Research Article

Dual Effects of Cigarette Smoke Extract on Proliferation of Endothelial Progenitor Cells and the Protective Effect of 5-aza-2'-deoxycytidine on EPCs against the Damage Caused by CSE

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1. Introduction

Cigarette smoke (CS) is well known to be a risk factor for pulmonary diseases including chronic obstructive pulmonary disease (COPD), lung cancer, asthma, pulmonary hypertension, and vascular diseases such as atherosclerosis [1]. It is a mixture of more than 4,000 different chemical compounds, such as free radicals, toxins, and electrophiles [2, 3]. The CS extract (CSE) contains almost all of the compounds inhaled by cigarette smokers, including nicotine that is one of the most active pharmacological compounds in CSE.

The bone-marrow-derived endothelial progenitor cells (EPCs) provide an alternative source of endothelial cells (ECs) that contributes to neovessel formation in endothelium.
structure [4, 5]. The EPCs play a critical role in postnatal vasculogenesis through pivotal bioactivities of mobilization, homing, migration, differentiation, and proliferation in angiovasculogenic tissues [6]. Normal proliferation of EPCs is essential to maintain the efficient count of EPCs in postnatal vasculogenesis [7]. However, the EPCs were greatly reduced in patients with severe COPD and the reduction was correlated with COPD severity [8]. Our previous study also showed decreased and dysfunctional circulating EPCs in patients with COPD [9]. The EPCs seem to be impaired and thus lead to the repair of capacity of the lung tissue reduced in patients with COPD.

Through the studies of evaluating the effects of CSE on various types of cells, such as pulmonary endothelial cell [10], lung fibroblasts [11], epithelial cells [12], airway smooth muscle cells [13] and alveolar macrophages [14], the pathogeneses of COPD relevant to cellular level were gradually elucidated. However, little information is known about the effects of CSE on EPCs concerning the pathophysiology of COPD. The method of stimulating isolated cells with CSE in vitro has been explored and frequently applied to determine the direct causes in the relationships between cigarette smoking and cellular functions [15]. In addition, the in-depth study on genome-wide epigenetics gained more comprehensive understandings of epigenome in many diseases. Soria et al. demonstrated that a total of 32% of the bronchial brush samples from former cigarette smokers had methylation [16]. Kikuchi et al. reported that hypermethylation of genes were associated with cigarette smoke [17]. In this study, in attempt to provide fresh information about the impact of CS on proliferation of EPCs to elucidate the pathophysiological mechanisms of the diseases relevant to CS in cellular level, we assessed the proliferation of EPCs after interfering the cells with a series of concentrations of CSE for various times of exposure in vitro. In the meantime, we examined whether a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-AZA-CdR), was able to reverse the suppression of EPCs caused by CSE exposure. This study was prospected to provide a new vision into the pathogeneses of pulmonary diseases relevant to smoke in cellular level.

2. Methods

2.1. Animals. Total of 36 male C57BL/6 mice aged four to six weeks old were randomly enrolled in this study. All animals were purchased from Shanghai laboratory animal center of Chinese Academy of Sciences (Shanghai laboratory animal center of Chinese Academy of Sciences, SLACCAS, Shanghai, China) and fed in a cleaning unit with 23–25 degrees of Celsius (°C), 50–60% humidity, and 12 hours (h) rhythm of night and day. The mice were sacrificed by cervical dislocation. The study was approved by the Institutional Review Board of Central-South University and conformed to the guiding principles for research involving animals and human beings (World Medical Association and American Physiological Society, 2002).
Figure 1: The morphological changes of endothelial progenitor cells (EPCs) during culture. (a) representative photomicrographs of EPCs on day 1 of the culture. The EPCs formed round, the sizes of cells were almost same, and the cells were suspended in the culture medium (arrow 1); (b) on day 4 of the culture, the cells were attached to each other, the sizes were getting enlarged, and the shapes became oval, spindle, or polygonal (arrow 2); (c) on day 7 of the culture, the cells shaped to fusiform or polygon patterns and contacted each other to attempt to form capillary structure (arrow 3). Scale bar represents 50 μm.

were involved to calculate the positive rate of amphophilic cells. Thirdly, the cells concurrently with surface markers of CD34+/CD133+/Flk-1 were identified as EPCs [19, 22]. The cells (2 × 10^5/mL) on day 7 of the culture were incubated in dark for 30 min at 4°C and then detected the corresponding surface markers by fluorescence-activated cell sorting (FACS) cytometry using FITC-conjugated anti-mouse CD34 antibody (FITC-CD34, Becton, Dickinson, USA), PE-conjugated anti mouse CD133 antibody (PE-CD133, eBioscience, USA), and APC-conjugated anti-mouse Flk-1 antibody (APC-Flk-1, Becton, Dickinson, USA) according to the instructions. Cells of coexpressing FITC-CD34, PE-CD133, and APC-Flk-1 were identified as EPCs (Figure 5).

2.6. Detection of the Proliferation of EPCs Incubated with a Series of Concentrations of CSE for Different Times. The proliferation of EPCs was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA) assay according to previously described method with a minor modification [23]. The EPCs were trypsinized (Amresco, USA) and centrifuged at 1,500 rpm for 5 min. After being washed, the cells were resuspended with vehicle control. Then the EPCs (1 × 10^4 in 200 μL volume) were transplanted to 15 wells in each of three 96-well plates. All three plates were incubated under standard cell culture condition at 37°C, 5% CO₂, and 95% humidity for 48 h. Then culture media were removed and replaced by 200 μL/well of the vehicle control, 1.0% CSE, 2.5% CSE, 5.0% CSE, and 10.0% CSE correspondingly for each of 3 wells in all the three 96-well plates. The three 96-well plates were incubated for 3 h, 6 h, and 24 h, respectively. After incubating for the corresponding periods, each well was added with 20 μL MTT (5 mg/mL) and incubated for another 4 h. Culture media were removed and replaced by 150 μL dimethyl sulfoxide (DMSO) (Sigma, USA). Then the EPCs were shaken for 10 min to dissolve crystal before optical density (OD) measurement at 490 nm for proliferation of EPCs (ELX800, Bio-Tek, USA).

2.7. Detection of the Proliferation of EPCs Incubated with CSE in Presence of 5-AZA-CdR. In order to search for any treatment agents in protecting the proliferation of EPCs against depression caused by CSE, a DNA methyltransferase
Figure 2: The identification of EPCs by double positive staining with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil) labeled acetylated low density lipoprotein (Dil-acLDL) and FITC labeled ulex europaeus agglutinin-1 (FITC-UEA-1). The laser scanning confocal microscope (LSCM) illustrated that the cells on day 7 of the culture displayed red cytoplasm when stained with Dil-acLDL (a), green cytomembrane when combined with FITC-UEA-1 (b), orange when double positively stained with Dil-acLDL and FITC-UEA-1) (c), and blue when stained with DAPI in nuclear localization (d). Scale bar represents 10 μm.

2.7. Proliferation of EPCs

Inhibitor, 5-AZA-CdR, was applied to the EPCs exposed to 1.0% CSE for incubation of 24 h and then the proliferation of EPCs was measured by MTT assay. Considering the clinical significance of cigarette smoke in the development of COPD, the exposure concentration of CSE was determined to be a relatively low at 1.0% CSE and the exposure time was relatively long for 24 h in this experiment. In addition, this exposure condition did not deadly depress the proliferation of EPCs according to our previous pilot study.

The EPCs were trypsinized (Amresco, USA) and centrifuged at 1,500 rpm for 5 min. After being washed, the EPCs (1 × 10^4/mL in 200 μL volume) were transplanted to one 96-well plate. The control and CSE wells were added with 200 μL of vehicle control, respectively, and the 5-AZA-CdR wells were added with 200 μL of 5-AZA-CdR in concentrations of 2.0 μmol/L, 5.0 μmol/L, and 10.0 μmol/L, respectively. The plate was incubated under standard cell culture condition at 37°C, 5% CO₂, and 95% humidity for 48 h. Then the culture media were removed and replaced by 200 μL of vehicle control in the control wells, of 1.0% CSE in the CSE wells and all 5-AZA-CdR wells. After incubating for another 24 h, each well was added with 20 μL MTT (5 mg/mL) and incubated for another 4 h. Culture media were removed and replaced by 150 μL DMSO. Then the EPCs were shaken for 10 min to dissolve crystal before OD measurement at 490 nm for proliferation of EPCs (ELX800, Bio-Tek, USA).

2.8. Statistical Analysis. Analyses were performed using SPSS for Windows 16.0 (SPSS Inc., Chicago, IL, USA). All data were expressed as means ± standard deviation (SD). Analysis of differences among groups was performed using analysis of variance (one-way ANOVA), followed by post hoc analysis as appropriated. Values of P < 0.05 were considered statistically significant.

3. Results

3.1. Identification of EPCs. On day 1 of the culture, the MNCs isolated from the murine bone marrow formed round, the sizes of cells were almost same, and the cells were suspended
in the culture media (Figure 1(a)). On day 4 of the culture, the cells were attached to each other, the sizes were getting enlarged, and the shapes became oval, spindle, or polygonal. The cells in this stage seemed to tend to gather together to form ball-like structure (Figure 1(b)). On day 7 of the culture, the cells shaped to fusiform or polygon patterns and contacted each other to attempt to form capillary structure (Figure 1(c)). The shapes of cells in this stage were displayed well in the culture media. In addition, the LSCM illustrated that the cells on day 7 of the culture displayed red color in cytoplasm when stained with Dil-acLDL (Figure 2(a)), green color in cytomembrane when combining with FITC-UEA-1 (Figure 2(b)), and orange color in confocal image when double positively stained with Dil-acLDL and FITC-UEA-1 (Figure 2(c)). The positive rate of amphophilic cells was 94.67 ± 4.16% on day 7 of the culture. Moreover, the rate of cells concurrently expressed with surface markers of FITC-CD34, PE-CD133, and APC-Flk-1 was 95.07 ± 1.73% on day 7 of the culture (Figure 3). Therefore, the cells harvested on day 7 of the culture were believed to be the characteristically biological EPCs and used for the present study.

3.2. The Effects of CSE on Proliferation of EPCs. As shown in Figure 4, the OD levels were significantly increased in the EPCs exposed to both 1.0% CSE (0.1935 ± 0.0168) and 2.5% CSE (0.2136 ± 0.0203) but decreased in the cells exposed to both 5.0% (0.0278 ± 0.0041) and 10.0% CSE (0.0009 ± 0.0001) compared to the OD levels of controls (0.1401 ± 0.0141, P < 0.01) for acute exposure to 3h. In addition, it seems that the higher exposure concentration of the CSE, the lower OD values of the EPCs, showing that the OD levels were significantly lower in the EPCs exposed to 10.0% CSE than the cells exposed to 5.0% CSE (P < 0.05), and the later was significantly lower than the cells exposed to 2.5% CSE (P < 0.01).

When exposing the EPCs to CSE for 6h (subacute exposure), the OD levels were significantly increased in the EPCs incubated with 1.0% CSE (0.1777 ± 0.0130) but decreased in the cells incubated with 2.5% CSE (0.0555 ± 0.0087), 5.0% CSE (0.0102 ± 0.0021), and 10.0% CSE (0.0007 ± 0.0002) compared to those of controls (0.1463 ± 0.0143, P < 0.01). In addition, the OD values were significantly lower in the EPCs incubated with 5.0% and 10.0% CSE than the cells exposed to 2.5% CSE (P < 0.01).

Our results also showed that the OD levels were significantly decreased in the EPCs exposed to 1.0% CSE (0.0837 ± 0.0051), 2.5% CSE (0.0035 ± 0.0007), 5.0% CSE (0.0008 ± 0.0002), and 10.0% CSE (0.0007 ± 0.0001) for 24h (chronic exposure) compared to those of controls (0.1591 ± 0.0108, 0.0986 ± 0.0051, 0.0035 ± 0.0007, and 0.0008 ± 0.0001), respectively (P < 0.01).
These astonishing results suggested that the acute stimulation by a relatively low concentration of CSE might not depress the proliferation of EPCs. However, the OD levels were significantly lower in the EPCs exposed to 1.0% CSE compared with those of the controls (\(P < 0.01\)).

3.3 \textit{The Effect of 5-AZA-CdR on the EPCs Exposed to CSE.} Considering the clinical significance of cigarette smoke in the development of COPD, we observed the effect of 5-AZA-CdR on the EPCs exposed to a low concentration of CSE (1.0%) for a chronic exposure (24 h). In addition, this exposure condition did not notably depress the proliferation of EPCs according to the results described above.

As shown in Figure 5, the OD levels were significantly decreased in the EPCs exposed to 1.0% CSE (0.1020 ± 0.0140), 1.0% CSE + 2.0 \(\mu\)mol/L 5-AZA-CdR (0.1308 ± 0.0118), 1.0% CSE + 5.0 \(\mu\)mol/L 5-AZA-CdR (0.1310 ± 0.0124), and 1.0% CSE + 10.0 \(\mu\)mol/L 5-AZA-CdR (0.0093 ± 0.0023) compared to those of the controls (0.1595 ± 0.0125, \(P < 0.05\) for all). However, the OD levels were significantly higher in the EPCs exposed to 1.0% CSE + 2.0 \(\mu\)mol/L 5-AZA-CdR and 1.0% CSE + 5.0 \(\mu\)mol/L 5-AZA-CdR than the EPCs exposed to 1.0% CSE (\(P < 0.05\), respectively, suggesting that 5-AZA-CdR in concentrations of 2.0 \(\mu\)mol/L and 5.0 \(\mu\)mol/L significantly protected against the depression of proliferation of EPCs caused by CSE exposure. There was no significant difference of the OD levels between the EPCs exposed to 1.0% CSE + 2\(\mu\)mol/L 5-AZA-CdR and the cells exposed to 1.0% CSE + 5\(\mu\)mol/L 5-AZA-CdR. It seems that 5-AZA-CdR in concentration higher than 10.0 \(\mu\)mol/L showed toxic effect on the proliferation of EPCs. Because the OD levels were significantly lower in the EPCs exposed to 1.0% CSE + 10.0 \(\mu\)mol/L 5-AZA-CdR (0.0093 ± 0.0023) than the EPCs exposed to vehicle control of FBS-free EGM-2 (0.1595 ± 0.0125, \(P < 0.05\)) and 1.0% CSE (0.1020 ± 0.0140, \(P < 0.05\), respectively.

4. \textit{Discussion} The most important finding of this study was that the proliferation capacity of EPCs was significantly enhanced when acute exposing (3 h) the cells to relatively low concentrations of CSE (1.0–2.5%) but depressed when acute exposing the cells to relatively high concentration of CSE (5.0–10.0%) compared to that of EPCs exposed to control medium. These astonishing results suggested that the acute stimulation by a relatively low concentration of CSE might...
activate certain “danger signals” in the EPCs to motivate the endogenous repair mechanisms of EPCs by which the proliferation of EPCs could be compensated and maintained to struggle against the damages caused by CSE exposure. These protective phenomena can still be observed in the EPCs when they are sub-acute ly exposed (6 h) to a relatively low concentration of 1.0% CSE but vanished when chronically exposed (24 h) to any level of CSE, indicating fatal damages in the proliferation of EPCs after a chronic CSE exposure. A DNA methyltransferase inhibitor, 5-AZA-CdR, might partly protect the proliferation of EPCs from damages by chronic CSE exposure.

EPCs were isolated primarily from peripheral blood in 1997 by Asahara et al. [24] and now can also be isolated from bone marrow and umbilical cord blood [25, 26]. EPCs were localized mainly in the bone marrow of postnatal life [27]. In previous studies, EPCs were identified based on their morphology and growth characteristics [28] or characterized by dual staining with Dil-acLDL and FITC-UEA-1 through LSCM observation [21] or defined as three-color fluorescence flow cytometry using antibodies against CD133, CD34, and KDR (Flk-1 in animal) [19]. In recent studies, EPCs were identified by morphology, dual staining by Dil-acLDL and FITC-UEA-1, and surface markers of CD34+/CD133+/Flk-1+ simultaneously. The current EPC product demonstrated that the highly purified EPCs could be obtained by culturing the isolate MNCs of mice bone marrow with EGM-2 through ficoll density gradient centrifugation.

The endothelium regulates vascular homeostasis and is responsible for angiogenesis in physiological and pathological tissues of humans as well as animals. EPCs are the precursors of endothelial cells and play a fundamental role in the maintenance of endothelial integrity and function by both developing into endothelial cells and secreting vasoactive substances [29, 30]. Accumulating evidences indicated that EPCs derived from bone marrow contributed to reendothelialization of injured vessels as well as neo-vascularization of ischemic lesions in either direct or indirect pathways under physiological or pathological conditions [31, 32]. The proliferation of EPCs is required for tissue repairing and airway remodeling in lungs [33–35]. It was demonstrated that the number and/or function of EPCs were inversely correlated with risk factors of cardiovascular and pulmonary diseases [36, 37]. Cigarette smoke is a major risk factor of cardiovascular and pulmonary diseases. It reduces the number and function of EPCs [38] probably through oxidative stress induced by reactive oxygen species (ROS) from CS [39, 40]. Indeed, the present results demonstrated that a high concentration of CSE harmfully depressed the proliferation of EPCs. Supportably, a study on the proliferation, migration, cytokine release, and contraction of human bronchial smooth muscle cells (SMCs) similarly showed that treatment of the cells with 10.0% CSE induced cell death, reduced migration, and contraction through an increased ROS production [41].

Surprisingly, we found that the proliferation capacity of EPCs was enhanced with stimulation of relatively low concentrations of CSE (1.0–2.5%) for an acute exposure of 3 h. Up to date, the effects of CSE on the cell proliferations were controversially reported. Ambalavanan et al. [42] reported that CSE caused a dose- and time-dependent decreases in neonatal porcine vascular SMCs. Studies also evidenced that CSE induced apoptosis of human airway SMCs [43, 44], human pulmonary endothelial cells [10], and vascular endothelial cells [45]. In controversy, Xing and his colleagues [46] reported that stimulation of rat pulmonary artery SMCs with CSE significantly increased cell proliferation and promoted cell cycle progression. Promotions of cell prolif erations stimulated by CSE were also observed on bovine tracheal SMCs [47] and human aortic SMCs [3]. Such controversial results were theoretically explained by discrepancies among the methods applied to those studies, such as the animals, the cell types, and the CSE preparations.

The present finding about the dual effects of CSE on proliferation of EPCs was also supported by clinical observations that the circulating EPCs number was increased and the EPCs were motivated to contribute to vascular repairing and reconstruction of lung vessels in patients with early stage of COPD [48] and that the circulating EPCs number was decreased in patients with later stage of COPD [8, 49].

5-AZA-CdR is a deoxynucleoside analog of cytidine in which the carbon 5 position of the pyrimidine ring is substituted by nitrogen [50]. It is an S-phase specific inhibitor of DNA methyltransferase, which triggers demethylation leading to a consecutive reactivation of epigenetically silenced tumor suppressor genes in vitro and in vivo. The sparking interest of 5-AZA-CdR is using it as a potential therapeutic agent. In this study, 5-AZA-CdR in concentrations of 2.0 µmol/L and 5.0 µmol/L partly protected the proliferation of EPCs from damage caused by CSE; whereas, 5-AZA-CdR in a high concentration of 10.0 µmol/L showed toxic effect on the proliferation of EPCs. It might be because of a dual mechanism of demethylation in 5-AZA-CdR with reactivation of silenced genes at low doses and cytotoxicity at high doses [51]. Additionally, a novel dose schedule of 5-AZA-CdR for treatment in patients with cancer is suggested due to the myelosuppression induced by high doses [52]. The 5-AZA-CdR protected the proliferation of EPCs from damage caused by 1.0% CSE exposure for 24 h, which could rule out the possibility of the enhancement of proliferation of EPCs resulted from the 1.0% CSE exposure for 3 h observed in this study (Figure 5). In the meantime, this finding suggested that DNA methylation may be involved in the dysfunction of EPCs induced by CSE. Clinically, hypermethylation was detected in cigarette smokers [53–55]. Moreover, the oxidative stress induced by CS destructed the lung tissue in COPD leading to acquired genetic changes including DNA methylation due to inefficient DNA repair mechanism [56].

5. Conclusions

In summary, we demonstrated for the first time that CSE showed dual effects on proliferation of EPCs and a DNA methyltransferase inhibitor, 5-AZA-CdR, partly protected the proliferation of EPCs from damage caused by CSE. These findings would be helpful in understanding of different voices from various studies that focused on roles of EPCs in cigarette smoke-induced diseases.
Conflict of Interests
The authors declare that they have no conflict of interests.

Authors’ Contribution
Zhi-Hui He performed the laboratory work and statistical analyses and drafted the paper. Ping Chen and Yan Chen are the coprincipal investigator of the study, and they supervised the study and helped to draft the paper. Ying-Qun Zhu helped to conceive the study and participated in its design. Sheng Dong He and Ji-Ru Ye performed the laboratory work and statistical analyses. Da Liu and Yue Yang contributed to the statistical analyses and helped to draft the paper. Every author read and approved the final paper.

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