significant increase in MIF protein expression. (B) Pretreatment of HPAECs with recombinant human MIF (rMIF, 100 ng/ml) efficiently suppressed apoptosis in response to CSE exposure (open bar), in contrast to vehicle (BSA) pretreatment (solid bars). (C) Transfection of duplex RNA targeting MIF (MIF Si) versus control duplex (ctrl Si) was associated with efficient suppression of MIF protein to less than 20% of control levels after exposure to CSE, as assessed by Western blotting of total cell lysates and densitometric analysis. (D) Suppression of MIF by RNAi (MIF Si, dark gray bars) significantly sensitized HPAECs to apoptogenic effects of CSE compared with ctrl Si transfectants (solid bars), but had no appreciable effect on baseline cell death. Pretreatment of MIF Si transfectants with recombinant MIF protein (rMIF) significantly reduced apoptosis after CSE exposure (open bar), compared with MIF Si transfectants exposed to CSE and carrier. Results are representative of at least three independent studies. * $P < 0.05$ versus vehicle treatment. ** $P < 0.05$ versus ctrl Si transfectants exposed to CSE. *** $P < 0.05$ versus MIF Si transfectants exposed to CSE.

Figure 5. Loss of the MIF binding partner, Jab-1, sensitizes endothelial cells to CS-induced apoptosis. Transfection of duplex RNA targeting Jab-1 (Jab-1 Si) versus control duplex (ctrl Si) or mock (no duplex) transfected cells was associated with efficient suppression of Jab-1 protein to less than 30% of control levels, as assessed by Western blotting (inset). Jab-1 Si RNA transfection significantly sensitized HPAECs to apoptogenic effects of CSE exposure (gray bars) compared with ctrl Si, but had no appreciable effect on baseline cell death (solid bars). Results are representative of at least three independent studies. * $P < 0.05$ versus vehicle treatment. ** $P < 0.05$ versus ctrl transfectants exposed to CSE.

was also abrogated by the forced expression of AdDN9, compared with cells transduced with Adnull (Figure 6C). Thus, the CSE-induced death of MIF-deficient and Jab-1-deficient cells requires both p53 and caspase 9. These results suggest that the functions of p53 and caspase 9 either lie distal to the protective effects of MIF and Jab-1, or operate through an obligatory parallel pathway in the activation of the apoptotic machinery by CSE.

MIF Antagonizes Cigarette-Mediated p53 Expression

To determine if MIF functions to inhibit CSE-induced death via direct effects on p53 expression, we analyzed the effects of exogenous MIF on p53 expression in response to CSE. HPAECs were pretreated with rMIF or vehicle before challenge with CSE, and total cell lysates were analyzed by Western blotting for p53. As demonstrated in Figure 7A, exogenous MIF efficiently suppressed the p53 protein expression induced by CSE. Quantification via densitometric analysis normalized to β -actin indicated that p53 expression remained at baseline in the presence of rMIF.

Based on the capacity of MIF deficiency to sensitize cells to p53-dependent death, we reasoned that the induction of p53 would be increased in MIF Si transfectants exposed to CSE relative to control Si transfectants. Control Si and MIF Si cells

were exposed to vehicle or CSE, and analyzed for p53 protein expression via Western blotting (Figure 7B). Baseline levels of p53 protein were not appreciably different between control Si and MIF Si transfectants. As previously demonstrated, exposure to CSE was associated with increased p53 expression in control Si transfectants. MIF Si transfectants demonstrated a significant increase in the magnitude of change in p53 expression after CSE compared with ctrl Si transfectants, providing additional evidence that MIF functions to suppress the CSE-mediated induction of p53. This finding, coupled with our data demonstrating the CSE-induced death of MIF-deficient

Figure 6. Apoptosis of MIF and Jab-1-deficient cells is p53-dependent. HPAECs were transfected with either ctrl, MIF, or Jab-1-directed duplex RNA (Si RNA), as detailed in MATERIALS AND METHODS. After transfection, cells were preincubated with the p53 inhibitor, PFTα (10 μ M, open bars), or its carrier (DMSO, solid bars), challenged with CSE, and analyzed for apoptotic morphologic changes. The enhanced apoptosis after exposure to CSE demonstrated by MIF Si transfectants (A) and Jab-1 Si transfectants (B) was abrogated by pharmacologic inhibition of p53 and not vehicle. (C) HPAECs were infected with adenovirus encoding dominant negative caspase 9 (AdDN9) versus empty vector (AdNull), in accordance with MATERIALS AND METHODS, before exposure to CSE (2×10^{-2} cigarette/ml), followed by analysis of apoptotic morphology. Cells expressing DN9 (gray bars) were protected from CSE-induced apoptosis compared with control (AdNull) lines (solid bars). Results are representative of at least three independent studies. * $P < 0.05$ versus ctrl transfectants exposed to CSE and carrier. ** $P < 0.05$ versus MIF Si transfectants treated with carrier. *** $P < 0.05$ versus Jab-1 Si transfectants treated with carrier. **** $P < 0.05$ versus cells transduced with AdNull before CSE treatment.

Figure 7. MIF antagonizes induction of p53 by cigarette smoke. (A) Pretreatment of HPAECs with recombinant human MIF (rMIF) efficiently suppressed increases in p53 expression in response to CSE exposure, in contrast to vehicle-treated cells (inset). Densitometric analysis indicated that p53 protein (normalized to β -actin) increased more than twofold after exposure to CSE (solid bars). Expression of p53 protein in cells treated with both CSE and rMIF (open bar) approached levels in vehicle-treated cells. HPAECs were transfected with either ctrl or MIF-directed duplex RNA (Si RNA). (B) After transfection, cells were challenged with CSE where indicated, and analyzed for expression of p53 protein via Western blotting. Treatment of MIF Si transfectants was associated with decreased induction of p53 protein expression after exposure to CSE. Results are representative of at least three independent studies. * $P < 0.05$ versus vehicle treatment. ** $P < 0.05$ versus cells exposed to CSE.

cells, is dependent of p53 expression/function places MIF upstream of p53.

DISCUSSION

The emerging appreciation for the role of apoptosis in the development of emphysema has raised interest in the identification of molecular determinants of CS-induced pulmonary endothelial cell death as potential targets for intervention. Here, we show that p53 is induced in HPAECs exposed to CSE, consistent with both preclinical animal studies and recent clinical work linking increased p53 expression with the development of emphysema and COPD in patients who smoke (17–19). p53 functions in the regulation of the cell cycle, DNA repair, and apoptosis, leading us to hypothesize that p53 contributes to CS-induced apoptosis. Pretreatment of HPAECs with PFT- α , an antagonist of p53, efficiently blocks CSE-induced apoptosis, implicating p53 functionally in CSE-mediated cell death. Furthermore, we provide evidence that p53 is required for CSE-induced apoptosis, as demonstrated by the resistance of p53-deficient cells to death. We also provide evidence that the expression of the pluripotent cytokine MIF is increased by CSE, and exogenous MIF antagonizes CS-induced apoptosis, suggesting a cytoprotective role for MIF. Moreover, the capacity of exogenous MIF to suppress p53 protein induction, and conversely, the enhanced induction of p53 in MIF-deficient cells, indicate that MIF acts upstream of p53. Thus, we provide mechanistic evidence supporting a causal link between the increased expression of p53 reported *in vivo* and CS-mediated injury in the form of endothelial cell apoptosis.

This study was limited by its focus on human pulmonary endothelial cells *in vitro*. Other studies characterizing emphysematous human lungs or rats exposed to chronic smoke support the contention that cigarette smoke increases p53 expression, but those studies failed to identify the cellular sources within the intact lung, and could only provide a corollary between disease and protein expression. In view of this limitation, our *in vitro* model allowed us to address the contributions of p53 to human endothelial apoptosis directly in response to cigarette smoke.

Further, we provide evidence that the observed p53-dependent death is also caspase-dependent, the biochemical hallmark of apoptosis. Classic apoptotic cell death is dependent on the activity of these cysteine proteases, which act as initiators of the pathway (initiator caspases) or are ultimately responsible for the disassembly of the cell (executer caspases). Using a panel of substrate analogues that irreversibly bind their respective target caspases, we defined the initiator caspase required for CSE-induced apoptosis. The inhibition of caspase 9 efficiently blocks the CS-induced activation of executioner caspases 3 and 7, indicating that it is required for the CS-mediated activation of caspases 3 and 7. Further, the antagonism of caspase 9 activity, through either a peptide inhibitor (z-LEDH-fmk) or the forced expression of a dominant-negative mutant of caspase 9 (AdDN9), efficiently blocks CSE-associated apoptosis. The requirement for caspase 9 function is consistent with cell death via the mitochondrial or intrinsic apoptotic pathway (37). Although the intrinsic pathway can be indirectly activated via a signal initiated in the extrinsic or death receptor pathway, the lack of efficacy of z-IEDT-fmk, a caspase 8 inhibitor, favors a direct activation of the mitochondrial pathway by CSE. In addition to defining the requirement for caspase 9 in cell death, we demonstrate that CS is sufficient to activate Bax, a major regulator of MOMP (25). Exposure to CS also results in the release of cytochrome C from the mitochondria, a requirement of apoptosome formation and caspase 9 activation (24). These molecular events are stereotypic of death via the mitochondrial

apoptotic pathway. Further, we demonstrate that p53 is required for the CS-induced activation of Bax, thus mapping p53 function upstream of conformational changes in Bax. The capacity of p53 to trigger death via the mitochondrial pathway is well-established in other models, and can occur through the transcriptional effects of p53 on both proapoptotic molecules (including Bax and Puma) and antiapoptotic molecules (such as Bcl-xl) (16). Moreover, p53 is known to activate the intrinsic pathway via a transcriptionally independent mechanism in which cytoplasmic p53 translocates to the mitochondria and interacts with Bax to mediate changes in mitochondrial permeability, ultimately leading to the activation of caspase 9 (16). Studies are underway to determine which, if any, of these known p53-dependent events are required for CS-induced apoptosis.

CSE is sufficient to increase the expression of MIF in HPAECs in culture. Importantly, exogenous MIF antagonizes CS-induced apoptosis. Thus, the induction of MIF by CSE may represent a cytoprotective response. Although multiple receptors and binding partners were identified for MIF, we focused our analysis on the role of Jab-1 in the response of HPAECs to CS. We could not detect the expression of an alternative MIF receptor, CD74, in HPAEC lysates (data not shown), but cannot definitively exclude a role for this receptor in our model. Jab-1, also known as CSN5, is a component of the COP9 signalosome (33). The COP9 complex was shown to regulate the degradation of p53 (37). Recent work by Zhang and colleagues provided evidence that Jab-1/CSN5 can physically interact with and alter the stability of murine double mutant 2 (MDM2), a known negative regulator of p53 involved in its proteasome-mediated degradation (38). Thus, Jab-1 was shown to alter p53 protein expression and turnover indirectly, via effects on MDM2. Further, recent studies demonstrated that MIF can dynamically interact with p53 and stabilize the association of a p53–MDM2 complex (39). The capacity of MIF and Jab-1 to interact physically is well-established, and exogenous MIF is known to gain access to and bind this cytoplasmic receptor/binding partner via an unknown mechanism (32). The phenotypic similarities between MIF-deficient and Jab-1-deficient cells (e.g., their enhanced sensitivity to p53-dependent CSE-induced death) are consistent with these molecules acting in concert to antagonize p53-mediated death in response to CSE. Based on reports indicating that the manipulation of MIF or Jab-1 individually modified the expression of p53 via effects on MDM2, a model may be envisioned in which MIF and Jab-1 act in conjunction to stabilize MDM2 and enhance the MDM2-mediated degradation of p53, preventing the accumulation of p53. Exposure to CS may induce the expression of p53 via the disruption of these physical and functional interactions, thereby repressing MDM2-mediated degradation. We are investigating the effects of CSE on the associations of MIF with Jab-1, MDM2, and p53, to address this possibility.

Because CS is known to contain detectable levels of LPS (40, 41) and chronic LPS exposure is sufficient to induce both apoptosis and emphysematous changes in mice (42), LPS within CS may contribute causally to the CSE-induced apoptosis of HPAECs. Our previous work demonstrated that MIF is an endogenous determinant of endothelial cell sensitivity to LPS-induced apoptosis (21). MIF is required for the induction of FLIPshort in response to LPS. This MIF-dependent expression of FLIPshort is necessary to antagonize caspase 8 and ultimately apoptosis. In contrast to LPS (21), CSE-induced apoptosis occurs in the presence of the caspase 8 inhibitor, z-IEDT-fmk (Figure 1D), suggesting a caspase 8-independent mechanism. In addition, we were unable to detect changes in FLIPshort expression after treatment with CSE, and the suppression of FLIPshort expres-

sion did not appreciably sensitize HPAECs to CSE (data not shown). Such negative results, although not definitive, do not support a role for FLIPshort in the regulation of CSE-mediated cytotoxicity. Our work suggests that although MIF functions to antagonize both LPS-induced and CSE-induced endothelial apoptosis, it does so via distinct mechanisms. In the case of LPS, MIF inhibits apoptosis by functionally antagonizing, via effects on FLIPshort, the death receptor pathway (i.e., its is caspase 8-dependent). Here, we provide evidence that MIF, via its effects on p53, antagonizes the intrinsic or caspase 9-dependent pathway. Because the expression of both p53 and FLIPshort are dynamically regulated via proteasome-mediated degradation (43, 44), we postulate that the capacity of MIF to inhibit apoptosis via both the extrinsic and intrinsic death pathways stems from its ability to influence the turnover of critically antiapoptotic and proapoptotic proteins. Additional studies are necessary to test this potentially unifying hypothesis formally.

In conclusion, the present study shows that p53 mediates CSE-induced endothelial cell death via the caspase 9-dependent intrinsic apoptotic pathway, acting upstream of Bax activation. We further demonstrate that MIF and its binding partner, Jab-1, act to antagonize p53 induction and apoptosis, and thus are endogenous determinants of endothelial cell sensitivity to the toxic effects of CSE. It remains to be determined if chronic exposure to CS alters the expression and function of MIF in animal models or humans. We predict that genetic variants in the MIF promoter associated with altered MIF expression (45) will affect clinical responses to chronic CS in patients. This work, coupled with preclinical and clinical observations (17–19), supports a causal link between increased p53 expression and the increased apoptosis of emphysema, and implicates altered MIF expression and function as potential determinants of disease.

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