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Up-regulation interleukin-6 and interleukin-8 by activated protein C in lipopolysaccharide-treated human umbilical vein endothelial cells

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Abstract: Objective: To investigate the effect of activated protein C (APC) on inflammatory responses in human umbilical vein endothelial cells (HUVEC) stimulated with lipopolysaccharide (LPS). Methods: The second passage of collagenase digested HUVEC was divided into the following groups: serum free medium control group (SFM control), phosphate buffer solution control group (PBS control), LPS group with final concentration of 1 μg/ml (LPS group), APC group with final concentration of 7 μg/ml, Pre-APC group (APC pretreatment for 30 min prior to LPS challenge), and Post-APC group (APC administration 30 min after LPS challenge). Supernatant was harvested at 0, 4, 8, 12 and 24 h after LPS challenge. Interleukin-6 (IL-6) and Interleukin-8 (IL-8) levels were analyzed with ELISA. Cells were harvested at 24 h after LPS challenge, and total RNA was extracted. Messenger RNA levels for IL-6 and IL-8 were semi-quantitatively determined by RT-PCR. Results: Compared with control group, IL-6 and IL-8 levels steadily increased 4 to 24 h after LPS stimulation. APC treatment could increase LPS-induced IL-6 and IL-8 production. The mRNA levels of IL-6 and IL-8 exhibited a similar change. Conclusion: APC can further increase the level of IL-6 and IL-8 induced by LPS. The effect of these elevated cytokines is still under investigation.

Key words: Activated protein C (APC), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Sepsis, Human umbilical vein endothelial cell (HUVEC)

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INTRODUCTION

Sepsis is the prime factor contributing to death in the intensive care unit (ICU), and it is the eleventh most common cause of death in America. Research in the past two decades suggests that the imbalance between pro- and anti-inflammatory reactions is the main mechanism of organ injury in septic patients. Sepsis is defined as infection-induced systemic inflammatory reaction syndrome (SIRS) in 1992 by the Society of Critical Care Medicine (SCCM).

From the above theory, anti-inflammatory therapy has become focus of the sepsis research. Although good results have been obtained from animal experiments, 28 multi-center-anti-inflammatory clinical therapy trials have failed. Three of those trials were suspended because of higher mortality in the treatment group, which suggests the difficulty to lower the sepsis mortality further simply by reducing the inflammatory reaction.

Furthermore over 50% of patients diagnosed with sepsis associated with disseminated intravascular coagulation (DIC) do not survive. Using an active-site blocked factor Xa (DEGR-FXa) to interfere with the procoagulant events in septic animals, Taylor *et al*.(1991) failed to prevent shock and organ injury from occurring. This suggests that sepsis cannot be more effectively managed only by adding anti-coagulation therapy.

Recently the pathophysiology of sepsis was studied intensively, revealing a complex network of interactions between coagulation and inflammation (Esmon, 2000). Coagulation is an important part of the innate immunity, and shares its biologic evolution

with inflammation (Opal, 2000). Mononuclear and endothelial cells play vital role not only in inflammation but also in coagulation. When challenged by endotoxin, they not only produce inflammatory factors, but express tissue factors and initiate the extrinsic coagulation pathway as well (Lawson *et al*., 1994).

APC (actived protein C), one of the most important endogenous inhibitors of coagulation, has recently been shown to exhibit anti-inflammatory activities. A large phase III clinical trial has shown that APC can down-regulate plasma IL-6 and D-dimer level in severe sepsis patients, lowering the mortality (Bernard *et al*., 2001). But the exact molecular mechanism of this result is still unclear. APC can down-regulate the TNF-α production on LPS (lipopolysaccharide) stimulated mononuclear cell through NF-κB activation pathway (Grey *et al*., 1998). Compared with the experiments on mononuclear cells, there are few on endothelial cells which also play an important role in the sepsis. Using gene chip methods, Joyce *et al*.(2001) demonstrated TNF can lower the NF-κB activity on the APC pretreated human umbilical vein endothelial cells (HUVEC). Hooper *et al*.(1998) demonstrated APC can up-regulate IL-6 and IL-8 in HUVEC. Owing to inconsistent results, in the present study we have chosen to investigate the role of APC in LPS-induced inflammatory events in HUVEC.

MATERIALS AND METHODS

Cell culture and drug treatment

Endothelial cells were isolated from human umbilical cords obtained less than 8 h after delivery, essentially as described by Jaffe *et al*.(1973) with minor modifications. In brief, the umbilical vein was cannulated, perfused with 1×PBS and treated with 0.5 mg/ml collagenase A (Hyclone, America) for 15 min at 37 °C. Cells were centrifuged at 500 r/min for 8 min, and resuspended in complete medium containing M199 (Sigma, America), 20% FBS (Hyclone, America), L-glutamine 2 mmol/L, penicillin 50 IU/ml, streptomycin 50 μg/ml (Sigma, America), and ECGF 20 μg/ml (Roche, America). The resuspended cells were seeded in 25-cm^2 flasks precoated with 0.1% gelatin (Sigma, America) and then incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 24 h the medium was refed. Confluent cultures were subcultured at a ratio 1:3 by 1 ml trypsin-EDTA at 37 °C for 3 min.

All studies in HUVEC were performed with confluent cultures on the second passage. HUVEC was divided into the following groups: SFM control (serum free medium control group), PBS control (phosphate buffer solution control group), LPS group (LPS, *Escherichia coli* 055:B5; Sigma, America; with final concentration of 1 μg/ml), APC group (Sigma, America; with final concentration of 7 μg/ml), Pre-APC group (APC pretreatment for 30 min prior to LPS challenge), and Post-APC group (APC administration 30 min after LPS challenge). Twenty-four hours before stimulation cells were washed three times with Hank's balanced salt solution and then were fed with fresh SFM for further experiment.

Enzyme-linked immunosorbent assay (ELISA)

To investigate the protein levels of IL-6 and IL-8, supernatants of the above cultures were harvested at 0, 4, 8, 12 and 24 h after LPS challenge. Supernatants were centrifuged at 250 r/min and frozen at −70 °C for further analysis. IL-6 and IL-8 concentrations were detected by ELISA (Genzyme, America) according to the manufacturer's protocol. All experiments were performed in five different original HUVECs.

Preparations of RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

To determine the mRNA expression of IL-6 and IL-8, we performed reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted with Trizol reagent (Gibco BRL, America) according to the manufacturer's protocol.

For cDNA synthesis, 5 μg of total RNA was reverse-transcribed in 30 μl of reaction mixture containing 2.5 mmol/L each dNTP, 20 units of RNase inhibitor, 50 units of moloney murine leukemia virus reverse transcriptase, 6 μl 5× first strand buffer, 0.2 μl oligo(dT)15 (all the above reagents were purchased from Promega). The reaction mix was incubated at 37 °C for 1 h and then denatured at 70 °C for 15 min. For each sample, a control reaction not containing the reverse transcriptase enzyme was also performed.

Specific primers for IL-6 (forward: 5′-TgACA AACAAATTCggTACATCC-3′; reverse: 5′-ATCTg

AggTgCCCATgCTAC-3′), IL-8 (forward: 5′-TgCCA AggAgTgCTAAAg-3′; reverse: 5′-TCTCAgCCCTC TTCAAAA-3′) and GAPDH (forward: 5′-CgCTgAg TACgTggAg-3′; reverse: 5′-gAggAgTgggTgTCgC TgTT-3′) were designed from sequences in the GenBank database using Primer Oligo 6.0 Software. The gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as endogenous control to standardize the amount of RNA in each reaction. For each sample tested, PCR reaction was carried out in a 50-μl volume containing 2 μl of cDNA reaction, 2 μl 1 μmol/L of GAPDH primer, 2 μl 10 μmol/L IL-6 or IL-8 primer and 2.5 units of *Taq* DNA polymerase.

PCR was performed with the following amplification profile: 3 min at 94 °C, then 30 cycles (45 s denaturation at 94 °C; 45 s annealing at 56.5 °C for IL-6 and 52.2 °C for IL-8; 50 s extension time at 72 °C) followed by a final extension at 72 °C 10 min.

Fifteen microlitres of the reaction mix were electrophoresed in 1.5% agarose gel and amplified bands were visualized by ethidium bromide. PCR band intensities were determined by densitometry analysis with the UVP (ultraviolet product) image-store-7500 fluorescent system and molecular analyst software for quantification of images (Gene, British). Values corresponding to IL-6 or IL-8 amplification were normalized with those for GAPDH amplification.

Statistical analysis

All statistical analyses were performed with one way ANOVA test by SPSS 10.0 statistical software (SPSS, Chicago, IL).

RESULTS

Supernatants collected at different times were tested for IL-6 and IL-8 levels, as shown in Fig.1. Twenty-four hours after the challenge(s), RNA was extracted to study the change at the transcriptional level with PCR product band densitometry analysis. The cytokines' RNA expression can be increased by APC or LPS. Their levels were enhanced further when APC and LPS were added together. Fig.2 shows the electrophoresis results of the IL-6 and IL-8 respectively. Fig.3 shows the results of densitometry analysis of PCR fragments corresponding to IL-6 and IL-8 respectively.

In our experiment, both LPS and APC powder were dissolved in PBS. We set up the PBS control group to examine whether PBS can have any effect on the cytokine production. Our experiment showed there was no difference between the SFM control and the PBS control in the production of cytokine (P=0.81 for IL-6, P=0.89 for IL-8).

LPS alone increased both mRNA expression and supernatant content of IL-6 and IL-8, as is well known. Its level at 1 μg/ml was a strong stimulator of cytokine production in HUVEC as evidenced in our experiment. As determined by ELISA, significant increases in both IL-6 and IL-8 production were detected as early as 4 h after the addition of LPS, and the cytokine levels continued to increase through 24 h. The levels of cytokine rose with increasing time of exposure to LPS. There was statistical significance between the two groups for IL-8 (P=0.04).

APC, at concentration of 7 μg/ml can increase the cytokine levels with increasing exposure time. But there was no statistical significance between APC group and the controls (for IL-6, P=0.39 between SFM control and APC, P=0.27 between PBS control and APC; for IL-8, P=0.44 between SFM control and APC, P=0.37 between PBS control and APC). APC alone does not influence IL-6 and IL-8 mRNA expression (Fig.3). This is in contrast to the experiments of Hooper *et al*.(1998) and needs to be repeated in different doses of stimulator.

The combination of LPS and APC has an additive effect on both mRNA expression and protein levels in supernatant content of IL-6 and IL-8. There was statistical significance for IL-6 between the controls and Pre-APC group (P=0.02 and 0.01 for SFM control and PBS control respectively). IL-8 level was statistically significant between the controls and the Pre-APC/Post-APC (P=0.01 between SFM control and Pre-APC, P=0.01 between PBS control and Pre-APC, P=0.01 between SFM control and Post-APC, P=0.01 between PBS control and Post-APC). There was statistical significance for IL-8 between APC and Pre-APC group (P=0.04). This information is interesting, since it is unexpected from the clinical trial data (Bernard *et al*., 2001).

We added APC 30 min before or 30 min after the LPS addition to explore whether there was a time effect on the cytokine production. There was no

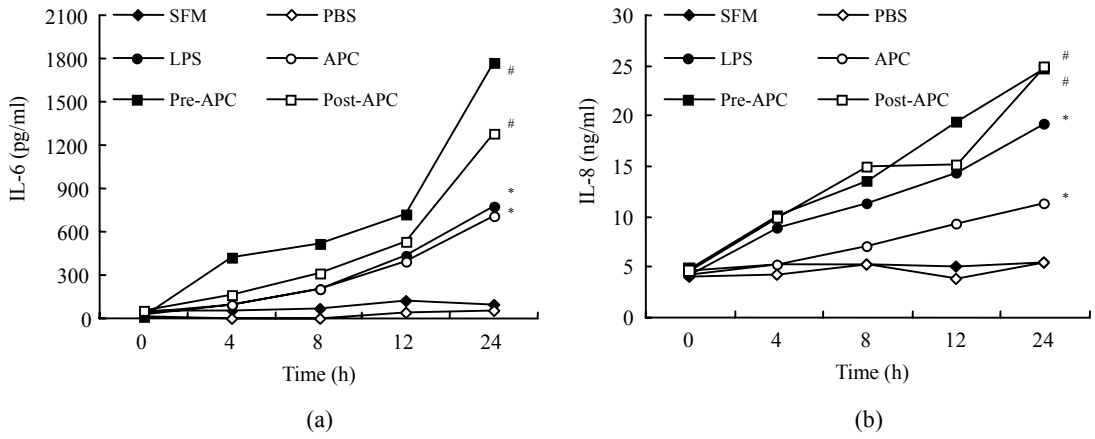

Fig.1 Effect of APC on the protein levels of IL-6 (a) and IL-8 (b) in LPS-treated HUVEC. HUVEC was stimulated by LPS (1 μg/ml) and/or APC (7 μg/ml), then supernatant was harvested and the protein levels of IL-6 (a) and IL-8 (b) were measured

(a) pg/ml: SFM: 82.46±8.56, PBS: 24.03±3.43, LPS: 312.88±8.26, APC: 289.01±7.31, Pre-APC: 689.55±56.26, ost-APC: 469.39±91.57 (n=5); (b) ng/ml: SFM: 5.07±0.45, PBS: 4.62±0.77, LPS: 11.62±0.26, APC: 7.58±0.84, Pre-APC: 14.57±0.80, Post-APC: 14.02±7.51 (n=5). * Versus control, P<0.05; # Versus LPS group, P<0.05

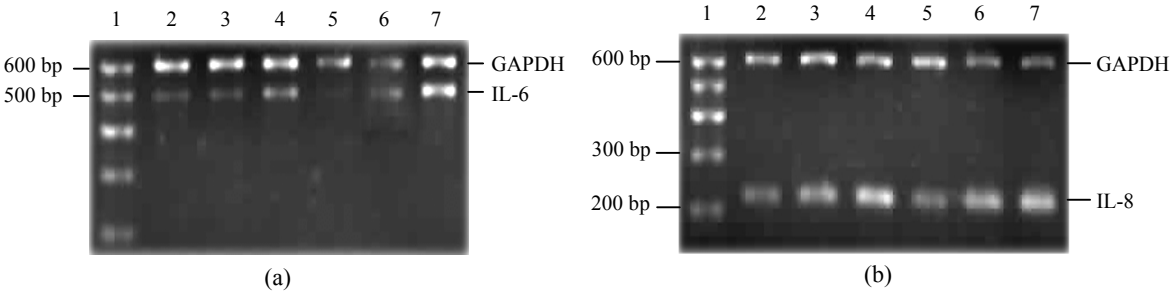

Fig.2 Electrophoretic analysis of effect of APC on the mRNA expression of IL-6 (a) and IL-8 (b) in LPS-treated HUVEC

GAPDH is used as endogenous control. 1: Marker; 2: SFM control; 3: PBS control; 4: LPS group; 5: APC group; 6: Pre-APC group; 7: Post-APC group

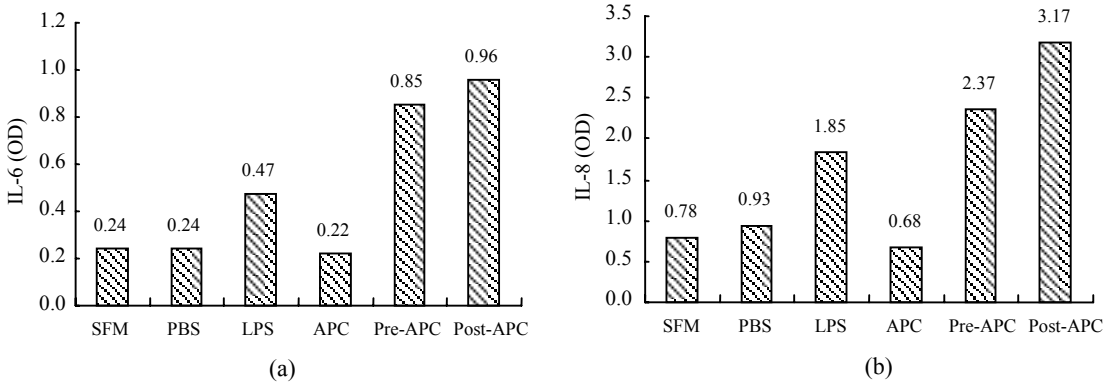

Fig.3 Effect of APC on the mRNA expression of IL-6 (a) and IL-8 (b) in LPS-treated HUVEC. The figures show the result of densitometry analysis that is the ratio of IL-6 (a) and IL-8 (b) PCR product to GAPDH PCR product. GAPDH is used as endogenous control

statistical significance between Pre-APC group and the Post-APC group (P=0.36 for IL-6 and P=0.87 for IL-8).

DISCUSSION

Sepsis was thought to be an imbalance between the inflammation and coagulation reactions. The protein C/protein S anticoagulant pathway has been proposed to serve as a link between inflammation and coagulation. Research on APC has become one of the focus areas. Although APC achieved satisfactory result in the clinical phase III trial, the molecular basis for the anti-inflammatory properties of APC has not yet been fully elucidated (Bernard *et al*., 2001).

The work reported here supports the concept that APC can be important in cytokine regulation. Our results demonstrate that (1) LPS as well as APC, with increasing time exposure, up-regulates the production of both IL-6 and IL-8 in HUVEC; (2) The increase was seen at both transcriptional and protein level; (3) APC enhanced LPS induced IL-6 and IL-8 productions.

LPS is an important part of the Gram-negative bacteria, which are used here to initiate the septic state. As many papers have reported, IL-6 and IL-8 were elevated after the LPS challenge. Though there are many researches on the APC role in sepsis, only one paper has focused on its pure effect on endothelium (Hooper *et al*., 1998). APC in our experiment was found to increase IL-6 and IL-8 in the supernatant of HUVEC culture. Though this is interesting, it does not represent a new finding as this had been reported previously. Why is there no change in the transcriptional levels? Maybe tests with different doses of APC are needed.

When HUVEC was challenged by APC and LPS successively, IL-6 and IL-8 were increased further. We added APC into the LPS challenged HUVEC at different time to exclude the time effect on the result and obtained a similar result. This observation is contrary to what we hypothesized from the clinical trial (Bernard *et al*., 2001). The cytokine level will decrease in all such cases after APC treatment. In vivo studies showed APC has anti-inflammatory effects. Using baboon models of sepsis, Taylor *et al*.(1987) demonstrated that APC results in a significant reduction in TNF-α and IL-6. APC can decrease the IL-6 level in severe sepsis patients (Bernard *et al*., 2001). The reasons for the difference between the in vivo and our in vitro can be explained by the presence of several other IL-6 and IL-8-producing cell types (such as monocytes, fibroblasts) in vivo compared with the only endothelial cells in vitro. Thus, the cytokine expressed by endothelium may not reflect its level in the body.

Some experiments show different cell model can give rise to diverse results, though they are challenged by the same stimulator. Grey *et al*.(1998) challenged endothelium and THP-1 monocyte with TNF-α. He found the former expresses less thrombomodular, while the latter expresses more. Our study result differed from that of the monocyte model. APC can inhibit the NF-κB nuclear translocation and TNF-α production induced by LPS in monocyte (White *et al*., 2000).

Even in similar model of human monocyte, different functions of APC on cytokine have been reported. Czeslick showed that even in monocyte model, APC cannot affect intracellular production of IL-6 and TNF-α. Conclusion is drawn that the possible anti-inflammatory activity of APC may not occur via the production of pro-inflammatory cytokines in human monocytes (Czeslick *et al*., 2005). The recently published result is contrary to that of White.

It seems that the exact mechanism of the APC benefit in human being is intricate. Long term work is needed to elucidate its role.

What do we make of the elevated cytokines?

Usually they are thought to maintain leukocyte trafficking and adherence at the site of injury, thereby amplifying the inflammatory response. Conversely under some circumstances, IL-6 and IL-8 can have anti-inflammatory functions. In vivo trials showed that IL-6 can inhibit the production of TNF-α and IL-1; decrease the number of neutrophils, increase the soluble TNF-α receptor; decrease the degradation of tissue matrix and inhibit cell apoptosis (Aderka *et al*., 1989; Tilg *et al*., 1994). Smith *et al*.(1993) used IL-8 to pretreat neutrophils, thus decreasing the IL-8 concentration gap between the inflammatory area and the cell with the result of attenuating the neutrophil migration.

Except in vitro studies, some in vivo trial demonstrated the anti-inflammatory function of both cy-

tokines. In the LPS inhalation mice model, Xing *et al*.(1998) found compared with the control group IL-6 gene knock-out mice have higher TNF-α, neutrophils and higher mortality. Hechtman *et al*.(1991) found intravenous infusion of IL-8 can decrease the number of neutrophils, while subcutaneous infusion increases its number. It suggests IL-8 have different function depending on the site of action. In view of the above results, IL-6 and IL-8 can play opposite roles at different sites. The effect of elevated IL-6 and IL-8 on the endothelium remains under investigation.

Besides APC, antithrombin is another important natural serine protease inhibitor, which has been shown to have anti-inflammatory properties in addition to their anticoagulation properties. But the clinical phase III trial on antithrombin showed no benefit in sepsis patients. The difference in the results obtained with the two endogenous anticoagulants, APC and antithrombin, has puzzled researchers (Warren *et al*., 2001). Using the same LPS stimulated HUVEC model, Souter *et al*.(2001) found antithrombin can inhibit the IL-6 production induced by LPS. The difference between the two in vitro experiments may lead us to explore the different clinical experiment results.

More recent trial raises doubts on the clinical usefulness of APC in patients with severe sepsis and an APACHE II score≥25 (Wiedermann and Kaneider, 2005; Abraham *et al*., 2005). All these controversies need our deeper work in the in vitro and in vivo study.

CONCLUSION

In summary, our results suggest that, in addition to the benefits reported by others, APC may also have an unknown effect on the host through the induction of IL-6 and IL-8. The exact consequences of the elevated cytokine remain under investigation. Our findings give us more evidence for the benefit of APC and the futility of antithrombin in the sepsis clinical phase III trial.

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