AP-2 α expression and cell apoptosis of the lung tissue of rats with COPD and ECV304 cells stimulated by cigarette smoke extract

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An increasing body of evidence suggests that apoptosis of structural cells in the lung might be an important upstream event in the pathogenesis of chronic obstructive pulmonary disease (COPD). AP-2 α is one of the important transcription factors involved in the modulation of apoptosis in carcinogenesis and idiopathic-dilated cardiomyopathy. The relationship between AP-2 α and apoptosis in COPD remains to be elucidated. The aim of the present study was to investigate the expression of AP-2 α in the lung tissues of rats with COPD induced by smoking and its possible protective effect on cigarette smoke extract (CSE) induced endothelial cell apoptosis. Sprague-Dawley rats ($n = 24$) were randomly assigned to normal and COPD groups. The COPD group was exposed to smoke from 20 commercial unfiltered cigarettes for 80 d before morphological assessment of the lung tissue was performed. The expression of AP-2 α in lung tissues was measured by Western blotting. To demonstrate the relationship between apoptosis and AP-2 α , *in vitro* cell experiments were carried out. Cells were treated with different concentrations of CSE before proliferation was measured by MTT. Apoptosis was then determined by Hoechst staining and the expression of cleaved caspase-3 and AP-2 α by Western blotting over time following treatment with 5% CSE. Cells were then infected with an AP-2 α adenovirus vector and the expression of cleaved caspase-3 and AP-2 α was compared to the control groups by Western blotting. The COPD group showed larger air spaces and significant decrease of FEV_{0.3}/FVC compared with the rats in the control group ($P < 0.05$). The expression of AP-2 α was significantly higher in the lung tissue of rats with COPD compared with those of controls ($P < 0.05$). In the ECV304 cells, CSE induced apoptosis ($P < 0.01$) and caspase-3 activation in a time-dependent manner and reduced the cell proliferation rate in a dose-dependent manner ($P < 0.005$). Moreover, 5% CSE treatment increased endogenous AP-2 α protein expression. AP-2 α overexpression inhibited 5% CSE-induced cell apoptosis and activated caspase-3 expression ($P < 0.05$). AP-2 α protects ECV304 cells against CSE-induced apoptosis and may play an important role in smoking induced-apoptosis in COPD.

AP-2 α , apoptosis, cigarette smoke extract, COPD, vascular endothelial cell

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Chronic obstructive pulmonary disease (COPD) is one of the major public health problems because of its high morbidity and mortality, but its pathogenesis remains enigmatic. Cigarette smoking is one of the major etiological factors of COPD, with the mechanisms including chronic inflammation [1], elastase/antielastase imbalance [2] and oxidant/antioxidant imbalances [3]. Recently, more and more studies

have shown increased apoptosis in the alveolar walls in COPD patients [4] and COPD animal models [5] induced by cigarette smoke exposure. Some studies related apoptosis in alveolar walls to emphysema in animal models. The pro-apoptosis mechanisms of cigarette smoke exposure were related to activated caspase [6], decreases in VEGF and VEGFR-2 [7], increased oxidative stress [8], inflammation and impaired repair responses [9].

Activator protein-2 (AP-2) is a family of cell type-specific and developmentally regulated transcription factors. To date,

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five members of the AP-2 family including AP-2 α , AP-2 β , AP-2 γ , AP-2 δ and AP-2 ϵ have been identified. AP-2 α has been implicated in vertebrate development, embryogenesis [10], carcinogenesis [11] and idiopathic-dilated cardiomyopathy [12]. As a transcription factor, AP-2 α was suggested to associate with apoptosis by modulating the expressions of many genes such as VEGF [13], P⁵³ [14], p21WAF/CIP [15], Bcl-2 [16] and manganese superoxide dismutase (MnSOD) [17].

Based on the important status of apoptosis in COPD and that AP-2 α is involved in apoptosis in many diseases, in this study we investigated the expression of AP-2 α in the lung tissue of smoking-induced COPD rats as well as the effect of AP-2 α over-expression in the CSE-induced apoptosis of ECV304 cells.

1 Materials and methods

1.1 Animals

Twenty-four male Sprague-Dawley rats, each weighing 186–240 g, were purchased from the Animal Center of the Second Xiangya Hospital, Changsha, China. The rats were randomly assigned to two groups of 12: normal control and COPD groups. The COPD group was exposed to smoke from 20 commercial unfiltered cigarettes (Furong, Changde Cigarette Factory, Changde, Hunan, China) for 1 h each day, 6 d a week, for a total of 80 d. The smoke exposure box was 69 cm×47 cm×38 cm. The control group were treated the same as the COPD group except with the absence of smoke exposure. Experiments were approved by the animal ethics committee and were performed following strict government and international guidelines.

1.2 Morphological assessment

After fixation, 5- μ m lung sections were stained with hematoxylin and eosin (HE) stain. As emphysema is a structural disorder characterized by destruction of the alveolar walls and enlargement of the alveolar spaces, enlargement of alveolar spaces was assessed by quantifying the mean linear intercept (MLI) and destruction of alveolar walls by measuring the destructive index (DI) [18]. MLI, a measure of inter-alveolar wall distance, was determined by light microscopy at a magnification of 100 $\times$. Fields that included large airways or vessels were excluded from the analysis. MLI was defined as the total length of the cross-line divided by the numbers of alveolar walls intersecting the test lines, as described by Xu et al. [19]. DI was calculated as a measure of parenchymal destruction using a microscopic point-count technique [20]. The analysis was performed in duplicate by randomly counting more than 3000 alveoli from 50 HE sections from each rat at a magnification of 200 $\times$. For each photomicrograph, a single observer who was blind to the related data performed the measurements.

1.3 Pulmonary function

Half of the rats from the two groups were randomly selected for lung function measurements, according to the method described by Chen et al [21]. After induction of anaesthesia by intraperitoneal administration of chloral hydrate (3 mL/kg), a Y-type endotracheal cannula was connected to a flow transducer (HX200, Beijing Xinghangxingye Corporation, Beijing, China) for measurement of FEV_{0.3}/FVC (Forced expiratory volume in 0.3 s/Forced vital capacity) and PEF (Peak expiratory flow).

1.4 Cell culture

ECV304 cells obtained from the Xiangya Type Culture Collection (Changsha, China), were cultured in RPMI medium 1640 supplemented with 10% heat-inactivated newborn bovine serum and antibiotic-antimycotic mix in a humidified incubator with 5% CO₂ and 95% air. At 70%–80% confluence, ECV304 cells were treated with CSE and/or adenovirus infection.

1.5 Preparation of cigarette smoke extract (CSE)

CSE was generated by bubbling smoke from 1 standard reference cigarette into 20 mL RPMI medium 1640 (Life Technologies, California, USA) through a 50-mL fritted impinger at a flow rate of 350 mL/min. The resulting extract was passed through a 0.22- μ m filter and was considered 100% CSE. The CSE was adjusted to pH 7.4 by the addition of NaOH before being diluted to 2.5%–20% concentration with RPMI medium 1640 and used within 1 h.

1.6 Adenovirus reagents and infections

The Ad-AP-2 α adenovirus vector and its control Ad-BgII were generously provided by Dr F. Domann of Iowa University (USA). Adenoviral infections were carried out as described [22]. Briefly, 5×10^5 cells were plated in a 60-mm culture dish. After 24 h of culture, when reaching 80% confluence, the cells were washed three times using PBS. Adenoviral titers were determined and transient infections were carried out using an MOI of 100. After another 24-h culture in free-serum medium, the cells were collected or 5% CSE was added for culturing another 24 h, then the cells were harvested for Hoechst staining and Western blot analysis.

1.7 Hoechst 33258 nuclear staining assay

Nuclear staining with Hoechst 33258 (Sigma Chemical Co, USA) was performed as described elsewhere [23]. Briefly, the floating and trypsinized-adherent CSE treated and non-treated cells were collected at appropriate time points, washed with PBS and then fixed with 4% paraformaldehyde in PBS for 30 min at 4°C. After washing, the cells were

smear onto microscope slides followed by incubation in Hoechst 33258 at a final concentration of 30 $\mu\text{g/mL}$ at room temperature for 5 min. Nuclear morphology was then examined under a fluorescent microscope (Imaging Source Europe GmbH, Bremen, Germany). The analysis was performed in duplicate by randomly counting more than 500 nuclei from each concentration at a magnification of 100 $\times$. For each photomicrograph, a single observer who was blind to the related data performed the measurements.

1.8 MTT assay

The MTT assay was carried out as described previously [24]. Cells were plated in a 96-well plate at $1.3 \times 10^4/\text{well}$. After 24 h of plating, the cells were treated with 0, 2.5%, 5%, 10%, 15% or 20% CSE for 24 h before MTT (20 μL of 5 mg/mL) was added. The medium was removed from the wells 4 h after MTT addition, and 100 μL of dimethyl sulfoxide was added to dissolve the formazan crystals before the absorbance was measured at 570 nm in an enzyme-linked immunosorbent assay reader.

1.9 Western blotting analysis

Nuclear and cytoplasmic extracts were prepared according to the method of Schreiber et al. [25]. Briefly, lung homogenate or the cell pellet was resuspended in 400 μL of cold buffer A (10 mmol L^{-1} HEPES pH 7.9, 10 mmol L^{-1} KCl, 0.1 mmol L^{-1} EDTA, 0.1 mmol L^{-1} EGTA, 1 mmol L^{-1} DTT, 0.5 mmol L^{-1} PMSF). The cells were allowed to swell on ice for 15 min, after which 25 μL of a 10% solution of NP-40 was added and the tube was vigorously vortexed for 10 s. The homogenate was centrifuged for 30 s in a microcentrifuge and the supernatant containing the cytoplasmic extract was removed. The nuclear pellet was resuspended in 50 μL of ice-cold buffer B (20 mmol L^{-1} HEPES pH 7.9, 0.4 mol L^{-1} NaCl, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} EGTA, 1 mmol L^{-1} DTT, 1 mmol L^{-1} PMSF) and the tube was vigorously rocked at 4°C for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min in a microcentrifuge at 4°C and the supernatant was frozen in aliquots at -80°C . The protein contents of the different fractions were determined by the Bradford assay method.

Proteins from the subcellular fractions were mixed with 2 $\times$ SDS sample buffer (100 mmol L^{-1} Tris-HCl (pH 6.8), 4% SDS (w/v), 20% glycerol, 200 mmol L^{-1} DTT, 0.1% bromophenol blue (w/v)) and boiled in a water bath for 5 min, before separation on 10% polyacrylamide gels and transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was then blocked in blocking buffer at room temperature for 6 h, incubated for 2 h at 25°C with primary antibody (mouse anti-AP-2 α monoclonal antibody (Santa Cruz, US; 1:1000); mouse anti-caspase-3 monoclonal antibody (Beyotime Institute of Biotechnology, China;

1:250); mouse anti-proliferating cellular nuclear antigen (PCNA) monoclonal antibody (Upstate, US; 1:1000)), followed by horseradish peroxidase-conjugated secondary antibody IgG (Jackson Biotech, US; 1:1000), for 1 h at 25°C. Proteins were visualized using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, UK) and X-ray film.

1.10 Statistical analysis

Data were presented as means $\pm$ SEM. Significance of differences between groups was determined using ANOVA with two-tailed Student's *t* test or LSD test. Multiple rate comparisons were examined by the χ^2 test. $P < 0.05$ was considered significant.

2 Results

2.1 Establishment of a rat model of COPD and the expression of AP-2 α in lung tissue of COPD rats

(i) Histological studies. HE staining of lung tissue sections from the COPD group showed histologically advanced emphysema. In contrast, histological markers of emphysema were absent in lung tissue sections obtained from the normal group (Figure 1). The MLI and DI in the COPD group (77 ± 29 mm and $59\% \pm 7\%$, respectively) were significantly higher than those in the normal group (44 ± 7 mm and $18\% \pm 5\%$). There was increased inflammatory cell infiltration in COPD rat lungs compared with normal rat lungs, as assessed by light microscopic examination of HE-stained slides.

(ii) Lung function. $\text{FEV}_{0.3}/\text{FVC}$ and PEF were lower in the COPD group ($65.1\% \pm 8.4\%$ and 18.8 ± 1.6 mL/s, respectively) compared with the normal group ($85.6\% \pm 5.9\%$ and 47.2 ± 7.3 mL/s) ($P < 0.05$).

(iii) Expression of AP-2 α in lung tissue of COPD rats. AP-2 α protein expression in lung nuclear extracts from rats in the two experimental groups was assessed by Western blotting. The expression of AP-2 α was increased in the COPD group compared with the normal group (Figure 2).

2.2 Cell proliferation, apoptosis and activation of caspase-3 in ECV304 cells treated with CSE

To assay the cytotoxic potential of CSE, the influence of different concentrations of CSE on the viability of ECV304 cells was measured by MTT assay. Compared with the control group, proliferation was significantly increased in the 2.5% CSE-treated group, while it decreased in a concentration-dependent manner in the remaining CSE-treated groups ($P < 0.01$) (Figure 3).

To examine the cell apoptosis in response to CSE treatment, both control and 5% CSE-treated cells were stained with the fluorescent Hoechst 33528 dye and visualized

Figure 1 HE staining of lung tissue sections from rats in the normal group (a) and the COPD group (b) (100 $\times$).

under a fluorescence microscope. Figure 4 shows the Hoechst staining results for 5% CSE treated-ECV304 cells over 48 h. ECV304 cells at 0 h remained uniformly stained. At 12 h, the cells began to display apoptotic morphology, such as enucleation and apoptotic bodies. At 24 h, nuclear condensation and fragmentation were more apparent, with the chromatin condensed into lumps, exhibiting punctated morphology typical of apoptotic cells. At 36 and 48 h, apoptotic

events culminated in the formation of apoptotic bodies, seen as smaller intensely stained spots. Shrinkage of cells, DNA condensation and even some cell destruction were observed.

The expression levels of activated caspase-3 were increased in ECV304 cells treated with 5% CSE in a time-dependent manner (Figure 5(a)). Compared with the untreated cells, caspase-3 levels increased to 135% at 12 h, 244% at 18 h, 289% at 24 h and 323% at 36 h, respectively (Figure 5(b)).

2.3 The expression of AP-2 α was induced by 5% CSE in ECV304

The expression of AP-2 α protein in ECV304 cells treated with

Figure 2 Western blot analysis of AP-2 α protein expression in lung nuclear extracts. Lanes 1 and 2, normal group; lanes 3–8, COPD groups. HeLa cell nuclear extract was used as a positive control.

Figure 3 ECV304 cells were treated with a concentration of 0, 2.5%, 5%, 10%, 15% and 20% of CSE for 24 h, and the cell viability was quantified using the MTT assay. The absorbance for 0% CSE group (control group) was considered as 100%. The abscissa indicates different groups; The Y-axis indicates the ratio of treatment group A/Ctrl group A. *, $P < 0.05$ compared with the control group.

Figure 4 Nuclear staining of ECV304 cells with Hoechst 33258. Cells were treated with 5% CSE. (a) Untreated (control) ECV304 cells remained uniformly stained with round and unpunctuated nuclei; (b) 5% CSE at 12 h; (c) 5% CSE at 24 h; (d) 5% CSE at 36 h; (e) 5% CSE at 48 h. 5% CSE-treated ECV304 cells showed apoptotic morphology: cell shrinkage, DNA condensation and nuclear fragmentation.

Figure 5 The expression of activated caspase-3 was analyzed by Western blotting in ECV304 cells treated with 5% CSE for 0, 12, 18, 24 and 36 h, respectively. The nuclear and cytoplasmic fractions were isolated and the level of activated caspase-3 was assayed by Western blotting (a). β -actin was used as an internal control. (b) Compared with the control, the expressions of activated caspase-3 at 18, 24 and 36 h were significant ($P < 0.001$, $n = 3$).

5% CSE was detected by Western blotting. The expression level of AP-2 α increased in a time-dependent manner (Figure 6(a)), with levels increasing to 106% at 6 h, 125% at 12 h, 139% at 24 h and 161% at 48 h, respectively, compared to untreated controls (Figure 6(b)).

2.4 Overexpression of AP-2 α by adenoviral infection inhibited both apoptosis and caspase-3 activation induced by CSE in ECV304

(i) Expression of AP-2 α in Ad-AP-2 α -infected cells. To

Figure 6 The expression of AP-2 α induced by 5% CSE. The cells were stimulated with 5% CSE for 0, 6, 12, 24 and 48 h. Nucleoproteins were isolated and the levels of AP-2 α were assayed by Western blotting. HeLa cells were used as the positive control. PCNA was used as an internal control. (a) There was a time-dependent increase in the expression level of AP-2 α ; (b) compared with Ctrl (0% CSE), the expression levels of AP-2 α at 12, 24 and 48 h were increased significantly ($P < 0.05$ for all, $n = 3$).

detect the induced expression of AP-2 α protein in Ad-AP-2 α -infected ECV304 cells, AP-2 α protein levels were monitored and compared to the levels in cells infected with the control virus Ad-BgII. At 24 h after infection, nuclear extracts were collected and the expression of AP-2 α protein was assessed. Upon Ad-AP-2 α infection, ECV304 cells showed twice the amount of AP-2 α protein in comparison with Ad-BgII transfection (Figure 7(a)).

(ii) AP-2 α overexpression inhibited the apoptosis induced by CSE in ECV304. To study the effect of AP-2 α on apoptosis induced by CSE, we stimulated ECV304 cells with 5% CSE 24 h after infection with Ad-BgII and Ad-AP-2 α , before assessing the morphological changes 24 h later. ECV304 cells infected with Ad-BgII displayed apoptotic morphology, such as enucleation and apoptotic bodies, whereas ECV304 infected with Ad-AP-2 α showed fewer apoptotic cells than with Ad-Bg-II ($P < 0.005$, $n = 3$) (Figure 7(b)).

(iii) AP-2 α overexpression inhibited caspase-3 activation induced by CSE in ECV304. To study the effect of AP-2 α on the activation of caspase-3 induced by CSE, ECV304 cells were treated as described in section (ii) before the protein level of activated caspase-3 (17 kD) was measured by Western blot (Figure 7(c)). Compared with the Ad-BgII 24 h group, the expression of activated caspase-3 is significantly decreased in the Ad-AP-2 α group ($P < 0.001$).

Figure 7 The effect of AP-2 α over-expression on CSE-induced apoptosis. (a) The induced expression of AP-2 α protein in Ad-AP-2 α -infected ECV304 cells. (b) Nuclear staining of ECV304 cells with Hoechst 33258. ECV304 cells were infected with AP-2 α (1) or Ad-BgII (2) for 24 h, and then stimulated with 5% CSE for 24 h. (c) Effect of AP-2 α overexpression on the activation of caspase-3 (17 kD) induced by CSE in ECV304 cells. β -actin was used as an internal control. Lane 1, ECV304 cells were infected with Ad-BgII for 24 h, and then treated with 5% CSE for 24 h; lane 2, ECV304 cells were infected with Ad-AP-2 α for 24 h, then treated with 5% CSE for 24 h; lane 3, ECV304 cells were cultured with a serum-free medium for 24 h, and then treated with 5% CSE for 24 h.

3 Discussion

COPD is characterized by chronic inflammation of the airways and progressive destruction of lung parenchyma. Emphysema is a major component of COPD and cigarette smoking is the single most important factor in the development of emphysema. The present study showed that exposure of rats to cigarette smoke decreased their FEV_{0.3}/FVC and PEF, consistent with the clinical observations in COPD patients. There were obvious emphysematous changes in the COPD group, including enlargement of alveolar spaces and destruction of alveolar walls, which were quantified by measuring MLI and DI, respectively. MLI and DI were increased in the lungs of rats in the COPD group compared with those in the normal group. These results suggest that a rat model of COPD was successfully established.

Recently, an increasing body of evidence suggests that apoptosis of structural cells in the lung might be an important upstream event in the pathogenesis of COPD. There is an increase in the number of apoptotic cells in the lungs of COPD patients. Because this is not counterbalanced by an increase in proliferation of these structural cells, the net result is destruction of lung tissue and the development of emphysema.

Increasing evidence demonstrates that AP-2 α is involved in apoptosis. In some tissues the expression of AP-2 α is decreased or lost resulting in apoptosis increase. In addition, AP-2 α functions as an anti-apoptotic factor in epithelial ovarian cancer [26], colorectal adenomas and adenocarcinomas [27], human gliomas [28], prostate cancer cells [29] and melanoma [30]. In contrast, AP-2 α expression is increased and functions as a pro-apoptotic factor in human failing myocardium with idiopathic-dilated cardiomyopathy and in human breast cancer [31]. We show that AP-2 α protein was expressed and increased in the lung of a rat model of COPD induced by cigarette smoke exposure, and was associated with increased cell apoptosis. It is unclear whether there was a direct relationship between the increase in AP-2 α and the increase in cell apoptosis.

Lung alveolar walls cells includes epithelial cells, endothelial cells and interstitial cells. Taraseviciene-Stewart et al. [32] developed a model of autoimmune emphysema in adult rats by intraperitoneal injection of human umbilical vein endothelial cells (HUVECs). In this model, anti-VEGF receptor antibodies were generated and apoptosis, especially of endothelial cells, occurred in the lung. This suggests the importance of endothelial cell apoptosis in COPD.

The ECV304 cell line, although identified as a bladder carcinoma cell line, displays some endothelial characteristics including tubule formation (differentiation) following appropriate stimulation [33,34]. We used ECV304 cells to study the role and mechanisms of AP-2 α in vascular endothelial cell apoptosis induced by cigarette smoke exposure. Compared with the control group, proliferation was significantly

increased in cells treated with 2.5% CSE, while it decreased in a dose-dependent manner at concentrations higher than 2.5%. Apoptosis was also increased in a time-dependent manner in cells exposed to 5% CSE. At higher concentrations of CSE (15%–20%) necrosis was evident. The increased proliferation at low concentration or short-period exposure to CSE may be because the low levels of damaging factors present were unable to induce apoptosis but could induce oncogenic mutation. This may be one reason why lung cancer is prone to occur in smokers. Higher CSE concentration or longer periods of stimulation with CSE leads to apoptosis or even necrosis, consistent with the results from other researchers [35,36]. Besides treatment factors, cell type [37] is also one factor that can affect cell apoptosis or necrosis when stimulated.

The expressions of cleaved caspase-3 and AP-2 α in ECV304 cells were increased in a time-dependent manner following 5% CSE treatment. The activation of caspase-3 is considered an important index of cell apoptosis. To probe the relationship between the expression of AP-2 α and the activation of caspase-3, we successfully infected ECV304 cells with Ad-AP-2 α . As the results show, over-expression of AP-2 α inhibited ECV304 cell apoptosis and caspase-3 activation induced by CSE. This suggests that AP-2 α might protect ECV304 cells from CSE. There is no report showing that AP-2 α directly modulates the expression, activation or cleavage of caspase-3. Possible mechanisms for AP-2 α inhibition of apoptosis include inhibiting the expression of Mn-SOD [38], down-regulating the expression and inhibiting the activation of MMP-9 [39], negative regulation of c-MYC activity [40] activating some oncogene such as HER-2/neu [31] and interfering with the cytokine network [41].

This is the first report to demonstrate that the expression of AP-2 α is increased in lung tissue of COPD rats and ECV304 cells stimulated by cigarette smoke exposure. Adenoviral-mediated over-expression of AP-2 α protects against ECV304 cell apoptosis and caspase-3 activation induced by CSE. These results suggest that AP-2 α might protect endothelial cells against CSE and rat lung tissue against cigarette smoke exposure. Knocking out the AP-2 α gene in rats would confirm these observations.

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