Cytotoxic effects of cigarette smoke extract on an alveolar type II cell-derived cell line

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Hoshino, Yuma, Tadashi Mio, Sonoko Nagai, Hiroyuki Miki, Isao Ito, and Takateru Izumi. Cytotoxic effects of cigarette smoke extract on an alveolar type II cell-derived cell line. Am J Physiol Lung Cell Mol Physiol 281: L509–L516, 2001.—Injury of the alveolar epithelium by cigarette smoke is presumed to be an important process in the pathogenesis of smoking-related pulmonary diseases. We investigated the cytotoxic effects of cigarette smoke extract (CSE) on an alveolar type II cell-derived cell line (A549). CSE caused apoptosis at concentrations of 5% or less and necrosis at 10% or more. When CSE was exposed to air before application to A549 cells, the cytotoxic effects were attenuated. CSE caused cell death without direct contact with the cells. Acrolein and hydrogen peroxide, two major volatile factors in cigarette smoke, caused cell death in a similar manner. Aldehyde dehydrogenase, a scavenger of aldehydes, and N-acetylcysteine, a scavenger of oxidants and aldehydes, completely inhibited CSE-induced apoptosis. CSE and acrolein increased intracellular oxidant activity. In conclusion, apoptosis of alveolar epithelial cells may be one of the mechanisms of lung injury induced by cigarette smoking. This cytotoxic effect might be due to an interaction between aldehydes and oxidants present in CSE or formed in CSE-exposed cells.

apoptosis; oxidant; aldehyde

cigarette smoking is one of the most clearly proven etiologic factors in the development of bronchogenic carcinoma and chronic obstructive pulmonary disease (10). There are also significant relationships between cigarette smoking and the incidence and progression of pulmonary diseases such as respiratory bronchiolitis and eosinophilic granuloma (10), although the precise mechanisms of interaction remain unclear.

Alveolar epithelial cells are known to have diverse functions. They produce surfactant proteins to reduce surface tension and protect lung constituents from external stimulants; they release cytokines and growth factors to regulate inflammation and cell growth; and they release matrix proteins, proteinases, and proteinase inhibitors to regulate turnover of alveolar structure (11, 19, 21). Thus damage to alveolar cells may increase in epithelial permeability and decreases in surfactant synthesis, ultimately leading to pulmonary edema and collapse of the alveoli. Moreover, the damage may elicit remodeling of the alveolar structure due to inappropriate production of cytokines and growth factors.

Cigarette smoke is known to have various injurious effects on alveolar epithelial cells. For example, it suppresses proliferation, attenuates attachment, and augments detachment of the cells (14, 22). It can also cause DNA single-strand breaks in the cells and suppression of surfactant secretion and collagen production (14, 16). Thus injury of alveolar epithelial cells might contribute to the development of the lung diseases induced by cigarette smoking.

Cigarette smoke contains >4,000 kinds of constituents (2, 6). Many factors in both the nonvolatile and volatile phases of cigarette smoke are known to be carcinogenic and cause lung injuries (4, 7). Oxidants and aldehydes, major constituents in the volatile phase of cigarette smoke, are suggested to induce the death of lung cells (13, 18). However, it remains unclear which constituents in cigarette smoke play the most important roles in cell injuries.

In this study, we investigated the mechanisms by which cigarette smoke induced cell death in an alveolar type II cell-derived cell line. The results indicated that volatile factors in cigarette smoke cause mainly apoptosis of the cells at lower concentrations and necrosis at higher concentrations. It also appeared that aldehydes and oxidants are major factors in these processes.

MATERIALS AND METHODS

Chemicals and reagents. The following chemicals and reagents were purchased for this study: aldehyde dehydrogenase (ALDH; Biogenesis), benzoylcarbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone (ZVAD-fmk; Enzyme Systems Products, Livermore, CA), 5-(and-6)-choloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Molecular Probes, Eugene, OR), fetal bovine serum (FBS; Whittaker BioProducts, Walkersville, MD), and Dulbecco's modified Ear

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The double staining with Hoechst 33342 and ethidium bromide and Hoechst 33342 was performed immediately before each experiment except when otherwise indicated.

Cell culture. A549 cells (alveolar type II cell-derived cell line; American Type Culture Collection, Manassas, VA) were cultured with DMEM supplemented with 10% FBS, 100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B and subcultured weekly at a 1:4 ratio. The pH of the CSE was between 7.4 and 7.5 when diluted for each experiment.

The concentration of nicotine, one of the relatively stable constituents, was assessed with high-performance liquid chromatography. The mean concentration of three separated CSE preparations was 1.00 ± 0.09 (SD) mg/ml. The CSE was prepared immediately before each experiment except when otherwise indicated.

Cell treatment. After culture in 48-well tissue culture plates until confluence, the cells were washed three times with serum-free DMEM and incubated for 16 h to minimize the effects of FBS. The cells were then washed another three times with HEPES-buffered DMEM and incubated separately with CSE, hydrogen peroxide (H₂O₂), or acrolein (Acr) at the concentrations indicated for 48 h (except in the time-course experiments and the examinations of intracellular oxidant activities).

Intracellular oxidant activities. The viability, morphological changes, DNA fragmentation, and intracellular oxidant activity of the incubated cells were then assessed as described below. Effects of the oxidants were further examined by simultaneous addition, separately, of their scavengers, N-acetylcysteine (NAC), oxyhemoglobin (HbO₂), superoxide dismutase (SOD), catalase (Cat), uric acid (UA), or mannitol (MTL), at the concentrations indicated with the CSE. Effects of the aldehydes were also examined by separately adding their scavenger, ALDH, at the concentration indicated with the CSE in the presence of 0.5 mM NAD⁺.

A broad-spectrum caspase inhibitor, ZVAD-fmk, was used to examine the involvement of caspase in CSE-induced cell death. The cells were incubated with 20 mM ZVAD-fmk for 2 h and then exposed to 5% CSE. After 48 h of incubation, the viability and morphological changes of the cells were assessed as described below.

Assay of cell viability and morphological observations. The viability of the cells treated by the above-described procedures was quantified by three separate methods: i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the trypan blue exclusion method, and double staining with ethidium bromide and Hoechst 33342.

The MTT assay was performed as described in a previously published report (20). Briefly, CSE-treated or untreated cells cultured in 48-well tissue culture plates (Corning, Corning, NY) were incubated with 8 mg/ml of MTT, and the precipitated formazan was dissolved by adding three volumes of ethanol. Optical density was quantified with a spectrophotometer (MCC/340, Titertek Instruments, Huntsville, AL).

The double staining with Hoechst 33342 and ethidium bromide was performed with a modification of a previously published method (5). Cells cultured in eight-well Lab-Tek chamber slides (Nunc, Naperville, IL) were stained with 4 μg/ml of Hoechst 33342 and 4 μg/ml of ethidium bromide and were then observed with fluorescence microscopy. The number of living, apoptotic, and necrotic cells was quantified, starting with minimum cell counts of 400 cells.

Qualitative assay of DNA fragmentation. Apoptotic cell death was also confirmed by revealing DNA fragmentation with a modification of a previously published method (12). After 48 h of incubation with CSE, the cells were lysed with 0.1% Triton X-100 and centrifuged at 30,000 g for 30 min. The supernatants were incubated with 20 μg/ml of RNase A at 37°C for 60 min and then with 100 μg/ml of proteinase K at 50°C for 3 h. The samples obtained were purified with phenol-chloroform extraction, precipitated by ethanol, and applied to a 1% agarose gel for electrophoresis at 50 V for 3 h. The gels were stained with ethidium bromide and visualized under ultraviolet transillumination.

Assay of intracellular oxidative activity. Intracellular oxidative activity was evaluated with a fluorogenic substrate, CM-H₂DCFDA, as described in a previously published report (9). The chloromethyl group of the substrate binds with intracellular glutathione, and the subsequent oxidation yields fluorescent adducts that are trapped inside the cell.

The cells were incubated with either 5% CSE (in the presence and absence of either 1 mg/ml of NAC or 1 U/ml of ALDH), 200 μM Acr, or 1 mM H₂O₂ for 3 h. The cells were then trypsinized, diluted to 10⁵ cells/ml in PBS, and incubated with 10 mM CM-H₂DCFDA for 15 min at 37°C. After the cells were washed twice with PBS, 10⁴ cells were analyzed by FACSScan (Becton Dickinson, Franklin Lakes, NJ) to determine their fluorescence intensity. To confirm that the uptake of CM-H₂DCFDA was equal in the three conditions, the cells were further incubated with 10 mM H₂O₂ for 30 min. There were no significant differences in fluorescence intensity between untreated cells and treated cells after the incubation.

Data analysis. Statistical analyses were performed with StatView (SAS Institute, Cary, NC). Data are expressed as means ± SD. Analysis of variance (ANOVA) was performed.
followed by Fisher’s protected least significant difference as a post hoc test. A P value of <0.05 was considered significant.

RESULTS

Effects of CSE on cell viability. CSE reduced cell viability in a time- and concentration-dependent manner (P < 0.001 by two-way ANOVA; Fig. 1). In the presence of 5% CSE, cell viability was reduced to 58 ± 2 and 19 ± 4% at 48 and 72 h of incubation, respectively. After 48 or more hours of incubation, CSE at concentrations of 10% or more reduced cell viability to <5%. In the subsequent experiments, cell viability was measured after 48 h of incubation. The cell viability in this study was assessed by MTT assay and trypan blue exclusion. Because the results by the two methods were almost identical, only the results by MTT assay are given.

Morphological and molecular changes of A549 cells induced by CSE. After 48 h of incubation, 5% CSE induced chromatin condensation in ~44.7% of the cells, suggesting apoptotic cell death. At the same time point, 99.0% of cells incubated with 10% CSE were necrotic and 99.2% of untreated cells were alive (Fig. 2).

Electrophoresis of the DNA fraction also confirmed that incubation with 5% CSE induced apoptotic cell death. The DNA fraction of the cells treated with 5% CSE revealed a ladder formation of an ~200-bp fragment and its multiples (Fig. 3).

For further confirmation, apoptotic cell death was assessed after treatment with a broad-spectrum caspase inhibitor. However, ZVAD-fmk did not eliminate the morphological changes in A549 cells (Fig. 2D) or the reduced cell viability (51 ± 3% of the control cells) induced by 5% CSE.

Fig. 2. Morphological changes in A549 cells induced by CSE. After 48 h of incubation, culture medium, 5 or 10% CSE, or both 5% CSE and 20 μM benzoyloxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone (A–D, respectively), the cells were stained with Hoechst 33342 and ethidium bromide and observed with fluorescence microscopy. Living cells have normal-shaped nuclei that are faintly stained with Hoechst 33342 and ethidium bromide and observed with fluorescence microscopy. Living cells have normal-shaped nuclei that are faintly stained with Hoechst 33342. Apoptotic cells have shrunken nuclei with chromatin condensation. Necrotic cells have normal-shaped nuclei that are brightly stained with ethidium bromide. Percentages of living (solid bars), apoptotic (hatched bars), and necrotic cells (open bars) were determined by counting as described in MATERIALS AND METHODS (E). At least 400 cells were counted for each sample.
Treatment of CSE and its effects on cytotoxicity. To determine which cytotoxic effects of CSE were present in the volatile and nonvolatile phases, aliquots of CSE were exposed to air in a clean bench for the indicated hours and applied to the cells. As shown in Fig. 4, air exposure reduced the cytotoxic effects of CSE in a time-dependent manner ($P < 0.001$ by ANOVA). Air exposure for 24 h or more completely abolished the effects.

When the cells were incubated in a large dish with CSE without direct contact (Fig. 5A), the CSE exerted a cytotoxic effect in a concentration-dependent manner ($P < 0.001$ by ANOVA; Fig. 5B).

**Modulation of cytotoxic effects of CSE.** As shown in Fig. 6, NAC (a nonspecific antioxidant) completely abolished the cytotoxic effects of CSE. HbO$_2$ (a nitric oxide scavenger), Cat (a H$_2$O$_2$ scavenger), UA (a peroxynitrite scavenger), and MTL (a hydroxyl radical scavenger) also significantly reduced the cytotoxic effects of CSE. When the cells were treated with a combination of NAC and HbO$_2$, the cytotoxic effects were almost completely abolished.

Fig. 3. DNA fragmentation of A549 cells induced by CSE. A549 cells were incubated with culture medium or 5 or 10% CSE for 48 h and lysed. DNA fractions were collected, treated with RNase A and proteinase K, and applied to gel electrophoresis. Lane 1, DNA marker of HindIII-digested phage DNA (top to bottom: bands of 9,416, 6,557, 4,361, 2,322, 2,027, and 564 bp). Lanes 2–4, DNA fractions of cells treated with culture medium or 5 or 10% CSE, respectively.

Fig. 4. Effects of exposure to air on cytotoxicity of CSE. CSE was separated into 4 aliquots. Three aliquots were exposed to air in a clean bench for 1, 3, or 24 h before being applied to the cells. One aliquot was applied immediately to the cells. After 48 h of incubation, cell viability was evaluated. • Culture medium; ♦, 5% CSE; ▲, 10% CSE. *$P < 0.05$ compared with the cells treated with CSE immediately after preparation.

Fig. 5. Indirect contact of CSE with A549 cells. A549 cells cultured in a 35-mm dish and CSE or culture medium cultured in a separate 35-mm dish were placed together in one 100-mm dish (A). After 48 h of incubation, cell viability was evaluated by MTT assay (B). *$P < 0.05$ compared with medium-alone condition.
scavenger) partially attenuated the effects. SOD (a superoxide scavenger) alone did not attenuate the effects, but a combination of SOD and Cat did. ALDH (an aldehyde scavenger) almost completely abolished the effects. All of the effects observed were concentration dependent ($P < 0.05$ by ANOVA).

Cytotoxic effects of aldehyde and oxidant. Because aldehydes and oxidants seemed to be the major contributors of the cytotoxic effects in CSE, the effects of Acr and $H_2O_2$ were examined. Both reagents induced apoptosis at a lower concentration (200 $\mu$M and 1 mM, respectively) and necrosis at a higher concentration (500 $\mu$M and 10 mM, respectively; Fig. 7).

Effects of oxidant, aldehyde, and CSE on intracellular oxidative activity. Treatment with $H_2O_2$, Acr, or CSE at a concentration that induced mainly apoptosis (1 mM, 200 nM, and 5%, respectively) increased the fluorescence intensity of A549 cells treated with CM-H$_2$DCFDA to 5.6-, 5.3-, and 2.1-fold, respectively, of that of untreated cells (Fig. 8). The addition of NAC or
ALDH significantly reduced the CSE-induced increase in fluorescence intensity to 47.3 and 49.3%, respectively (Fig. 8, C and D). The results indicate that 1 mM H₂O₂, 200 mM Acr, or 5% CSE increased the intracellular oxidative activity in A549 cells and that the effects of CSE were inhibited by NAC and ALDH.

**DISCUSSION**

In this study, we showed that CSE induced apoptosis of an alveolar type II cell-derived cell line (A549) at lower concentrations and necrosis of A549 cells at higher concentrations. These effects were induced mainly by the volatile phase of CSE and were attenuated by both aldehyde scavengers and oxidant scavengers. Similarly, Acr and H₂O₂ also induced apoptosis and necrosis of the cells. Both CSE and Acr enhanced intracellular oxidative activity.

The concentration of nicotine in the 5% CSE used in the present study was ~50 μg/ml according to the high-performance liquid chromatography measurements described in materials and methods. Based on the results shown in Figs. 2D and 7, the cytotoxic effects of 200 μM Acr can be presumed to be equal to those of 5% CSE, assuming that all of the cytotoxic effects of CSE are due to the Acr contained therein. Previously published reports (8, 11) suggested that the concentrations of nicotine and Acr in the alveolar lining fluid are assumed to be 1–10 μg/ml and 80 μM, respectively, after smoking one cigarette. However, the concentrations of these substances could be higher in chronic smokers because they accumulate in the alveolar lining fluid (14). Therefore, the concentrations of nicotine and Acr in the 5% CSE used in this study may be similar to those in the epithelial lining fluids of chronic smokers.

CSE seems to induce apoptosis of freshly prepared human type II pneumocytes at lower concentrations compared with A549 cells (data not shown). The result was preliminary, and further examinations are required to establish the effects on the primary cells.

In this study, apoptotic cell death was quantified by two methods, the MTT assay and Hoechst 33342-ethidium bromide staining. A previous report (1) suggested that the MTT assay underestimated apoptosis, but in the present study, we found a significant correlation between the measurements with Hoechst 33342-ethidium bromide staining and the MTT assay (data not shown). This may have been because the incubation time was long enough for most of the apoptotic cells to exhibit features of late-phase apoptosis (e.g., stained by ethidium bromide). Therefore, we decided to use the data obtained by the MTT methods to represent the results.

Although morphological and molecular changes of the cells clearly showed that CSE concentrations of 5% or less induced apoptosis, a broad-spectrum caspase inhibitor was unable to inhibit CSE-induced apoptosis. This result suggests that cigarette smoke might induce apoptosis through a caspase-independent pathway.

Cigarette smoke consists of ~4,000 constituents (2), each present in varying amounts depending on the smoking conditions and kind of cigarette. Although both volatile and nonvolatile phases of cigarette smoke are known to contain cytotoxic constituents (7), our results suggested that the cytotoxic effects of CSE are mainly present in the volatile phase. Aldehydes and oxidants, two major constituents of cigarette smoke, have been reported to induce apoptosis of lung cells (13, 18). Our present study also indicated that either aldehyde or oxidant alone had cytotoxic effects similar to those of CSE, and this was consistent with the previous reports. In this study, ALDH almost completely abolished the apoptosis induced by CSE. Oxidant scavengers such as NAC, HbO₂, and Cat significantly attenuated the cytotoxicity, but all three of these substances are also capable of scavenging aldehydes (7). Thus the aldehydes appear to be the most important toxicants in the volatile phase of CSE. On
the other hand, because the cytotoxicity was also partly attenuated by UA and MTL, other oxidant scavengers that have no ability to scavenge aldehydes also appear to be involved, albeit to a lesser extent.

In view of the interaction between aldehydes and oxidants, we know that aldehydes can be produced by oxidant-induced lipid peroxidation processes and are known to be the main causative agents of oxidant injury (17). Our results also suggest that enhanced intracellular oxidative activity might be one of the mechanisms of aldehyde cytotoxicity. Thus we presume that the aldehydes and oxidants present in the CSE or formed in the CSE-exposed cells induce apoptosis of alveolar epithelial cells by acting synergistically.

In this study, physiologically attainable concentrations of soluble cigarette smoke induced apoptosis of A549 cells. Because alveolar lining fluid contains antioxidant activity, it may prevent the cytotoxic effects of cigarette smoke in healthy subjects. However, this activity is known to be decreased in disease-related conditions or by cigarette smoking (3). Thus cigarette smoke may cause apoptosis of alveolar epithelial cells in patients with inflammatory lung diseases and chronic smokers. Although it remains unclear how CSE-induced apoptosis of alveolar epithelial cells leads to lung injury, cell death and deprivation could denude the alveolar structure and augment its destruction. These events might be involved in the development of chronic obstructive pulmonary disease or the progression of pulmonary fibrosis. In addition to smoking cessation, the clinical use of aldehyde or oxidant scavengers such as NAC may be beneficial for the prevention of smoking-related lung injuries.

In conclusion, we demonstrated that CSE induces apoptosis of an alveolar type II cell-derived cell line. This cytotoxic effect was attributed to a complex interaction of aldehydes and oxidants that were either present in the CSE or formed in the cells exposed to the CSE. These results may contribute to the development of
of a new strategy to prevent cigarette smoke-induced lung injury.

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