Anti-apoptotic effect of activated protein C on lipopolysaccharide-stimulated human umbilical vein endothelial cells is associated with the inhibition of the caspase-3 pathway

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Abstract. Activated protein C (APC) is an anticoagulant polypeptide that plays a critical role in coagulation, inflammation and anticoagulation interactions; in addition, it decreases the mortality rate of patients with severe infectious disease. However, the exact mechanisms of these effects remain unclear. The aim of this study was to investigate the effect of APC on human umbilical vein endothelial cell apoptosis upon lipopolysaccharide (LPS) stimulation and to elucidate its underlying mechanisms. Apoptotic changes in cells were determined by examining cell ultra-structure, DNA fragmentation and Annexin V/PI staining. Cell viability and the expression of apoptotic-related factors were measured in LPS-stimulated cells with and without APC treatment. The results showed that the administration of APC decreased the apoptosis of cells by inhibiting the activity of the caspase-3 apoptotic-signaling pathway in LPS-stimulated cells. This study supports the notion that APC treatment may be beneficial for the attenuation of endothelial cell injury caused by severe infectious disease via the regulation of apoptotic pathways. Furthermore, APC may be useful in the treatment of infectious disease.

Introduction

In recent years, certain areas of clinical research have focused on elucidating the mechanisms involved in the dysfunction of the structure of vascular endothelial cells, as the disruption of the homeostatic functions of vascular endothelial cells acts as a substrate for end-organ damage and the occurrence of vascular events (1). It has been suggested that the injury of vascular endothelial cells plays a critical role in excessive inflammation, since it causes tissue-lesion formation and coagulation disorders, such as disseminated intra-vascular coagulation (2,3). Therefore, research with the aim of reaching a better understanding of how this process is regulated may lead to the development of potential therapies for pathological conditions that involve the injury of vascular endothelial cells.

Lipopolysaccharide (LPS) is a constituent of the outer membrane of the cell wall of Gram-negative bacteria and is a biologically active component of Gram-negative bacteria involved in infectious disease. LPS induces vascular endothelium injury, which leads to an increase in vascular permeability; this condition leads to edema of organs and then to organ failure (4,5). Therefore, LPS is a primary factor in the development of disease due to infection.

Studies have shown that the infusion of recombinant human activated protein C (APC), an anticoagulant polypeptide, reduces circulatory dysfunction, length of respirator-dependence, and the mortality rate of patients with severe infectious disease (6); these results suggest that APC may be used as a novel therapeutic agent. However, the exact mechanisms of its action remain unclear. The aim of this study was to investigate the anti-apoptotic effect of APC on human umbilical vein endothelial cells (HUVECs) upon LPS stimulation, and furthermore to explore its mechanisms of action.

Materials and methods

Cell culture and cell stimulation. The study was performed on HUVECs (American Type Culture Collection, Rockville, MD, USA). Cell culture was conducted in medium containing DMEM (Gibco BRL, Gaithersburg, MD, USA) and 10% fetal bovine serum (Gibco BRL) at a 1:1 ratio, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA) at 37°C in 5% CO_2. LPS and APC were obtained from Sigma.

Cells were randomly divided into four groups. Three of the groups were treated with either PBS, 10 ng/ml APC or 50 ng/ml APC, respectively, and induced with LPS (1.0 μg/ml); one group of cells was left untreated as the normal control group. The samples were harvested at 24 h after the onset of induction for further study.

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3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated at a density of 2×10^3/well in 96-well plates, and 20 μl MTT (5 mg/ml) was added to each well. After 4 h, the medium was removed, and 100 μl of dimethyl sulfoxide was added to each well. Cell viability was determined by the absorbance (A) at 570 nm relative to the control group: % viability = (A of study group)/(A of control group) x 100%.

Electron microscopy. The cells were trypsinized, fixed with 2.5% glutaraldehyde for 2 h at 4°C, washed with 0.1 M dimethyl arsenic trioxide buffer, fixed with 1% osmium acid for 2 h, dehydrated and embedded in 618 domestic epoxy resin followed by polymerization for 24 h. The embedded blocks were cut into ultra-thin sections. The sections were double-stained with uranyl acetate and lead citrate and examined with a JEM 100 CX electron microscope (Jeol, Peabody, NY, USA).

DNA agarose-gel electrophoresis. The cells were washed twice with PBS, and DNA was extracted with an apoptotic DNA ladder detection kit according to the manufacturer's instructions (Biyuntian Company, Shanghai, China). The extracted DNA was maintained at 4°C overnight. The DNA sample (8.5 μl) was then mixed with 1.5 μl of 6X loading buffer, electrophoresed at 40 V on 1% agarose gels containing ethidium bromide, and finally visualized using the Bio-Rad gel image analysis system (Bio-Rad, Baltimore, MD, USA).

Annexin V/propidium iodide (PI) assay. Cells were washed twice with PBS at 4°C then re-suspended in 250 μl of a combination buffer solution, and the cell concentration was adjusted to 1×10^5/ml. Annexin V/FITC (5 μl) and 5 μl PI were subsequently added. After incubation in the dark for 15 min, the cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR, USA).

Western blotting. The cells were homogenized in RIPA buffer (Biyuntian Company) for 10 min at 4°C, followed by centrifugation at 20,000 x g and 4°C for 10 min. The supernatant was collected and stored at -80°C, and the protein concentration of each sample was determined by bicinchoninic acid assay (Biyuntian Company). Proteins (40 μg) from each sample were denatured in loading buffer and separated by electrophoresis on a 6% SDS polyacrylamide gel at 100 V for 120 min. The separated proteins were transferred to a polyvinylidene difluoride membrane using transfer buffer at 200 mA for 90 min. The membranes were blocked with 5% non-fat dry milk in 0.1% Tween in Tris-buffered saline (TBS-T) for 1 h at room temperature and washed three times for 10 min in TBS-T. Then, the membranes were incubated with a primary antibody, including proliferating cell nuclear antigen (PCNA), P53, Bax, Bcl-2, cytochrome C or cleaved caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:500 dilution in TBS-T overnight at 4°C. The membranes were washed twice with TBS-T and incubated with a secondary antibody, horse-radish peroxidase-conjugated goat anti-rabbit immunoglobulin G or rabbit anti-mouse immunoglobulin G (Kangcheng Inc., Shanghai, China), for 1 h at room temperature. The
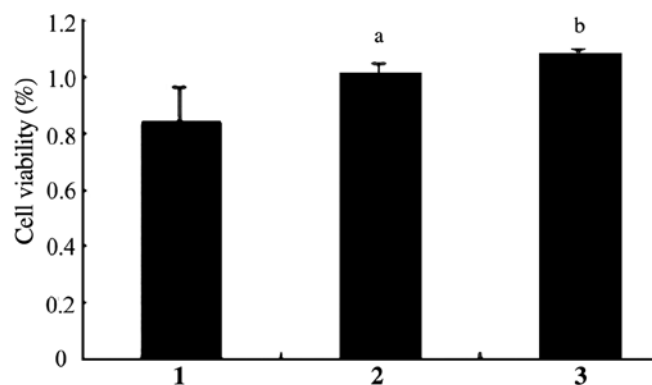

Figure 1. Cell viability determined using the MTT array. Bar 1, LPS 1 μg/ml group/control group; 2, (LPS 1 μg/ml + 10 ng/ml APC-treated group)/control group; 3 (LPS 1 μg/ml + 50 ng/ml APC-treated group)/control group. [a]P<0.05, [b]P<0.01 vs. 1.

membranes were analyzed by an enhanced chemiluminescence kit according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ, USA). β-actin was determined using a similar protocol with the β-actin antibody (Santa Cruz Biotechnology), and served as an endogenous control for the presence of other proteins.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using TRIzol reagent kits (Gibco BRL). The primers for P53, Bax, Bcl-2, caspase-3 and β-actin were constructed based on published human P53, Bax, Bcl-2, caspase-3 and β-actin nucleotide sequences, and were synthesized by Shenggong Technology Inc. (Shanghai, China). The RT-PCR reactions were performed in a 25-μl reaction volume and run in the Gene Amp 9600 instrument (Perkin-Elmer Inc., Wellesley, MA, USA). The primers were as follows: Bcl-2, 5'-AGA CAG CCA GGA GAA ATC AAA CAG-3' and 5'-TGC ACC TGA CGC CCT TCA C-3'; Bax, 5'-ACA AAG ATG GTC ACG GTC TG-3' and 5'-ACC AAG AAG CTG AGC GAG TGT C-3'; P53, 5'-TCT GGG ACA GCC AAG TCT GT-3' and 5'-GGA GTC TTC CAG TGT GAT GA-3'; caspase-3, 5'-GCT AGC TAG CAT GGA GAA CAC TGA AAA CTC AGT G-3' and 5'-GCT ACT CGA GGT GA T AAA AAT AGA GTT CTT TTG TG-3'; β-actin, 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' and 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'.

The RT-PCR conditions were as follows: 1 cycle for 5 min at 95°C; 35 cycles for 45 sec at 94°C, for 45 sec at 55°C (Bcl-2, β-actin), for 45 sec at 54°C (Bax), for 45 sec at 56°C (P53), for 45 sec at 57°C (caspase-3) and for 45 sec at 72°C; 1 cycle for 10 min at 72°C. The PCR products were separated by electrophoresis in 1.2% agarose gels and stained with ethidium bromide. The densities of the cDNA bands were analyzed by scanning densitometry using GelDoc 2000 (Bio-Rad). The band densities were normalized to the β-actin band densities, and the results were expressed as a ratio of densities relative to the β-actin band density.

Statistical analysis. All data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed with SPSS software version 10.5. One-way analysis of vari-
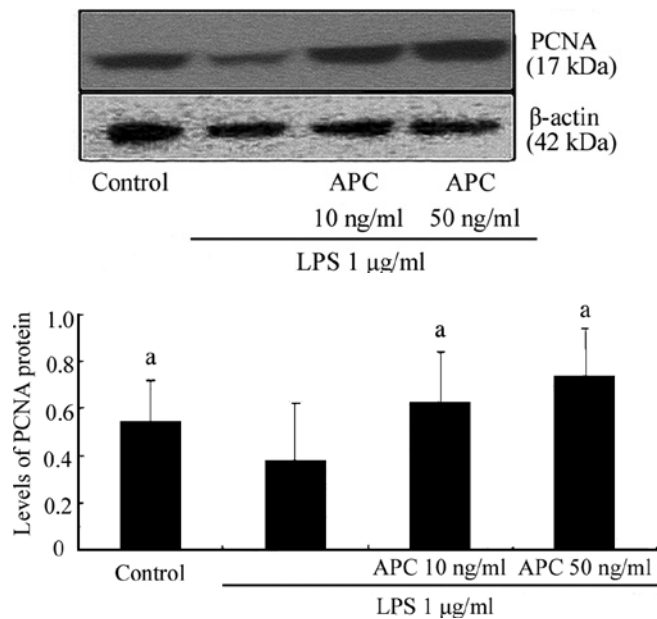
Figure 2. Representative Western blotting of proteins in the cells detected with PCNA and β-actin antibodies in each group. The graph shows the ratio of PCNA to β-actin band density in each group. [a]P<0.05 vs. the LPS 1 μg/ml group.

Figure 4. Expression of DNA fragments in each group by agarose gel electrophoresis. A typical ladder pattern of inter-nucleosomal DNA fragmentation was detected in the LPS-stimulated cells, which was attenuated by treatment with APC at both concentrations.
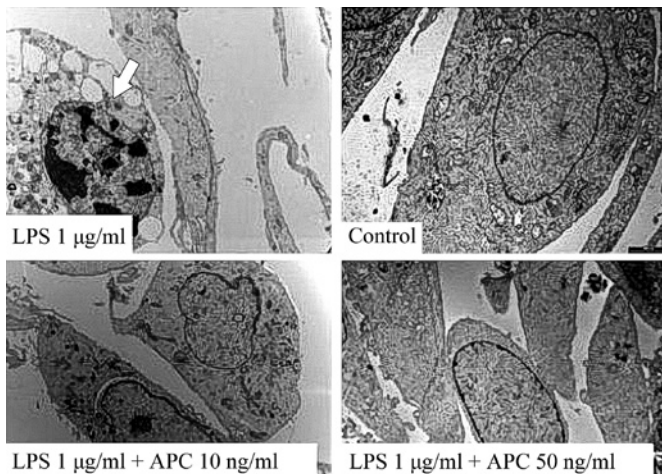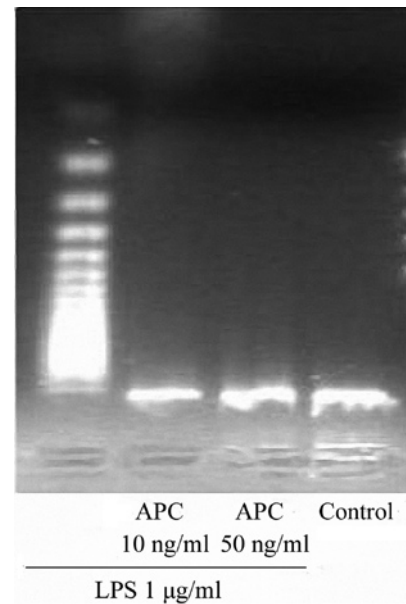

Figure 3. Cell ultra-structure visualized by electron microscopy for each group (x7,200). LPS-stimulated HUVECs exhibit shrinkage and condensation of the nucleus and cytoplasm, the margination of chromatin and nuclear membrane and the formation of apoptotic bodies (open arrow point); no apparent changes were observed between the LPS-stimulated HUVECs treated with either dose of APC and the control cells.

ance (ANOVA) with Dunnett's multiple comparison tests was used for comparisons. A P-value of <0.05 was considered statistically significant.

Results

Detection of cell viability using the MTT array. The viability of cells in the two APC-treated groups was significantly increased compared to that of the LPS group: 1.00±0.03 (P<0.01) for the 10 ng/ml APC-treated, 1.07±0.01 (P<0.05) for the 50 ng/ml APC-treated and 0.84±0.11 for the LPS groups (Fig. 1).

Detection of PCNA protein levels using Western blotting. PCNA protein levels were up-regulated significantly in the two groups treated with different doses of APC compared to the LPS group (P<0.05 for both) (Fig. 2).

Detection of cell ultra-structure using electron microscopy. As shown in Fig. 3, LPS stimulation for 24 h caused the shrinkage and condensation of the nucleus and cytoplasm, the margination of chromatin and the nuclear membrane, and the formation of apoptotic bodies, whereas no apparent changes were observed when comparing the two groups treated with different doses of APC and the control groups.

Detection of cell apoptosis using agarose gel electrophoresis. As shown in Fig. 4, a typical ladder pattern of inter-nucleosomal DNA fragmentation was detected in the cells stimulated with LPS. This ladder was eliminated upon the 24-h treatment with either dose of APC.

Detection of the cell apoptosis rate using flow cytometry. After stimulation with LPS for 24 h, the apoptotic rates of cells as shown by flow cytometry were found to be markedly increased (18.91±6.16%) as compared to the control group (7.19±4.90%) (P<0.01). After treatment with 50 or 10 ng/ml APC, the apoptotic rates of cells were decreased to 11.09±5.27 and 14.01±6.65%, respectively, with the higher-dose (APC 50 ng/ml) group displaying a greater effect (Fig. 5).

Detection of cytochrome C protein levels using Western blotting. In the control group, faint expression of cytochrome C protein was observed, wwhile LPS stimulation increased the expression in the cells (P<0.05). In the 10 and 50 ng/ml APC-treated groups, a gradual decrease in cytochrome C protein expression was observed. Statistic analysis demon-
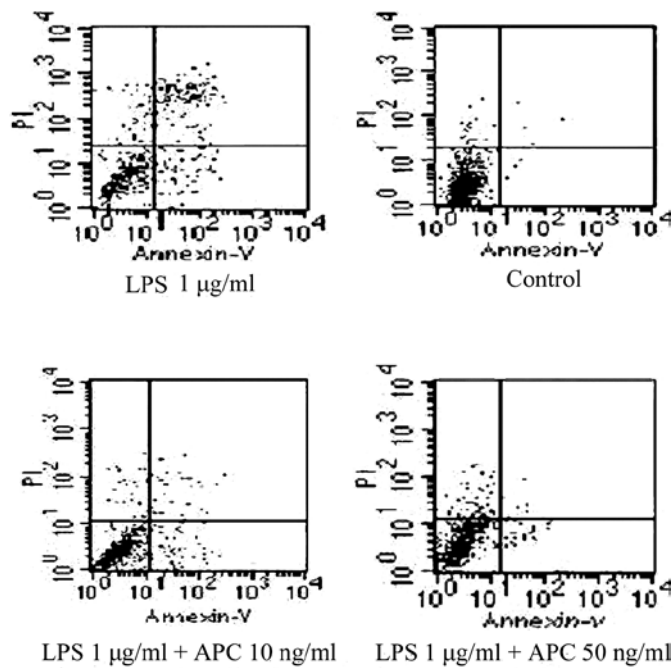
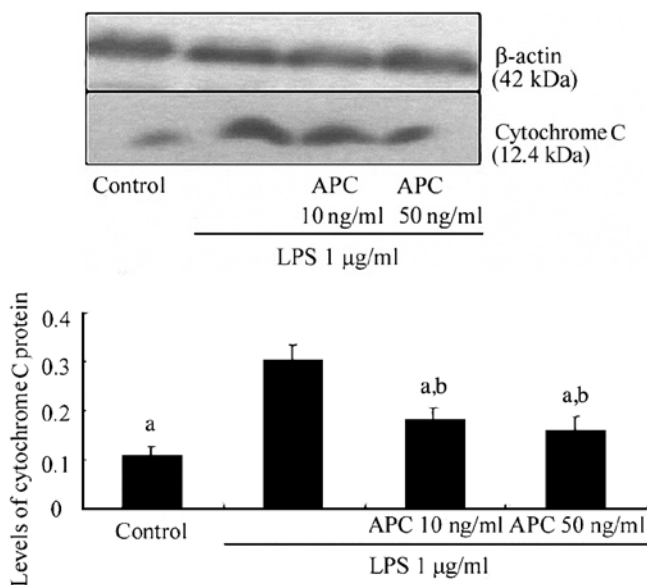Figure 5. Apoptotic rates of cells in each group measured by flow cytometry.

Figure 6. Representative Western blotting of proteins in the HUVECs detected with cytochrome C and β-actin antibodies in each group. The graph shows the ratio of cytochrome C to β-actin band density in each group. [a]P<0.05 vs. the LPS group; [b]P<0.05 vs. the control group.

strated a significant difference in cytochrome C protein levels between the LPS group and the 50 ng/ml APC-treated or control groups (P<0.05, respectively). There was no difference in protein expression between the two groups treated with different doses of APC (P>0.05) (Fig. 6).

Detection of P53, Bax, Bcl-2 and caspase-3 protein and mRNA levels using Western blotting and RT-PCR. In the LPS group, P53, Bax and cleaved caspase-3 protein levels were markedly

increased, while Bcl-2 protein levels were decreased. However, the expression levels were differed from those of the 50 ng/ml APC-treated and control groups (P<0.01 and P<0.05, respectively). The 10 ng/ml APC-treated group showed no difference in expression levels compared to the LPS group, suggesting a dose-dependent effect of APC treatment. The expression of P53 protein displayed a dose-dependent effect for APC treatment (P<0.01), and there was also a difference in expression between the 10 ng/ml APC-treated and control groups (P<0.01). The levels of the above-mentioned markers showed no difference between the 50 ng/ml APC-treated and control groups (P>0.05). The ratio of Bax to Bcl-2 protein levels was down-regulated in the APC-treated groups: 1.146±0.33 in the 10 ng/ml APC-treated group and 0.68±0.265 in the 50 ng/ml APC-treated group, compared to 2.28±0.375 in the LPS group. There was also a significant difference in this ratio between the APC-treated and control groups (0.45±0.425, P<0.05) (Fig. 7).

Next, the possible role of APC in the regulation of P53, Bax, Bcl-2 and caspase-3 mRNA expression was investigated (Fig. 8). As expected, P53, Bax and caspase-3 were highly expressed in the LPS group, with a marked difference compared to the mRNA levels in the 50 ng/ml APC-treated and control groups (P<0.01 or <0.05, respectively). The mRNA levels of the above-mentioned markers did not differ between the 50 ng/ml APC-treated and control groups (P>0.05) nor between the 10 ng/ml-APC treated and LPS groups (P>0.05). Bcl-2 mRNA levels were down-regulated in the LPS group, but up-regulated in the 50 ng/ml-APC treated and control groups compared to the LPS group (P<0.05 and <0.01, respectively). The expression of Bcl-2 and caspase-3 mRNA also showed a dose-dependent effect upon APC treatment (P<0.05, respectively), and there was a significant difference in expression between the 10 ng/ml APC-treated and control groups (P<0.05, respectively). By contrast, the ratio of Bax to Bcl-2 mRNA levels was greatly down-regulated in the APC-treated groups: 1.33±0.25 in the 10 ng/ml APC-treated and 0.72±0.15 in the 50 ng/ml APC-treated group, compared to 4.23±0.44 (P<0.05) in the LPS group. Again, there was a difference in mRNA levels between the APC-treated and control groups (0.42±0.26; P<0.05).

Discussion

Infections with Gram-negative bacteria are considered to be the most frequent cause of the onset of organ dysfunction (7,8). Furthermore, endothelial injury due to LPS stimulation is likely a major factor in disease progression for patients with severe infections, although other mechanisms may also be involved. Apoptosis, also called programmed cell death, is a tightly regulated form of cell mortality. Morphologically, apoptosis is characterized by chromatin condensation and cell shrinkage in the early stage of cell injury. In later stages, the nucleus and cytoplasm fragment, forming membrane-bound apoptotic bodies which can be engulfed by phagocytes. Recent studies indicate that apoptosis plays an key role in the detrimental injury of cells. Early treatment with apoptotic inhibitors may be beneficial for the attenuation of cell injury and subsequent injury development (9).
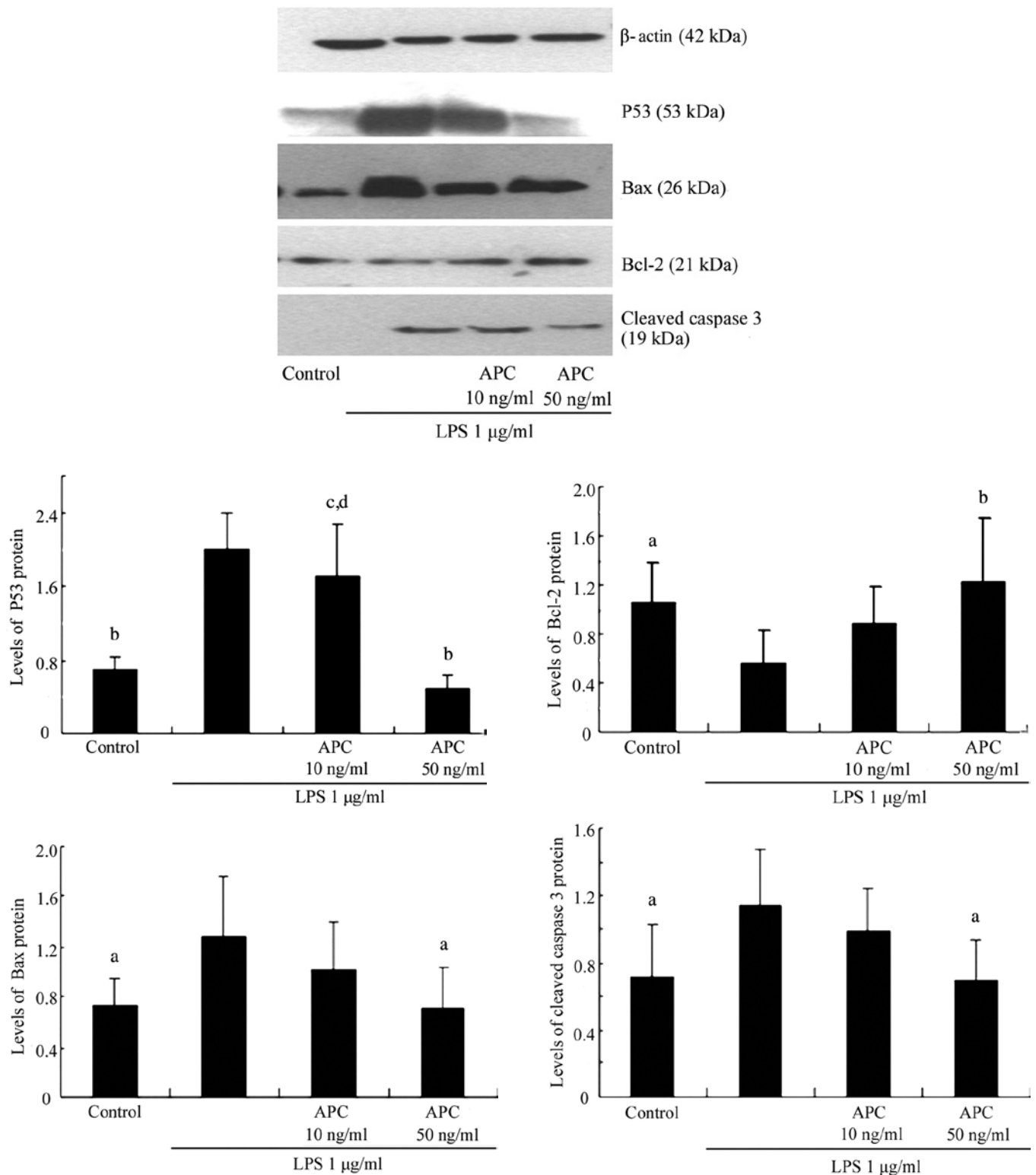
Figure 7. Representative Western blotting of proteins in HUVECs detected with P53, Bax, Bcl-2, cleaved caspase-3 and β-actin antibodies in each group. The graphs show the ratio of P53, Bax, Bcl-2 or cleaved caspase-3 to β-actin band density in each group. [a]P<0.05, [b]P<0.01 vs. the LPS group; [c]P<0.01 vs. the LPS 1 μg/ml + 50 ng/ml APC-treated group; [d]P<0.01 vs. the control group.

APC is an active precursor of protein C, which is activated by the action of the thrombin-thrombomodulin complex when protein C is bound to its endothelial receptor (endothelial cell protein C receptor). APC plays an important role in natural anti-coagulation by proteolytically cleaving activated factors V and VIII. APC also escalates fibrinolysis by inhibiting plasminogen activator inhibitor 1 and thrombin-activatable fibrinolysis inhibitor (10). Recent studies indicate that APC attenuates the inflammatory response by decreasing leukocyte rolling, and reduces the migration and accumula-
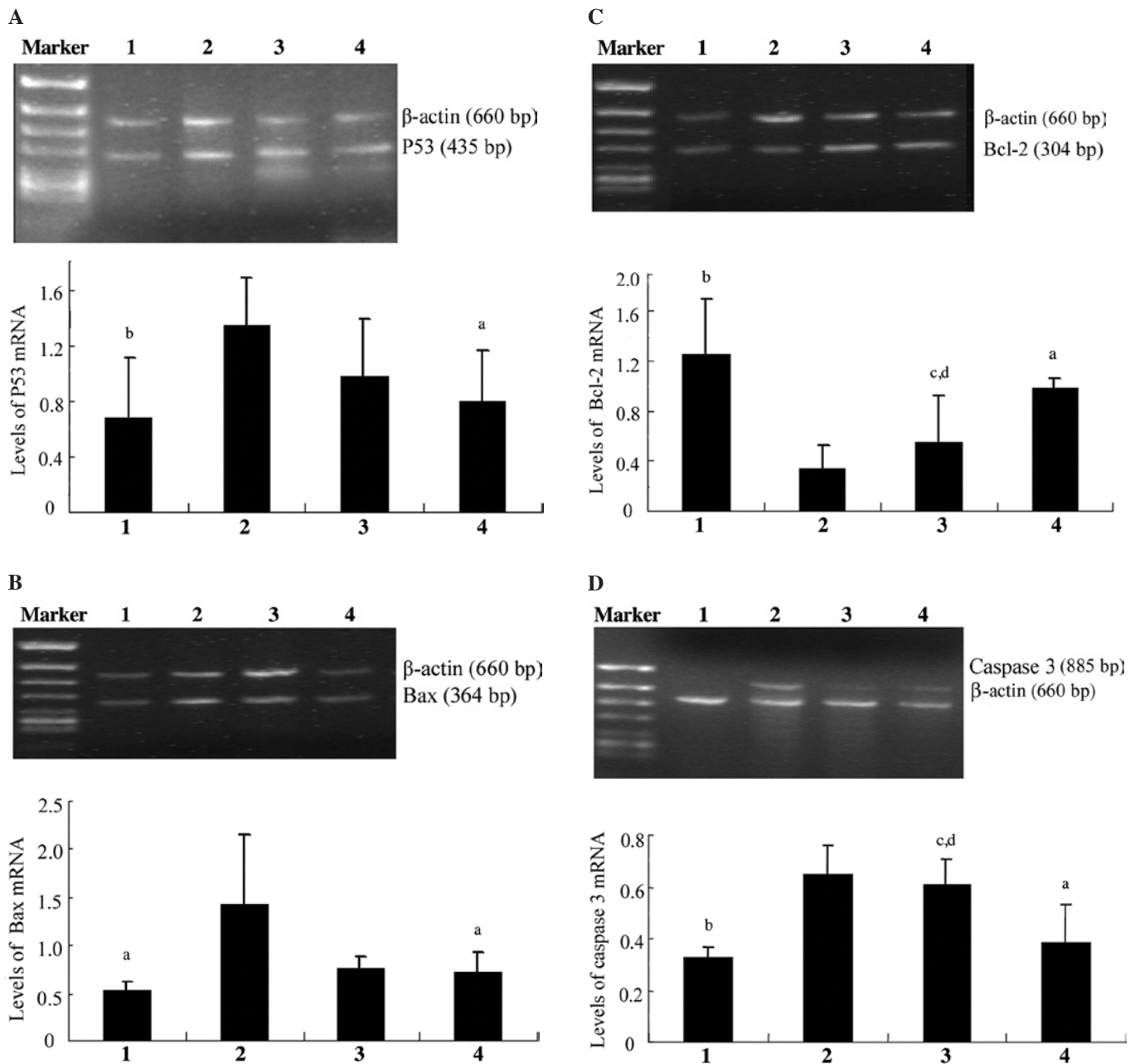
A

B

C

D

Figure 8. RT-PCR for P53, Bax, Bcl-2 and caspase-3 mRNA in each group. The mRNA levels of P53 (A), Bax (B), Bcl-2 (C) and caspase-3 (D) in the respective groups were analyzed and compared to β-actin as an internal control. The graphs show the ratio of P53, Bax, Bcl-2 or cleaved caspase-3 to β-actin band density in each group. Lane/bar 1, control group; 2, LPS group; 3, LPS 1 μg/ml + 10 ng/ml APC-treated group; 4, LPS 1 μg/ml + 50 ng/ml APC-treated group. [a]$P<0.05$, [b]$P<0.01$ vs. 2; [c]$P<0.05$ vs. 4; [d]$P<0.05$ vs. 1.

tion of leukocytes by inhibiting the translocation of nuclear factor κB in LPS-stimulated monocytes (11,12). Our previous studies suggested that APC inhibits the expression and activation of p38 mitogen-activated protein and c-Jun N-terminal kinases. These studies also found that APC up-regulated the expression and activation of extracellular signal-regulated kinases 1/2, leading to suppression of inflammatory cytokine release in pancreatic tissue and protecting against pancreatic tissue injury in rats with acute pancreatitis (13). In a study by Loubele *et al*, APC was administered in an animal model for myocardial I/R, and the results showed strong anti-apoptotic and anti-inflammatory effects of APC in the early phase of reperfusion (14). Furthermore, other studies have reported that

APC modulates apoptosis pathways, including the up-regulation of the endothelial Bcl-2 homolog (A1) (an inhibitor of apoptosis) and suppression of the apoptosis-associated gene calreticulin (15). Therefore, the effect of APC on the LPS-induced cell injury of endothelial cells and the identification of a novel effective mechanism of infectious disease involving APC treatment warrants investigation.

PCNA acts as a biologically essential factor that provides binding sites for DNA polymerase during DNA replication and repair. Researchers have reported that the levels of PCNA expression in the cell cycle increased at the G1 stage, peaked at the S stage and decreased at the G2 stage. Thus, PCNA serves as a marker of cell proliferation (16).

The present study showed that LPS inhibited cell proliferation by examining cellular viability and PCNA protein expression. LPS-induced HUVEC apoptosis was confirmed by cell ultra-structure, DNA fragmentation and the apoptotic rate. These results suggest that an early treatment strategy with an LPS inhibitor may be beneficial for the attenuation of cell injury and the promotion of cell growth. Consistent with the study of Joyce *et al* (15), the results of our study also showed that APC inhibited the apoptosis of HUVECs upon LPS stimulation, particularly with the higher 50 ng/ml APC dosage; however, the details of the effective mechanisms require further investigation.

Apoptosis is a complex process involving several genes, including apoptotic inhibiting genes (Bcl-2) and apoptotic promoting genes (P53, Bax, cytochrome C and caspase-3) (17). P53 limits cellular proliferation by inducing cell cycle arrest and apoptosis in response to cellular stresses, such as DNA damage and oncogene activation. In response to DNA damage, P53 mediates apoptosis through a linear pathway involving Bcl family members (18). Bcl-2 and Bax are both members of the Bcl-2 family, but play different roles in apoptosis. When Bax is overexpressed in cells, apoptosis in response to death signals is accelerated, which has led to its designation as a death agonist. When Bcl-2 is overexpressed, cell mortality is suppressed. Therefore, the ratio of Bcl-2 to Bax expression levels is an important factor in determining susceptibility to apoptosis.

Cytochrome C release from mitochondria is followed by the activation of caspase-3 (19), which in turn induces a series of biochemical reactions that result in caspase-3 activation and subsequent cell death (20). Caspase-3 serves as an executor caspase, playing a pivotal role in the progression of apoptosis. The activation of caspase-3 (cleaved caspase-3) leads to the classical morphological changes associated with apoptosis, including chromatin condensation, nuclear degeneration and cellular dehydration; therefore, caspase-3 activation is often considered the 'point of no return' in apoptosis (21,22). Our data clearly showed that the stimulation of HUVECs by LPS resulted in a significant up-regulation of cytochrome C expression, subsequently increasing the transcription of P53, the ratio of Bax to Bcl-2 expression and the activity of caspase-3. We also demonstrated that in LPS-stimulated cells, the up-regulation of apoptotic promoting factors, such as cytochrome C, P53, Bax and caspase-3, was markedly blocked by APC, and the down-regulation of apoptotic inhibiting factors, such as Bcl-2, was markedly increased by APC.

In summary, APC exerts a strong protective effect on vascular endothelial cells through the regulation of caspase-3-dependent gene expression, and thus may be beneficial for the attenuation of endothelial cell injury in severe infectious disease. This effect makes APC useful as a therapeutic agent against some of the harmful effects of infectious disease.

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