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Damaging Effect of Cigarette Smoke Extract on Primary Cultured Human Umbilical Vein Endothelial Cells and Its Mechanism

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Objective To investigate the cellular effects of cigarette smoke extract (CSE) on primarily cultured human umbilical vein endothelial cells (HUVEC). **Methods** The effects of CSE (5%-20%) and nicotine (10^{-4} mol/L) on HUVEC viability, proliferation, angiogenesis and apoptosis were observed. **Results** CSE decreased HUVEC survival rate and angiogenesis after 24 h as well as its proliferation after 48 h in a dose-dependent manner. Moreover, CSE induced apoptosis of HUVEC as indicated in condensation of nuclear chromatin and the presence of hypodiploid DNA. HUVEC incubated with CSE for 24 h gave a significant decrease in the expression of Bcl-2 as well as the decline in the Bcl-2/Bax ratio accompanied with the loss of mitochondrial membrane potential and excess cytosolic calcium. Our study also observed that p53 protein level decreased, rather than increased in cells treated with CSE. Nicotine had no discernible inhibitory effects on the above indices of HUVEC. **Conclusion** Exposure to CSE other than nicotine causes inhibition of viability, proliferation and differentiation of HUVEC. CSE-induced HUVEC injury is mediated in part through accelerated apoptosis but independent of p53 pathway. It appears that mitochondria have played a key role in the apoptosis of HUVEC induced by CSE.

Key words: Cigarette smoke extracts (CSE); Human umbilical endothelial cell (HUVEC); Viability; Proliferation; Angiogenesis; Mitochondrial membrane potential; Cytosolic calcium; Bcl-2; Bcl-2/Bax; p53

INTRODUCTION

Epidemiological studies revealed a strong correlation between cigarette smoking and the development and progression of atherosclerosis. However, the precise components of cigarette smoke responsible for this relationship and the mechanisms by which cigarette smoking exerts its effect have not been elucidated yet. Endothelial injury is considered to be a key initiating event in the pathogenesis of atherosclerosis. Components of cigarette smoke that gains access to the circulation will come in contact with vascular endothelial cells. There are some evidences that cigarette smoking can result in both morphological and biochemical disturbances to cardiovascular endothelium both in cell culture systems and *in vivo*[1].

In the present paper we have studied the effect of cigarette smoke water-soluble extracts

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(CSE) and nicotine on the viability, proliferation and differentiation of HUVEC. Recently, it was reported that cigarette smoking activated Caspase 3 to induce apoptosis of HUVEC [2], but the mechanism has not been elucidated. Endothelial cell apoptosis is particularly relevant to the maintenance of vascular wall integrity and promote the process of atherogenesis[3]. It has been shown that mitochondria plays essential roles in certain forms of apoptosis[4]. We have observed the mitochondrial structure and function injury in mice exposed to CSE[5]. It is so far unknown whether mitochondrial mechanism would plays a role in cigarette smoking-related vascular diseases. Therefore, we have attempted to determine whether CSE causes increased apoptosis of HUVEC and what its relevant cellular mechanism is.

MATERIALS AND METHODS

Reagents

M 199 culture medium was purchased from Gibco. Collagenase type IV, heparin，MTT, NP-40, PMSF, aprotinin, Triton X-100, propidium iodide (PI), Fura 2, Rhodamine 123, RNase A, Hoechst 33342, and L-glutamine were purchased from Sigma Co (St. Louis, MO). Endothelial cell growth factor (ECGF, 15 mg/3 mL) was obtained from Roche. Fetal bovine serum (FBS) was purchased from Hyclone. Nicotine ditartrate dihydrate was obtained from Acros. A 10^{-1} mol/L stock solution of nicotine was prepared in DMSO and stored at -20℃. All chemicals used for the experiments were of grade of analysis.

Preparation of CSE

Commercial cigarettes (Marlboro, Philip Morris, Inc., Richmond, VA) were smoked continuously by the smoking apparatus made by our laboratory. Smoke from three cigarettes was drawn through 10 mL of PBS to generate a CSE-PBS solution. Several variables must be carefully controlled: composition of tobacco, rate of cigarette combustion, smoke temperature, gas flow, and filtering mechanisms of the system.

Cell Culture

The method of preparing HUVEC was described previously[6]. Briefly, the human cord was severed from the placenta soon after birth, and placed in cord buffer at 4℃. The umbilical vein was cannulated with a 14-gauge needle and the needle was secured with a hemostat. The vein was perfused with PBS to wash out the blood and allowed to drain. The other end of the umbilical vein was then also cannulated. And both ends were shut with 10 mL syringes. Ten mL of 0.1% collagenase in Hank's solution was then infused into the umbilical vein. The umbilical cord, suspended by its ends, was placed at room temperature for 20-25 min. After incubation, the collagenase solution containing endothelial cells was flushed from the cord by perfusion with PBS. The effluent was collected in a sterile 50 mL glass centrifuge tube containing 2 mL FBS. Endothelial cells were centrifuged and maintained in M199 medium containing 10 mmol/L Hepes, supplemented with 50 μg/mL ECGF, 100 μg/mL sodium heparin, 20% FBS, 5 mmol/L glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The cell suspensions per cord were plated onto 1% gelatin-coated 25 cm^2 dishes (Nunc, Denmark) and maintained in the above medium at 37℃ and 5% CO_2 atmosphere in the incubator. The cells were fed every 2-3 days until confluence.

For subculture, the primary human umbilical vein endothelial cells (HUVEC) were

harvested with 0.05% trypsin-0.02% EDTA. All experiments were performed on endothelial cells of the second or third passage. The HUVEC were plated in 1% gelatin-coated 25 cm^2 dishes in most experiments except the viability and proliferation assay. Incubation for 36-48 h resulted in 70%-80% confluence. The culture medium was exchanged for ECGF-free medium containing 10% FBS and a range of concentrations of CSE (10%, 15% and 20%) or nicotine (10^{-4} mol/L) for a period of 24 h at 37℃, unless otherwise indicated. After treatment, cells were harvested and assayed.

Assessment of the Effect of CSE and Nicotine on Proliferation and Viability of HUVEC by MTT

Mitochondrial integrity was measured by reduction of mitochondrial enzyme succinate dehydrogenase by MTT[7]. Briefly, MTT was dissolved in culture medium at a concentration of 0.5 mg/mL and filtered to remove a small amount of insoluble residue. After cell treatment, MTT containing medium was added to each well in a volume of 0.1 mL and incubated for 4 h at 37℃. The supernatant was removed and 0.15 mL of DMSO was added to extract and solubilize formazan. After 10 min at room temperature, the absorbance of formazan was read at a wavelength of 570 nm determined with a BioRad reader. The amount of blue formazan reduced from tetrazolium salt was directly proportional to the number of viable cells.

For the assessment of viability, HUVECs were subcultured onto 96-well plates at a concentration of 2.5×10^4/100 μL and allowed to grow to confluence monolayers. For the assessment of proliferation, HUVECs were subcultured onto 96-well plates at a concentration of 5×10^3/100 μL and allowed to settle for 24 h before addition of the CSE being tested. Serial dilutions of fresh CSE made in M199 were added to each well at the final concentrations of 2.5%, 5%, 10%, 15% and 20% CSE. The final concentration of nicotine was 10^{-4} mol/L. The results are expressed as a percentage of the control cell. Each dose of CSE was tested in triplicate and experiments were repeated three times.

Apoptosis and Cell Cycle Analysis

CSE-treated and untreated HUVECs (1×10^6 cells) were collected with Trypsin-EDTA from the culture dishes by trypsinization followed by centrifugation, resuspended in 1 mL of 70% ethanol at 4℃ overnight. The cells were centrifuged, washed with 1 mL of PBS and resuspended in PBS containing RNase 60 μg/mL for 30 min at 37℃. The cells were then stained with 50 μg/mL of propidium iodide at 4℃ for 30 min. The stained cells were analyzed using Coulter EPICS XL flow cytometry. Apoptosis of HUVEC was quantified by determination of the proportion of cells with hypodiploid DNA. The distribution of cells in the cell cycle was determined simultaneously.

Observation of Cell Morphological Changes by Phase-contrast and Fluorescence Microscopy

The HUVECs incubated for 24 h as described above were observed by phase-contrast microscopy. Collected medium, the rinse, and trypsinized cells were pooled and collected by centrifugation at 800 rpm for 5 min at 4℃. The cell pellets were resuspended and incubated with membrane-permeable H33342 dye (10 μg/mL in PBS) for 30 min at 37℃. An aliquot of the incubated mixture was placed on a glass slide, covered with a glass cover-slip, and viewed under fluorescence microscopy. Individual nuclei were visualized at ×400 to distinguish the normal uniform nuclear pattern from the characteristically condensed coalesced chromatin pattern of apoptotic cells.

Angiogenesis

Matrigel (Collaborative Research, Inc. Waltham, MA), an extract containing basement membrane components, at 10 mg/mL, was applied to 96-well plates (Costar) and incubated at 37℃ for 1 h, which induced gelling. HUVECs at a density of 2×10^4 cells/mL were pipetted onto the Matrigel-coated 96-well culture plate. Changes in the morphology and reorganization of HUVEC were monitored by phase-contrast microscopy.

Rhodamine 123 Labelling of Mitochondria

Rhodamine 123, a specific cationic fluorescent dye to label the mitochondrial membrane potential, was used for the estimation of mitochondrial membrane potential[8]. After 24 h incubation in the presence or absence of CSE, HUVECs (1×10^6 cells) were separated from the incubation medium by 5 min centrifugation at 800 rpm and resuspended in PBS. Aliquots of the cell suspension (0.5 mL) were separated from the incubation medium by 4 min centrifugation at 800 rpm. The cell pellet was then resuspended in 2 mL of PBS containing 1.5 μmol/L rhodamine 123 and finally incubated at 37℃ for 10 min. After washed three times with PBS, the cells were resuspended at 0.5×10^6 cells/mL and kept at room temperature for a period no longer than 2 h before sampling. The accumulation of rhodamine 123 in the samples was measured using a Perkin-Elmer DL5 spectrofluorometer set at 490 nm excitation and 525 nm emission wavelengths.

Determination of Cytosolic Calcium by Fura 2

Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$) was performed using a Ca^{2+}-sensitive indicator fura 2 under flow cytometry[9]. HUVECs (1×10^6 cells/mL) were loaded with 5 μmol/L fura 2 for 40 min at 37℃. The cells were washed three times with PBS. The fura-2 fluorescent signals were determined at Ex=340/380 nm and Em=510 nm. All experiments were performed less than 90 min after loading.

Expression of p53, Bcl-2 and Bax by Flow Cytometry

The treated and untreated HUVECs (1×10^6 cells/mL) were harvested and washed in cold PBS. Expression of three proteins was measured by indirect immunofluorescence flow cytometric analysis with specific monoclonal antibody, respectively. HUVECs were fixed with 4% paraformaldehyde for 40 min at room temperature and rinsed twice in PBS. The cells were permeabilized with 0.2 % Triton X-100, 5% FBS in PBS for 10 min at 4℃ and washed twice. The cells were incubated with or without anti-human Bcl-2 mAb (SC-7382, C-2, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:25, p53 (SC-126, DO-1, WT+M) and Bax (SC-7480, B-9)1:40 in 200 μL of PBS for 40 min at 4℃ and washed twice with ice-cold PBS. The cells were incubated with FITC-conjugated goat anti-mouse IgG diluted 1:100 in 200 μL of PBS for 40 min at 4℃ in the dark and washed twice. Finally, they were resuspended in 0.5 mL 1% paraformaldehyde/PBS, and analyzed using a FACScan Coulter EPICS XL flow cytometer[10]. Data from the analysis are expressed as fluorescence intensity on a logarithmic scale. The mean fluorescence intensity for the samples incubated with stimulus was compared to that for cells incubated in PBS alone.

Bcl-2 Expression Using Western Blot Analysis

HUVECs were grown to subconfluence on 75 cm^2 culture dishes and incubated with CSE or nicotine for 24 h as detailed above. HUVECs were rinsed three times in ice cold

PBS and scraped off with a rubber scraper. HUVECs were lysed by addition of lysis buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, 100 μg/mL PMSF, 1% NP-40, 1 μg/mL aprotinin) for 30 min at 4℃, and the insoluble materials were removed by centrifugation at 12 000 g for 10 min at 4℃. The supernatant was collected and stored at -80℃. Samples containing 200 μg of total protein were mixed with 2×loading buffer (250 mmol/L Tris-HCl at pH 7.4, 20% glycerol, 4% SDS, 40 mmol/L DTT, 2 mmol/L EDTA, 0.1% bromophenol blue), boiled in a water bath for 4 min, and separated on a 12% SDS-polyacrylamide (PAGE) for 4-5 h. The proteins were electrophoretically transferred onto nitrocellulose membrane (NC) (Millipore, Bedfrod, MA) for 12 h. The blotted membrane was blocked for 2 h using 5% non-fat dried milk in TBS (20 mmol/L Tris-HCl, 150 mol/L NaCl, pH 8.0) and washed three times with TBS for 10 min each. Then it was hybridized for 4 h in a 1:30 dilution of mouse anti-human Bcl-2 antibody (SC-7382, C-2, Santa Cruz Biotechnology, Santa Cruz, CA) with gentle shaking at 4℃. The membranes were then washed three times with TBS for 10 min each to remove unbound antibody and probed with 1:200 dilution of goat anti-mouse IgG antibody conjugated with horseradish peroxidase (ZB-2305, Santa Cruz Biotechnology) in TTBS (20 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 8.0, 0.1% Tween 20) at room temperature for 2 h. The membrane was rinsed three times in TBS to remove unbound second antibody. Finally, the membrane was developed with DAB kit (Sino-American Biotechnology Co.) for 5 to 10 min. Photos were taken to note the results. The density of the bands was quantified with the BIO-RAD Multi-Analyst Version 1.1, image 1.47 alias software system.

RESULTS

Effect of CSE and Nicotine on Viability and Proliferation of HUVEC

The examination of cytotoxicity was an important aspect in understanding the effects of CSE on vascular function. As shown in Table 1, CSE decreased survival rates of HUVEC in a dose-dependent manner, with a maximal effect at 20% CSE. The survival rate of HUVEC at 24 h reached 28.1%±1.7% in medium with 20% CSE, as compared with that in normal control (P<0.01). 2.5%-5% of CSE showed no toxicity on HUVEC survival. HUVEC survived about 50% at 10%-15% CSE.

When HUVECs were treated with CSE in 20% FBS and 50 μg/mL ECGF medium for 48 h, the proliferation of HUVECs was inhibited in a dose-dependent manner. The concentration of CSE to inhibit proliferation was lower than that to reduce viability. The treatment with 5% CSE significantly reduced the cell number as compared with untreated cells. There was a ~50% reduction in the proliferation of HUVEC at the dose of 20% CSE after 48 h, as compared with control. However, no decrease in viability and proliferation was noticed at 10^{-4} mol/L nicotine, one of the main constitutes of cigarette smoke (Table 1).

Flow Cytometric Analysis of Apoptosis and Cell Cycle Distribution in HUVEC

Fluorescence-activated cell sorting (FACS) was used to determine apoptosis and distribution of HUVECs stained by PI in the cell cycle. Apoptotic nuclei were distinguished by the hypodiploid DNA contents, as compared with the diploid DNA content of normal cells. As shown in Fig. 1, a single peak of DNA, indicating the diploid DNA content, characterized the untreated HUVEC. In contrast, hypodiploid DNA was detected in CSE-treated HUVEC. When HUVECs were treated with increasing concentrations of CSE, there was an increase in the percentage of HUVEC with hypodiploid DNA. CSE-treated

HUVEC growth arrest occurred after 24 h with an increase from 4.7% to 33.2% of the cells in S phase. The percentage of cells in G0-G1 phase declined from 89.4% to 65.6% with the increase of CSE dose. At the concentration of 20% CSE, 33.2% of the cells were arrested in S phase coincident with the increase of apoptotic cell population, shown as sub-G1 peak (Table 2). However, nicotine at the concentration of 10^{-4} mol/L did not affect the apoptosis and cell cycle distribution of HUVEC.

TABLE 1

Effect of CSE and Nicotine on Viability and Proliferation of HUVEC

Normal Control	Viability[a] (% of Control)	Proliferation[b] (% of Control)
	100	100
CSE		
2.5%	98.6±12.3	95.7±10.4
5%	94.6±8.5	78.2±3.2**
10%	54.6±6.9**	63.1±0.9**
15%	48.2±4.1**	53.1±3.1**
20%	28.1±1.7**	48.3±1.6**
Nicotine 10^{-4} mol/L	94.6±9	96.9±1.9

Note. [a]Confluent HUVECs in 96-well plates in 10% FBS medium were exposed to different concentrations of CSE or 10^{-4} mol/L nicotine for 24 h. Cell counts are expressed as a percentage of the control by MTT assay. [b] HUVECs (5×10^3 cell/mL) were added into a 96-well. After adherence for 24 h, HUVECs were cultured at various concentrations of CSE or nicotine in 20% FBS and 50 μg/mL ECGF. After 48 h incubation, the proliferation rate was determined by MTT assay. The values determined in the absence of CSE were set to 100%. Each variable was tested in triplicate and the means and SD were given. $^{**}P<0.01$ compared with control.

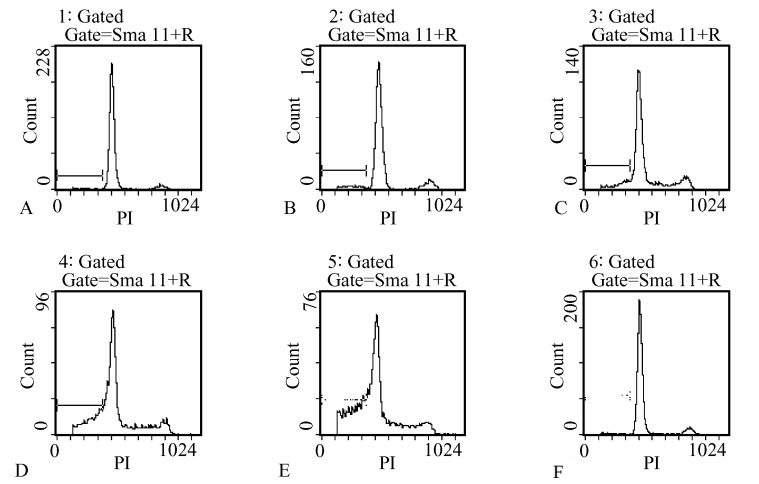

FIG. 1. Flow cytometric analysis of apoptosis in HUVEC in response to increasing concentrations of CSE or nicotine. Primarily cultured HUVECs were grown to approximately 70%-80% confluence and then treated with 5%-20% CSE or 10^{-4} mol/L nicotine for 24 h. Following incubation, the cells were harvested and stained with PI. The profiles are representative histograms of triplicate assays. (A) Normal, (B) 5% CSE, (C) 10% CSE,(D) 15% CSE, (E) 20% CSE and (F) 10^{-4} mol/L nicotine. Results are plotted as log fluorescence intensity versus relative cell number and are representative of three experiments, at least 10 000 cells were analyzed.

TABLE 2

Effect of CSE on Apoptosis and Cell Cycle of HUVECs

Group	Dip G0-G1 (%)	Dip S-Phase (%)	Dip G2-M:(%)	Apoptosis Sub-G1 (%)
Normal Control	89.4	4.7	6.0	4.22%
CSE (%)				
5	85.4	4.8	9.8	7.85%
10	69.6	22.3	8.0	11.6%
15	65.2	28.1	6.7	23.1%
20	65.6	33.2	4.2	32%
Nicotine 10^{-4} mol/L	85.8	6.8	7.4	4.97%

Note. Percentage of sub-G1 (%) represented the rate of apoptotic HUVEC nuclei with hypodiploid DNA content.

Morphology Changes of HUVEC Induced by CSE and Nicotine

Fig. 2 shows the morphology of HUVECs. After 24 h incubation with CSE, some cells started to become round and eventually detached from the plate and floated in the medium, leaving many holes in the confluent cells. Cell number was decreased greatly. The group treated with CSE had obviously less cells than the nicotine group and the normal control group.

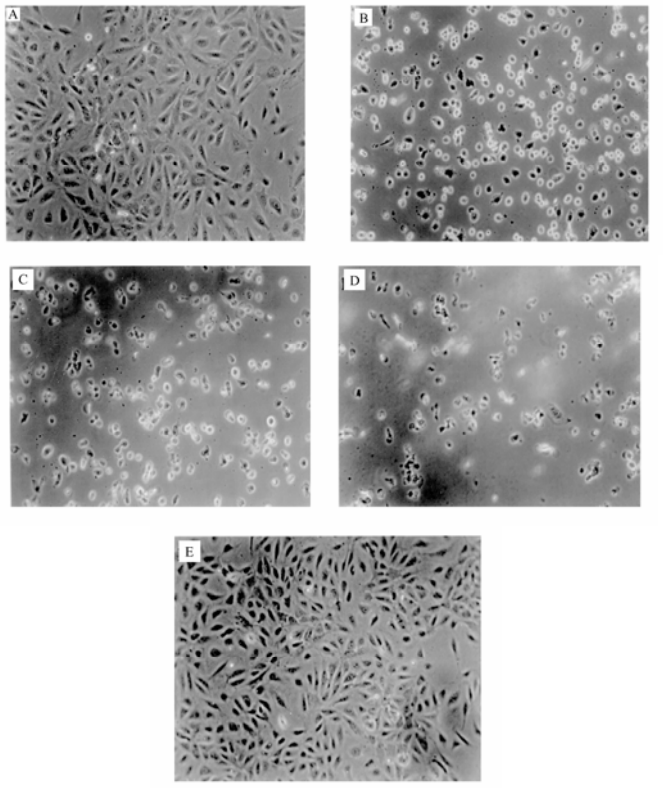

FIG. 2. Morphology changes of HUVEC induced by CSE and nicotine by light microscopy. (A) Normal control, (B) 10% CSE, (C) 15% CSE, (D) 20% CSE, (E) Nicotine.

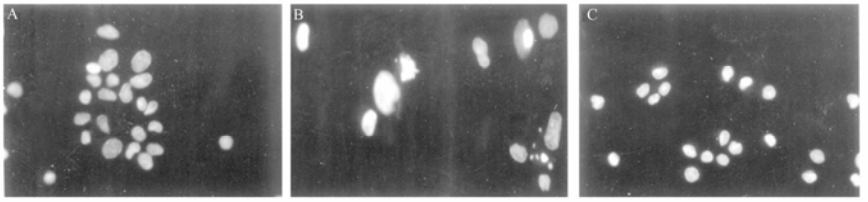

FIG. 3. Morphological changes of HUVEC stained with Hoechst 3332 in the presence and absence of CSE and nicotine. Cells were incubated with 20% CSE or 10^{-4} mol/L nicotine for 24 h. At the end of incubation, photomicrographs of the cells were taken. (A) Normal control cells showing Hoechst 33342 staining, (B) Cells exposed to 20% CSE for 24 h exhibiting the characteristic apoptotic small nuclei with condensed chromatin and bright Hoechst fluorescence, (C) Cells exposed to 10^{-4} mol/L nicotine (×400).

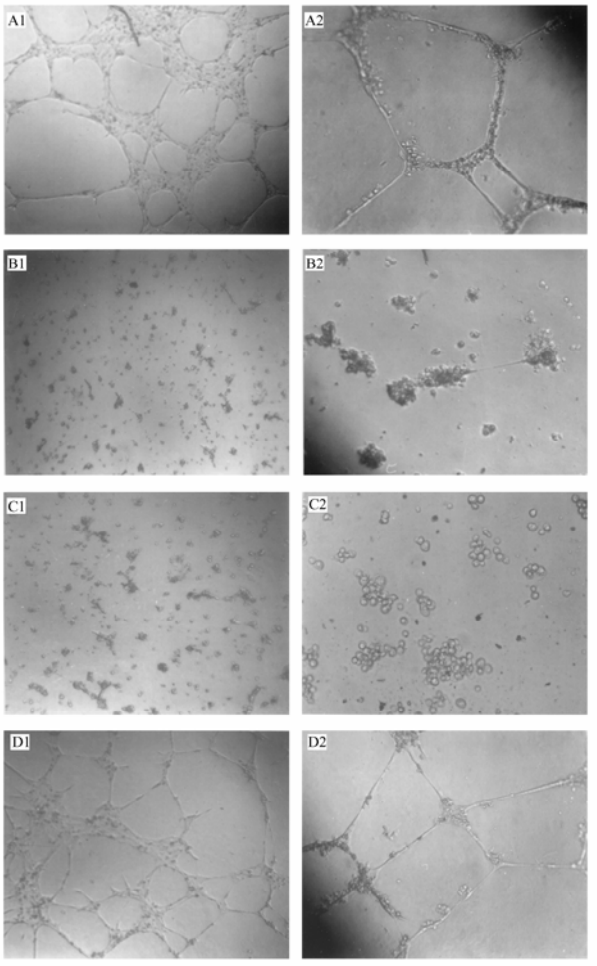

FIG. 4. Phase-contrast photomicrographs of HUVEC cells cultured on matrigel for 24 h. HUVEC at a density of 2×10^4 cell/mL cultured on a matrigel-coated 96-well plate for 24 h in the absence (a) or presence of 2.5% CSE (b), 5% CSE (c), or nicotine (d). (Original magnification×140 and ×280).

The nuclear membrane of apoptotic nuclei was permeable to Hoechst 33342, which intercalates the fragmented DNA. The fragmented apoptotic nuclei were observed under fluorescence microscope[11]. As shown in Fig. 3, apoptotic cells were identified based on nuclear pyknosis or chromatin condensation by Hoechst 33342 staining, a phenomenon that characterizes the apoptotic type of cell death. Nicotine caused no change in morphology of HUVEC.

Angiogenesis

HUVEC assumed typical "cobblestone" monolayer morphology when grown on gelatin-coated plates. As demonstrated by phase-contrast microscopy (Fig. 4), HUVECs began to align them end-to-end and to elongate in 2 h and formed three-dimensional, capillary-like structures by 24 h after initial plating on Matrigel. The angiogeneses of HUVEC were completely inhibited by CSE at noncytotoxic concentration. The lumen-containing structures were well detected in nicotine-treated HUVEC.

Rhodamine 123 Labelling of Mitochondria and Determination of Cytosolic Calcium by Fura 2 in HUVEC

We measured $\triangle\psi_m$ using the fluorescent dye rhodamine 123, which were incorporated into mitochondria driven by the $\triangle\psi_m$. As shown in Table 3, the control cells exposed to rhodamine 123 exhibited fluorescence with a mean intensity of 11.0. In contrast, exposure to CSE for 24 h caused a decline in Rh 123 fluorescence of 9.03, 8.47 and 7.80 in 10%, 15% and 20% CSE-treated HUVECs, respectively. The CSE-treated HUVECs exhibited two subpopulations, normal cell phase with fluorescence intensity of ~15 and small cell phase of ~2.5, which suggested the shrinkage of HUVEC induced by CSE. The percentage of cells in small cell phase increased from 6.84% in control to 9.7%, 29.2% and 47.8% in 10%, 15% and 20% CSE exposure, respectively (Fig. 5).

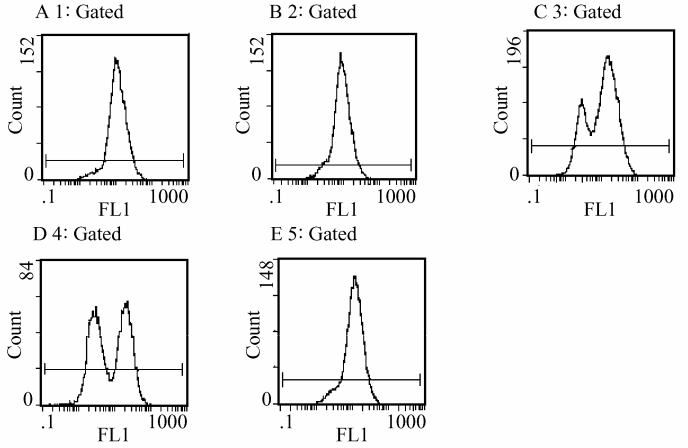

FIG. 5. Effect of CSE and nicotine on mitochondrial membrane potential. HUVECs were incubated in the absence (A) or presence of CSE (B: 10%, C: 15%, D: 20%) or nicotine (E: 10^{-4} mol/L) for 24 h. Cells were then stained with Rh 123 and analyzed by flow cytometry. CSE-treated HUVECs were divided into distinct large and small subsets. Peak 1, small cells; Peak 2, large cells. The decrease in the fluorescence intensity of HUVEC appeared to be restricted mainly to the small cells subsets. Results are plotted as log fluorescence versus relative cell number and are representative of three experiments, at least 10 000 cells were analyzed.

The fluorescence intensity of fura 2 was determined to indicate the $[Ca^{2+}]_i$ of HUVEC after exposure to CSE. As shown in Table 3, the mean fluorescence intensity increased from 5.71 (control) to 13.7 (20% CSE) in a dose-dependent manner after exposure to different doses of CSE. These results were indicative of CSE-dependent loss of $\triangle\psi_m$ and the rise in cytosolic free Ca^{2+}.

TABLE 3

Effect of CSE or Nicotine on p53 and Ratio of Bcl-2 to Bax Protein Levels in HUVEC by Flow Cytometric Analysis

Fluorescence Intensity	Normal	10% CSE	15% CSE	20% CSE	Nicotine
p53	4.79	3.8	3.67	1.64	5.08
Bcl-2/Bax	2.9	2.86	1.54	0.47	3.1
$\triangle\psi_m$	11.0	9.03	8.47	7.8	11.2
$[Ca^{2+}]i$	5.71	6.54	10.3	13.7	4.8

Note. HUVECs (1×106 cells) in 10% FBS were treated with various doses of CSE for 24 h at 37℃. Cells were labeled with FITC conjugated monoclonal antibody to p53, Bcl-2 or Bax, and rhodamine 123 or Fura 2. Then the cells were analyzed by flow cytometry as described in Materials and Methods. Fluorescence was determined by log amplification, at least 10 000 cells were analyzed. The fraction of positive cells present in each sample was calculated after subtracting the negative control histogram, respectively. The numbers in the figure indicate the mean fluorescence of cells in relative fluorescence units.

Expression of Apoptosis-related Protein on HUVEC in the Absence or Presence of CSE or Nicotine

As shown in Table 3, CSE treatment significantly decreased cellular p53 protein concentration as compared with that of the control cells. CSE stimulation failed to augment p53 protein expression of HUVEC, suggesting apoptosis mediated by p53-independent pathway. In contrast, the expression of Bcl-2 in HUVEC was down regulated by CSE in a dose-dependent manner. Fluorescence histograms also showed a decrease in the Bcl-2/Bax ratio in CSE-treated HUVEC.

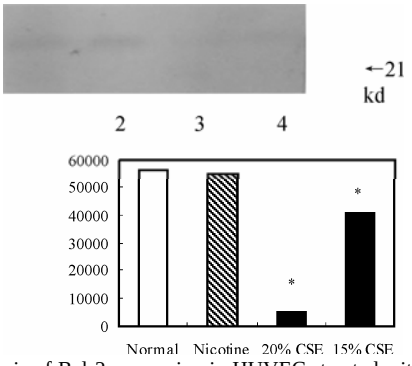

FIG. 6. Western blot analysis of Bcl-2 expression in HUVECs treated with CSE and nicotine. HUVEC were treated at various concentrations of CSE for 24 h. The cells were lysed and equal amounts of protein were subjected to SDS-PAGE on 12% polyacrylamide and immunoblotted by anti-Bcl-2 antibody. Lane 1: Control HUVEC, Lane 2: HUVEC treated with 10^{-4} mol/L nicotine, Lanes 3-4: HUVEC treated with 20%-15% CSE. The density of the bands was quantities in the below figure.

Statistical Analysis

In each experiment, experimental and control endothelial cells were matched for cell line, age, seeding density, number of passages, and number of days after confluence to avoid variation in tissue culture factors that can influence the results. Results are shown as $\bar{x}\pm s$. Student's *t* test was used to determine the significance of differences between samples. $P< 0.05$ was taken as significant.

DISCUSSION

The present study was the first one to demonstrate that exposure to CSE could cause inhibition of viability, proliferation, and differentiation of HUVECs. Moreover, the induction of apoptosis was confirmed by the condensation of nuclear chromatin and the presence of hypodiploid DNA in CSE-treated HUVECs, suggesting that CSE-induced injury of HUVEC is mediated in part through accelerated endothelial cell apoptosis which is independent of p53. A diverse range of changes in mitochondria induced by CSE includes the downregulation of Bcl-2 expression and decline in the Bcl-2/Bax ratio, excess intracellular free calcium and dissipation of inner transmembrane potential. It appears that mitochondria play a key role in the apoptosis of HUVEC induced by CSE. Nicotine has no discernible inhibitory effects on HUVECs.

Vascular endothelial cells played an important role in the process of angiogenesis, a critical process in wound healing, inflammation, and embryogenesis[12]. Endothelial cell injury might alter the capacity of the endothelium to function as a barrier. Matrigel provided a more authentic matrix substrate in which endothelial cells grew into three-dimensional, capillary-like structures. The dramatic morphological differentiation of endothelial cells on matrigel reflected the changes of gene expression and functions[13]. CSE inhibited HUVEC angiogenesis at noncytotoxic concentrations. The formation of specific cell-substratum interactions resulting from matrix remodeling was apparently essential for preserving cell viability in the context of angiogenesis[14]. It was speculated that this inhibition of differentiation might result in an attenuated repair response. Moreover, it would exacerbate CSE-induced vascular injury due to the inhibition of viability and proliferation of endothelial cells.

Mitochondria played an integral role in apoptotic signaling pathways. Exposure to stimuli might permeabilize the mitochondria, leading to release of cytochrome C into cytosol[15]. In the present study, CSE-induced nuclear fragmentation was also accompanied by mitochondrial dysfunction. The Mitochondrial membrane potential ($\triangle\psi_m$) formed as a result of the asymmetric distribution of ions across the inner mitochondrial membrane and was dependent upon its permeability. Since the normal inner membrane was negatively charged, rhodamine 123 was distributed in the mitochondrial matrix. The resulting fluorescence of the probes provided a measure of $\triangle\psi_m$. Our results clearly demonstrated that after 24 h of exposure to CSE, there was a reduction in HUVEC $\triangle\psi_m$, as evidenced by a decrease in fluorescence of rhodamine 123. Agents known to depolarize or deenergized mitochondria could decrease $\triangle\psi_m$[16]. It was reported that cigarette smoke contained uncouplers and mitochondrial respiratory inhibitors, which might contribute to the dissipation of $\triangle\psi_m$ in CSE-treated HUVEC[15].

Our data indicated that CSE-induced apoptosis of HUVEC was associated with dissipation of mitochondrial membrane potential and increased cytosolic calcium level. Ca^{2+} is a major second messenger implicated in signal transduction pathways regulating apoptosis.

The rise in cytosolic free Ca^{2+} has been considered to be involved in apoptosis through activation of calcium-dependent mitochondrial permeability transitional (MPT) pore opening, which in turn lead to the release of cytochrome C and initiate the apoptosis [17]. The mitochondrial membrane potential could detoxify excess accumulation of $[Ca^{2+}]_i$ by accelerating entry of Ca^{2+} to intracellular calcium pools to avoid the MPT pore opened. Our study suggested that the excess of $[Ca^{2+}]_i$ and decreased mitochondrial membrane potential caused by CSE were associated with apoptosis.

The decrease in Bcl-2 expression in CSE-treated cells also suggested that CSE exerted its inhibitory action at the level of mitochondria. Bcl-2 family members could regulate many of the apoptosis-related functions of the mitochondria. The down-regulation of Bcl-2 expression might be involved in apoptosis induction. Related to this finding was the observation that Bcl-2 was localized in mitochondrial membranes and protected cells from apoptosis, possibly by regulating MPT pore formation[18]. Mitochondrial membrane insertion of Bcl-2 might stabilize the mitochondrial outer membrane to inhibit mitochondrial calcium release[19] and MPT pore opening, maintain mitochondrial membrane potential ($\triangle \psi m$)[20], and block cytochrome C release[21]. The Bax homodimers were inhibited through heterodimerization with the survival factor Bcl-2 gene product. Bax/Bcl-2 ratio was thus the critical factor for susceptibility or inhibition of apoptosis[22]. These proteins could form pores in the mitochondrial membrane and in this way regulate ion transport across the outer mitochondrial membrane. The dose-dependent decline in Bcl-2/Bax mediated by CSE in our study was consistent with the finding that the mitochondrial pathway mediated CSE-induced endothelial apoptosis.

Moreover, we observed that CSE induced mitochondrial respiratory inhibition, swelling dysfunction and structure injury as reported in our previous studies[5]. Therefore, it is tempting to speculate that mitochondria damage is a key step in the pathogenesis of CSE-induced apoptosis in HUVEC.

Nicotine, one of the important components in cigarette smoke, was shown to be mitogenic for cells at lower concentrations by other investigators[23], but our experiments revealed that nicotine at concentrations up to 10^{-4} mol/L had no discernible inhibitory effects on HUVEC. It is unlikely that nicotine was the major constituent in cigarette smoke to induce damages of HUVECs.

Our study also demonstrated that p53 protein levels decreased, rather than increased, in HUVECs treated with CSE. Given the high p53 levels are associated with an increased apoptosis, our findings indicate that the CSE-induced HUVEC apoptosis may be p53 independent. The cell cycle analysis also demonstrated that CSE induced the S phase instead of G1 arrest in HUVEC. This finding was consistent with the earlier observation reported by Wang *et al.*[3].

In summary, the present study demonstrates that exposure to CSE can cause inhibition of viability, proliferation, and differentiation in HUVEC. HUVECs incubated with CSE give a significant decrease in the Bcl-2 and Bcl-2/Bax ratio accompanied by the loss of mitochondrial membrane potential and excess cytosolic calcium level. All these suggest that the mitochondrial events play a key role in CSE-mediated endothelium injury. Therefore, the mitochondrial pathway in HUVEC is responsible, at least in part, for the p53 independent apoptosis induced by CSE. However, it is not clear whether the regulation by CSE involves release of cytochrome C from mitochondria. Nicotine has no inhibitory effects on HUVEC. The results of the present study support the hypothesis that cigarette smoke-induced endothelial injury is mediated in part through accelerating endothelial apoptosis. The excessive apoptosis in endothelial cells induced by CSE may predispose smokers to cardiovascular complications and hinder the recovery. Our study may provide some new

information to explain cardiovascular dysfunction in cigarette smokers. Further studies will be necessary to explore the compounds in CSE to induceendothelial cytotoxicity and its related mechanism.

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