Acute cigarette smoke exposure induces apoptosis of alveolar macrophages

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Acute cigarette smoke exposure induces apoptosis of alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 281: L1392–L1401, 2001.—Alveolar macrophages (AMs) may play a critical role in cigarette smoke (CS)-related pulmonary diseases. This study was designed to determine whether CS induces apoptosis of AMs. In in vitro studies, mouse, rat, and human AMs and human blood monocyte-derived macrophages cultured with aqueous whole CS extracts underwent apoptosis that was detected by light and electron microscopy and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling. The gas phase of CSE did not cause apoptosis. The CS-induced apoptosis was associated with increased oxidative stress, Bax protein accumulation, mitochondrial dysfunction, and mitochondrial cytochrome c release but was independent of p53, Fas, and caspase activation. This apoptosis was inhibited by antioxidants such as glutathione, ascorbic acid, and α-tocopherol. In in vivo studies where rats were exposed to the smoke from 10 cigarettes over 5 h in an exposure chamber, ~3% of AMs obtained by bronchoalveolar lavage after 24 h showed apoptosis. These results suggest that acute CS exposure is capable of inducing apoptosis of AMs.

The toxicity of CS to AMs may be relevant to a clinical observation that cigarette smokers often suffer from nonspecific respiratory infections that may enhance mortality in this population (7, 8).

Despite the acute toxicity of CS, AMs are known to be increased in smokers compared with nonsmokers (3). This is thought to be due to enhanced recruitment of blood monocytes into the lung. However, it is currently unknown whether the rate of AM death is different in smokers and nonsmokers. Smokers’ AMs may become resistant to chronic and repetitive exposure to CS. Alternatively, AMs may be protected against the toxicity of CS in the microenvironment of the lung that contains large amounts of antioxidants. Further studies are needed to understand how CS induces AM death.

In the present study, we investigated whether acute exposure to CS induces apoptosis of AMs. To determine the direct effect of CS on AMs, we first conducted in vitro experiments in which AMs were exposed to aqueous CS extract (CSE). Next, we performed in vivo experiments in which rodents were acutely exposed to CS inhalation. From these experiments, we demonstrate that acute CS exposure induces apoptosis of AMs.

MATERIALS AND METHODS

Experimental animals. Ten-week-old male Sprague-Dawley rats and six-week-old male C57BL/6 wild-type, Fas-deficient lpr/lpr, and Fas ligand-defective gld/gld mice were purchased from SLC (Shizuoka, Japan). C57BL/6 p53-deficient [−/−] mice were obtained from Clea Japan (Tokyo, Japan). Animals were handled in accordance with Institutional Animal Care and Use Committee protocols approved by the animal facility of Tokyo Women’s Medical University (Tokyo, Japan). They were maintained under standard conditions, with a dark period from 8 PM to 8 AM, and water and food were provided ad libitum.

Preparations of macrophages. AMs were obtained by bronchoalveolar lavage (BAL) from rats, mice, and human healthy nonsmoking volunteers as previously described (5). Human blood monocyte-derived macrophages were prepared as previously described (19). Purity of the macrophage populations on May-Grünwald-Giemsa-stained cytosin slides

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was > 95%, and the cell viability as determined by trypan blue dye exclusion was > 98%.

Preparation of CSE solution. CSE was prepared as previously described (2). Commercial plain-ended cigarettes (Peace, Japan Tobacco, Tokyo, Japan) yielding 24 mg of tar and 2.4 mg of nicotine under a standard smoking regimen were used in this study. Mainstream smoke was generated from one cigarette by drawing consecutive puffs into a 20-ml plastic syringe, with a stopcock connected through one port to a glass vessel containing 10 ml of PBS (pH 7.4). A 20-ml puff drawn in 1 s was obtained at 10-s intervals, and each puff was held for 3 s and bubbled through PBS in 5 s. One cigarette yielded an average of 45 puffs by this procedure. To prepare gas-phase CSE, the smoke was drawn into the syringe through a 0.22-μm pore size filter (Milex-HA, Nihon Millipore, Tokyo, Japan) to remove the tar phase of CS. pH of the resultant solution was 7.4 in both whole and gas-phase CSE solutions. The CSE solutions were always prepared by the same person (K. Aoshiba) using exactly the same method and were used within 3 min after preparation.

Preparation of macrophages. For in vitro CS exposure, isolated macrophages (1 × 10⁶ cells/ml) were suspended in serum-free DMEM containing 100 U/ml of penicillin and 100 μg/ml of streptomycin with and without 0.5-10 vol% of freshly prepared CSE solutions. The cells were then cultured in either 96-well culture plates (Beckton Dickinson, Lincoln Park, NJ) or 1.5-ml graduated microcentrifuge tubes (Assist, Tokyo, Japan) at 37°C in a 5% CO₂ humidified atmosphere.

In vivo CS exposure. For in vivo CS exposure, three rats were placed in a plastic cage (27 × 27 × 18 cm) with a narrow orifice connected to a stopcock through which mainstream smoke puffs (45 puffs/cigarette, 20 ml/puff) were delivered. The CS was exhausted with four exhaust holes (1 cm) on the side panels. The animals were exposed to CS in the conscious state and breathed spontaneously in the exposure chamber. In brief, rats were exposed to CS from one cigarette for 20 min. Because the exhaust holes were narrow, the chamber was poorly ventilated so that the rats were expected to inspire high concentrations of CS. After the 20-min exposure to CS, the cage was ventilated by introducing fresh air for 10 min. A total of 10 cigarettes were smoked over 5 h at 30-min intervals. At the end of CS exposure, the rats were transferred to a new cage and allowed to inspire air. After 24 h, the rats were killed and BAL was performed.

Apoptosis assay by light and fluorescence microscopy. Cell samples were affixed to slides by cytospin (Cytospin 3, Shandon, Pittsburgh, PA) and air-dried. For light microscopy, cells were stained with May-Grünewald-Giemsa, and apoptotic cells were identified based on nuclear pyknosis or chromatin condensation together with cell shrinkage. For fluorescence microscopy, cells were fixed in 1% osmium tetroxide, dehydrated through absolute acetate and lead citrate. The samples of AMs were observed under an electron microscope (Hitachi H-7000) at a magnification of ×6,000.

Evaluation of oxidative stress in CS-exposed AMs. The level of 8-OHdG, which is a marker of oxidative DNA modification, was assessed by an immunocytochemical method with monoclonal anti-8-OHdG Ab (Japan Institute for the Control of Aging) as described in Immunocytochemistry. The cellular level of thiol antioxidants was determined with monochlorobimane (Molecular Probes), which passively diffuses across the plasma membrane into the cytoplasm where it forms blue fluorescent adducts with the reduced form of glutathione and other thiol-containing proteins (39). Cells (5 × 10⁴) were incubated with 50 μM monochlorobimane in 96-well plates for 40 min at 37°C, and then the plates were read on a Cytofluor II multiple plate fluorometer (Perceptive Biosystems, Framingham, MA) with excitation and emission wavelengths of 395 and 460 nm, respectively.

Immunoblot analysis. Cell lysates were solubilized in assay buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 0.5% Nonidet P-40, 0.1% SDS, 10 μg/ml of leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of aprotinin, and 1 mM sodium orthovanadate) and centrifuged at 10,000 g for 30
min at 4°C. The supernatant containing 50μg of protein was fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with 1μg/ml of Abs against Bcl-2, Bad (Transduction Laboratories, Lexington, KY), Bax, p53 (Calbiochem), and phosphorylated p53 at Ser392 (New England Biolabs, Beverly, MA). Primary Ab was detected by horseradish peroxidase-conjugated Ab (1:2,500), which, in turn, was visualized with enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL).

For analysis of poly(ADP)-ribose polymerase (PARP) degradation, cell lysates in 1× Laemmli sample buffer were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with 1μg/ml of anti-PARP Ab (Upstate Biotechnology).

Evaluation of mitochondrial function. Functional mitochondria were labeled by incubating cells with 250nM Mitotracker Red CMXRos (Molecular Probes) for 30 min. The cells were fixed with 3% paraformaldehyde in PBS and observed under an epifluorescence microscope. Mitotracker Red CMXRos is concentrated by active mitochondria, and its fluorescence is reduced and diffused during apoptosis-induced depolarization of mitochondrial inner membrane potential. Depolarization of mitochondrial inner membrane potential was also monitored with the mitochondrial membrane potential-sensitive dye 3,3’-dihexyloxacarbocyanine iodide [DiOC(3); Molecular Probes]. Briefly, 10^5 cells were incubated with 40nM DiOC(3) in 96-well plates for 15 min at 37°C, and then the plates were read on a Cytofluor II multimode fluorometer with excitation at 485 and 530 nm, respectively.

Assay for caspase-1- and caspase-3-like activities. Caspase proteolytic activities were measured essentially as previously described (20). Briefly, 10^6 cells were placed in lysis buffer [20mM Tris-HCl, pH 7.5, 2mM EDTA, 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 10μg/ml of aprotinin, 10μg of leupeptin, 1mM phenylmethylsulfonyl fluoride, and 3mM dithiothreitol] and were lysed with two cycles of freezing at −80°C and thawing at 4°C. After centrifugation for 10 min at 13,000 rpm, the supernatants were collected, diluted in 50mM Tris-HCl, pH 7.5, 0.1% CHAPS, 10% sucrose, and 10mM dithiothreitol, and then incubated with the fluorogenic substrates Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) or Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Pepstatin G) at a concentration of 50μM for 60 min at 37°C. Functional mitochondria were measured with excitation at 360 nm and emission at 460 nm with a spectrofluorometer.

Statistics. Results are presented as means ± SE. Comparisons were made by Student’s t-test.

RESULTS

CS induces AM apoptosis in vitro. To determine the direct effect of CS on AM apoptosis, we added aqueous CS to culture medium of in vitro AMs (38), which is a simple, reproducible, and widely used method. Treatment of rat AMs with CSE-induced apoptosis as demonstrated by light microscopy of May-Grünwald-Giemsa-stained cells (Fig. 1, A and B), fluorescence microscopy of acridine orange-stained cells (Fig. 1, C and D), TUNEL (Fig. 1, E and F), and transmission electron microscopy (Fig. 1, G and H). AMs exposed to CS clearly showed morphological hallmarks of apoptosis such as cellular shrinkage, cell surface smoothing, nuclear compaction, and chromatin condensation at the periphery of the nuclear envelope (Fig. 1, B and F). However, they rarely showed nuclear fragmentation, another feature of apoptosis that was observed in AMs exposed to diethyl maleate (5 × 10^{-5} M), tumor necrosis factor-α (1μg/ml) plus cycloheximide (1×10^{-4} M), or bleomycin (100μg/ml; data not shown), suggesting that AMs exposed to CS undergo a different mechanism of apoptosis. As shown in Fig. 2, apoptosis by CSE occurred in a dose- and time-dependent manner, with 95% of AMs showing apoptosis after 24-h exposure of AMs to 10 vol% of CSE solution. Gas-phase CSE, which was generated by removing the particle (tar) compo-
nent from whole smoke, induced apoptosis much less than whole CSE (8.5 ± 0.6 vs. 72.5 ± 2.1% apoptosis after 24-h exposure to gas-phase CSE and whole CSE, respectively), suggesting that some of the tar component is required for induction of apoptosis. Apoptosis of AMs was not induced by nicotine (10⁻⁸ to 10⁻⁸ M), a major tar component of CS (data not shown).

Similar to rat AMs, murine AMs, human AMs, and human blood monocyte-derived macrophages underwent apoptosis on exposure to CSE (Fig. 3), suggesting that CS-induced apoptosis of macrophages is not species or site specific.

**Oxidative stress mediates CS-induced AM apoptosis.** Because both CS, particularly its tar component, and CSE solutions contain ROS (25, 30), we evaluated the role of oxidative stress in CS-induced apoptosis of AMs. As shown in Fig. 4, A and B, exposure of rat AMs to CSE increased the level of 8-OHdG, a marker of oxidative DNA modification, and decreased the level of thiol antioxidants such as glutathione, indicating that CS imposes oxidative stress on AMs. Because ROS in the respiratory tract are effectively eliminated by many antioxidants in respiratory tract lining fluids (RTLFs) (4), we examined whether CS-induced AM apoptosis is inhibited by antioxidants. As shown in Fig. 4 C, CS-induced AM apoptosis was significantly inhibited by N-acetylcysteine (100 μM) and the RTLF antioxidants glutathione (100 μM), ascorbic acid (100 μM), and α-tocopherol (0.7 μM) at physiological concentrations (4).

**CS promotes accumulation of Bax protein.** The Bcl-2 protein family is a major class of intracellular regulators of apoptosis (1). Some, such as Bcl-2 and Bcl-XL, suppress apoptosis, whereas others such as Bax, Bad, and Bid promote it (1). The balance between proapoptotic and antiapoptotic members determines the fate of various types of cells. Therefore, we evaluated the effect of CS on the cellular levels of Bcl-2, Bax, and Bad proteins. CS promoted the accumulation of Bax protein in rat AMs (Fig. 5) but had no effect on the levels of Bcl-2 or Bad (Fig. 5 A). These results suggest that CS promotes the accumulation of Bax protein by AMs.

**CS-induced AM apoptosis is independent of p53 and Fas.** We then examined the mechanism of CS-induced apoptosis in a mouse model lacking the p53 gene. Because p53 has been demonstrated to induce Bax gene expression and ROS-dependent apoptosis (23), we asked whether CS-induced apoptosis is dependent on p53. We found that CS induced both Bax protein accumulation and apoptosis in AMs obtained from p53(−/−) mice (Fig. 6, A–D). In addition, immunoblot analysis showed that CS did not promote p53 protein accumulation and phosphorylation by rat AMs (data not shown). These results suggest that CS-induced AM apoptosis is independent of p53. We also asked whether Fas-Fas ligand interactions, which mediate apoptosis in various types of cells including macrophages, are required for CS-induced apoptosis of AMs. CS induced apoptosis in AMs obtained from...
Fas ligand-defective mice and those from Fas-deficient mice (Fig. 6, E–H), suggesting that CS-induced apoptosis is also independent of Fas-Fas ligand interactions.

Mitochondrial dysfunction and cytochrome c release during CS-induced apoptosis. Because Bax protein can trigger mitochondria to release caspase-activating proteins including cytochrome c (6), probably accelerating the opening of the voltage-dependent anion channel, we evaluated the effect of CS on mitochondrial function and cytochrome c release. MitoTracker Red CMXRos, a fluorescent dye that accumulates in functional mitochondria, was used to evaluate mitochondrial function (Fig. 7A). Rat AMs not exposed to CSE displayed a bright and punctuate MitoTracker Red CMXRos staining pattern. By contrast, when treated with 5 vol% CSE for 12 h, AMs showed diffuse MitoTracker Red CMXRos staining, suggesting that the mitochondrial inner membrane potential had decayed. A reduction in mitochondrial inner membrane potential in CSE-exposed AMs was also shown by a decline in the fluorescence level of DiOC6(3), which reflects depolarization of the mitochondrial membrane (12) (Fig. 7B). Immunocytochemistry demonstrated cytochrome c release from mitochondria to the cytoplasm on exposure to CSE (Fig. 7C). These results suggest that mitochondrial dysfunction and cytochrome c release occur during CS-induced apoptosis. CS-induced AM apoptosis is independent of caspase activation. Although caspases are key mediators of apoptosis, recent evidence has shown that Bax induces apoptosis by either a caspase-dependent or caspase-independent mechanism (1, 18, 22, 36). Therefore, we examined whether CS-induced AM apoptosis is depen-
dent on caspase activation. First, we tested several cell membrane-permeable peptide inhibitors of caspases for their ability to prevent CS-induced apoptosis. As shown in Fig. 8A, CS-induced apoptosis of rat AMs was not inhibited in the presence of Boc-D-FMK (general caspase inhibitor; Enzyme System Products, Liver-
more, CA), Tyr-Val-Ala-Asp-chloromethylketone (in-
hibitor of caspase-1 and -4; Bachem), or Asp-Glu-Val-
Asp-aldehyde (inhibitor of caspase-3, -6, -7, -8, and -10; BIOMOL Research, Plymouth Meeting, PA) at a con-
centration of 50 μM. We had previously confirmed that 
these inhibitors at a concentration of 50 μM had no 
toxicity to rat AMs by a colorimetric MTT assay (data 
not shown). Second, CS did not promote caspase-1-like 
or caspase-3-like proteolytic activities (Fig. 8B). Third, 
immunoblot analysis demonstrated that PARP, a nu-
uclear protein cleaved by multiple caspases to an 85-
kDa inactive form, was not degraded during CS-in-
duced apoptosis of AMs (Fig. 8C). Taken together, 
these results suggest that CS-induced apoptosis of 
AMs is independent of any known major caspases.

Induction of AM apoptosis by CS in vivo. To deter-
mine whether CS also induces AM apoptosis in vivo, 
rats were exposed to the smoke from 10 cigarettes over 
5 h at 30-min intervals in an exposure chamber. BAL 
fluid cells were obtained 24 h after CS exposure, and 
apoptosis was evaluated by light microscopy of May-
Grünnwald-Giemsa-stained cells (Fig. 9, A and B) and 
Giemsa-stained cells (Fig. 9, E and F), transmission 
electron microscopy (Fig. 9, C and D), and TUNEL (Fig. 
9, G and H). BAL fluid cells recovered from CS-exposed 
rats contained a small population of apoptotic cells 
(Fig. 9, B, D, F, and H). These apoptotic cells were 
positively stained with anti-macrophage Ab (Fig. 9, F 
and H), indicating that some AMs underwent apoptosis

![Image](http://ajplung.physiology.org/)

Fig. 7. Mitochondrial dysfunction and cytochrome c release during CS-induced apoptosis. A: functional mitochondria were labeled with Mitotracker Red CMXRos, a fluorescent dye that accumulates in functional mitochondria. Control rat AMs (Aa) have a punctate staining pattern. In contrast, cells exposed to 5 vol% CSE for 12 h (Ab) show cell shrinkage and diffuse staining throughout the cytoplasme, indicating mitochondrial dysfunction. Autofluorescence levels of control (Ac) and CSE-exposed (Ad) AMs prestained with Mito-Traker Red CMXRos are shown. Original magnification, ×400 for A, B, and E–H; ×200 for C and D.

![Image](http://ajplung.physiology.org/)

Fig. 6. CS-induced AM apoptosis is independent of p53 and Fas. AMs from p53-deficient mice (A–D), Fas-deficient mice (E and F), and Fas ligand-defective mice (G and H) were treated with (B, D, F, and H) or without (A, C, E, and G) 5 vol% CSE for 24 h. Then, cells 
were stained with May-Grünwald-Giemsa (A, B, and E–H) or immu-
nostained with anti-Bax antibody and DAB reaction (C and D). Arrows, Bax-overexpressing cells. Original magnifications: ×400 for 
A, B, and E–H; ×200 for C and D.

![Image](http://ajplung.physiology.org/)

Fig. 5. CS-induced AM apoptosis is independent of p53 and Fas. AMs from p53-deficient mice (A–D), Fas-deficient mice (E and F), and Fas ligand-defective mice (G and H) were treated with (B, D, F, and H) or without (A, C, E, and G) 5 vol% CSE for 24 h. Then, cells 
were stained with May-Grünwald-Giemsa (A, B, and E–H) or immu-
nostained with anti-Bax antibody and DAB reaction (C and D). Arrows, Bax-overexpressing cells. Original magnifications: ×400 for 
A, B, and E–H; ×200 for C and D.
in CS-exposed rats. However, the incidence of AM apoptosis in CS-exposed rats (3.2%) was much lower than expected based on the results of in vitro studies (Fig. 2). This may have been due to the long 24-h interval between CS exposure and BAL fluid collection. Alternatively, because antioxidants inhibited CS-induced AM apoptosis in vitro (Fig. 4C), antioxidants present in the respiratory tract may have prevented the effect of CS inhalation on AM apoptosis.

DISCUSSION

AMs represent the chief detoxifying mechanism for inhaled materials. Some inhaled toxic materials such as silica, asbestos, and other particulates are known to induce apoptosis of AMs (9, 14). The present study demonstrated that acute exposure to CS also induces apoptosis of AMs in vitro and, to some extent, in vivo. This apoptosis was associated with increased oxidative stress, Bax protein accumulation, mitochondrial dysfunction, and mitochondrial cytochrome c release but was independent of p53, Fas, and caspase activation. Importantly, the CS-induced apoptosis of AMs was inhibited by several antioxidants known to be present in the respiratory tract.

The findings of the present study implicate oxidative stress as a mechanism of CS-induced apoptosis. CS is a rich source of ROS and ROS inducers, and ROS, particularly at low levels, can induce apoptosis in some types of cells. However, the composition of ROS differs between different components of CS. The tar component of CS contains large quantities of stable and cell membrane-permeable radicals such as hydroquinones that redox cycle to form $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ and damage nuclear DNA (4, 25). In contrast, the gas-phase component of CS contains highly reactive, short-lived (<1-s) carbon- and nitrogen-centered species. This dif-
involvement. For example, infection of AMs by Chlamydia psittaci has recently been reported to induce apoptosis by a caspase-independent mechanism (21). Furthermore, Bax protein has been shown to induce both cytochrome c release and apoptosis-like cell death without caspase activation (16, 36). In this respect, recent evidence suggests that Bax and Bax-like proteins mediate caspase-independent death via channel-forming activity (1), which could promote mitochondrial permeability or puncture the mitochondrial outer membrane (8). In addition, CS may directly inhibit caspase functions through its oxidative activity because the caspases are cysteine-dependent enzymes that are sensitive to the redox status of the cells (10). This concept is supported by a recent report (29) documenting that CS directly inhibits caspase-3 activity. Taken together, these lines of evidence favor our conclusion that CS induces apoptosis of AMs through a mechanism other than caspase activation. However, we cannot exclude the possibility that CS-induced apoptosis is mediated by an as yet uncharacterized caspase or by other proteases. The mechanisms of apoptosis other than caspase activation may include activation of apoptosis-inducing factor, a newly identified mitochondrial protein that translocates into the nuclei and degrades DNA in response to apoptotic stimuli (15). However, this mechanism is unlikely involved in CS-induced AM apoptosis because an immunocytochemical study of CS-exposed AMs could not detect nuclear translocation of apoptosis-inducing factor from the mitochondria (data not shown).

The morphological features of CS-induced apoptosis resemble those of apoptosis previously reported to occur by a caspase-independent mechanism (22, 36). Caspase-independent apoptosis, some of which involves Bax, is associated with chromatin condensation, cell shrinkage, and mitochondrial dysfunction but not with chromatin fragmentation (22, 36). These changes are similar to those in CS-induced AM apoptosis found in this study. Both internucleosomal DNA fragmentation and chromatin fragmentation are known to require caspase activation. However, it has been reported that chromatin condensation during apoptosis does not require DNA fragmentation (28). In this context, agarose gel electrophoresis of DNA isolated from CS-exposed AMs in the present study could not detect any significant level of internucleosomal DNA fragmentation (data not shown).

Our finding of CS-induced AM apoptosis seems to be inconsistent with clinical observations in chronic cigarette smokers. The number of AMs in the lung is much greater in smokers than in nonsmokers (3). Indeed, macrophage alveolitis and respiratory bronchiolitis are thought to be the early changes in cigarette smokers, which, in susceptible individuals, probably lead to the alveolar wall destruction seen in pulmonary emphysema. However, if CS induces AM apoptosis in vivo, the number of AMs in the lung would decrease.

A simple explanation for the increased number of AMs in smokers is that the rate of monocyte recruitment from the blood into the lung exceeds the rate of AM death by apoptosis. Alternatively, there may be a mechanism in place to limit CS-mediated apoptosis of AMs in smokers’ lungs. First, AMs exposed to chronic CS in smokers’ lungs may become resistant to apoptosis. A very recent study (34) showed that expression of Bel-X<sub>L</sub>, an antiapoptotic protein, was increased in AMs from smokers compared with those from nonsmokers. Second, antioxidants present in the respiratory tract

Fig. 9. Induction of apoptosis of AMs by CS in vivo. Three rats were exposed to the smoke from 10 cigarettes over 5 h in an exposure chamber. After 24 h, rats were killed, and bronchoalveolar lavage (BAL) fluid cells were analyzed for apoptosis. A, C, E, and G: BAL fluid cells recovered from control rats. B, D, F, and H: BAL fluid cells recovered from CS-exposed rats. A and B: May-Grunwald-Giemsa staining (original magnification, ×400). C and D: transmission electron micrographs (original magnification, ×6000). E and F: immunostaining with anti-ED2 and DAB reaction followed by Giemsa staining (original magnification, ×200). G and H: TUNEL with 3-amino-9-ethylcarbazole reaction was followed by immunostaining with anti-ED2 and DAB reaction (original magnification, ×200). B, F, and H, arrows: apoptotic cells. BAL fluid recovered from CS-exposed rats contained a small proportion (~3%) of apoptotic cells positively stained with anti-ED2, which reacts with macrophages (F and H). Note that BAL fluid from CS-exposed rats contained some neutrophils and red blood cells (B). Photographs are representative of 3 separate experiments.
may prevent apoptosis of AMs by CS inhalation. The respiratory tract is covered with RTLEs that contain various antioxidants acting as a defensive shield against inhaled ROS and CS (4). In this context, the results of our in vitro studies show that glutathione, ascorbic acid, and α-tocopherol, which are considered to be important antioxidants in the respiratory tract (4), inhibited CS-induced apoptosis of AMs. Chronic smoke exposure in humans and hamsters has also been shown to increase antioxidant enzyme activities in AMs (17). This increased activity may serve as a mechanism to limit CS-mediated apoptosis in AM in smokers’ lungs. Although the life span of AMs in the normal lung is estimated to be ~80 days (33), studies are needed to determine the survival of AMs within smokers’ lungs.

The CS exposure experiments in vitro and in vivo in the present study have several limitations that may make them difficult to extrapolate to the clinical situation. First, the in vitro exposure to CSE does not simulate in vivo CS exposure. Although exposure to CSE is a standard procedure, its relevance to the in vivo state remains unclear. Second, the CS exposure in vivo in the present study seems to be very intense as evidenced by the occurrence of some degree of alveolar bleeding. This is never observed in human smokers. Third, because mice are obligatory nasal breathers, some toxic products that would be normally inhaled by humans may have been deposited in the nasal passage of the mice. Fourth, the CS exposure experiments in this study are a model of acute smoking and cannot be extrapolated to what occurs in smokers who inhale CS chronically and intermittently. Although these limitations must be taken into account when interpreting the results of this study in relation to cigarette smokers, the results demonstrate that CS has the ability to induce apoptosis of AMs.

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