行政院國家科學委員會專題研究計畫 成果報告

香煙萃物在人類臍靜脈內皮細胞造成膜上 E-selectin 及 ICAM-1 表達增加之分子機制探討

研究成果報告(精簡版)

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執 行 單 位 ：中山醫學大學生物醫學科學學系

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中 華 民 國 96 年 10 月 31 日

計畫成果自評

本研究內容主要探討香煙萃物增加人類內皮細胞黏著因子(ICAM-1 及 E-selectin)在膜上表達之可能機制為主，主要發現香煙萃物會透過一 MAPK-非依賴訊號傳遞路徑，活化轉錄因子 AP-1，進而造成人類內皮細胞黏著因子在膜上的表達增加，另外，在此過程中細絲骨架的重建也扮演一重要的調節功能，研究成果對吸煙造成心血管病變提供一新的參考資料，適合發表於學術期刊，目前已完文稿撰寫準備投稿中。研究成內容與原計畫內容大致相同也也達到預期目標。

Figure 6

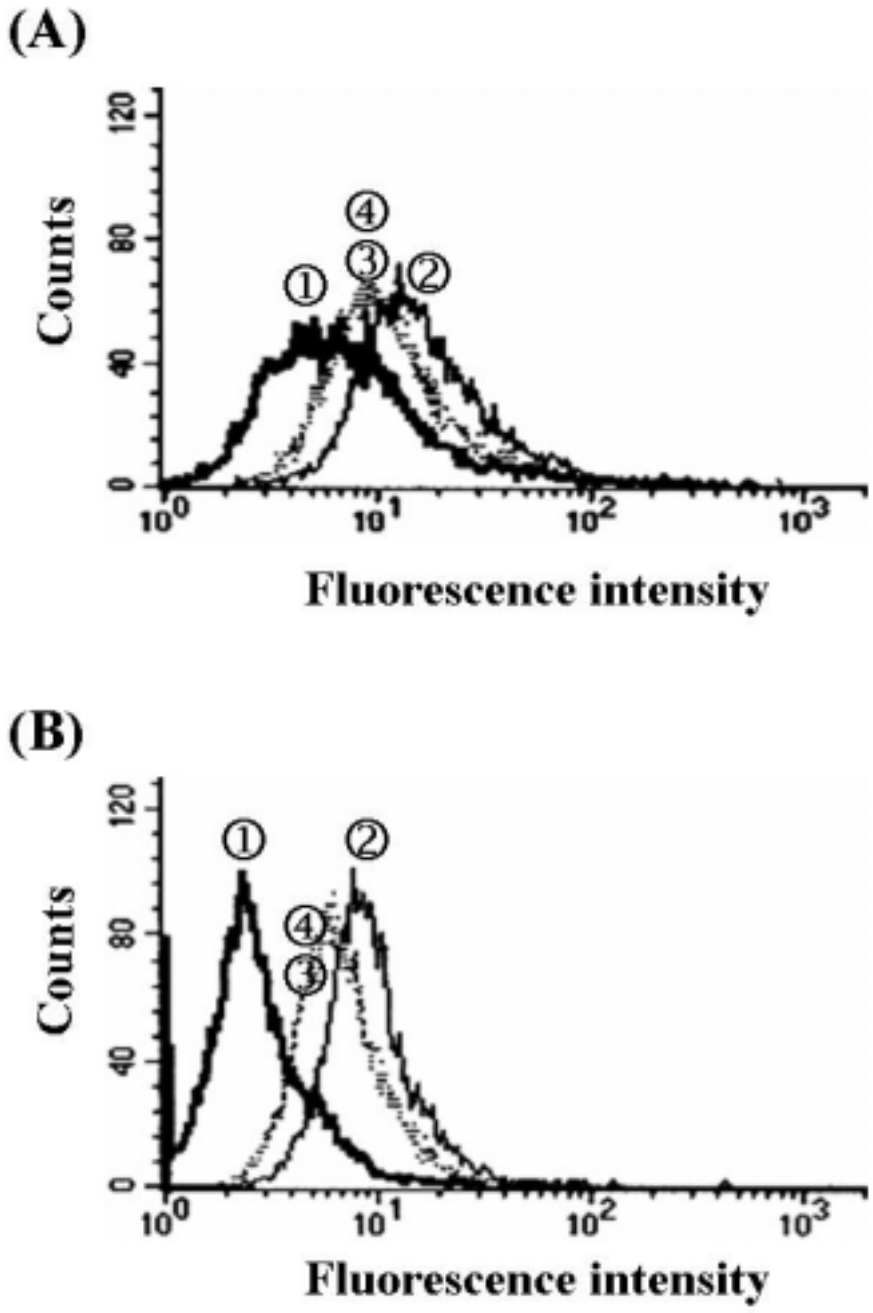
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Figure 5

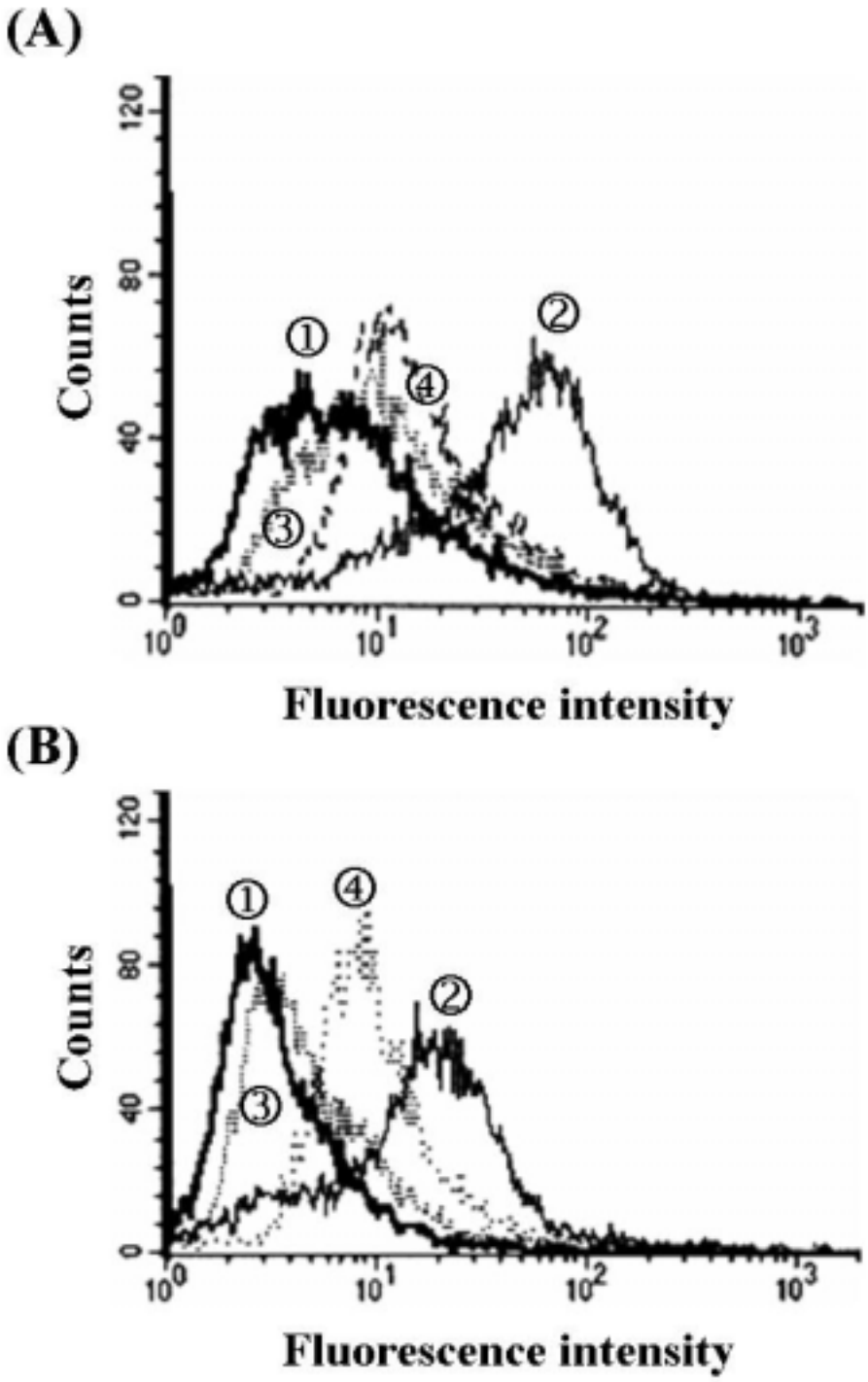
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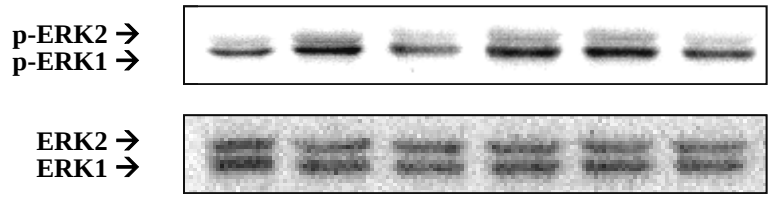
Figure 4

(A)

TNF-α (ng/ml)	**0**	**20**	**0**	**0**	**0**	**0**
CSE (%)	**0**	**0**	**1**	**1**	**1**	**1**
Time (min)	**0**	**15**	**5**	**15**	**30**	**60**



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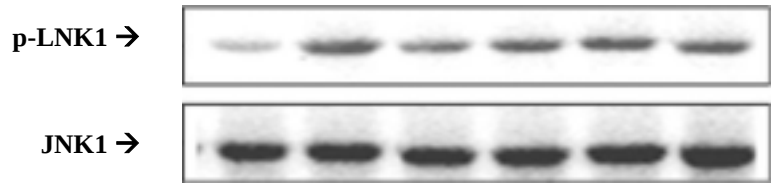

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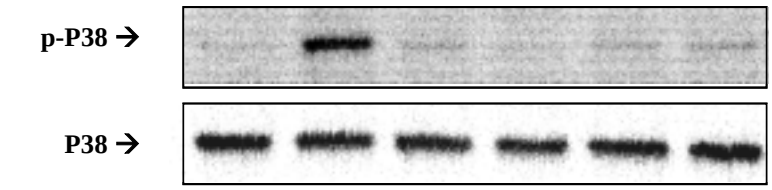


Figure 3

TNF-α (ng/ml)	**0**	**20**	**20**	**20**	**0**	**0**	**0**	**0**
Cold (ng)	**0**	**0**	**100**	**0**	**0**	**0**	**0**	**0**
Mutant (ng)	**0**	**0**	**0**	**100**	**0**	**0**	**0**	**0**
CSE (%)	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**
Time (min)	**0**	**30**	**0**	**0**	**5**	**15**	**30**	**60**

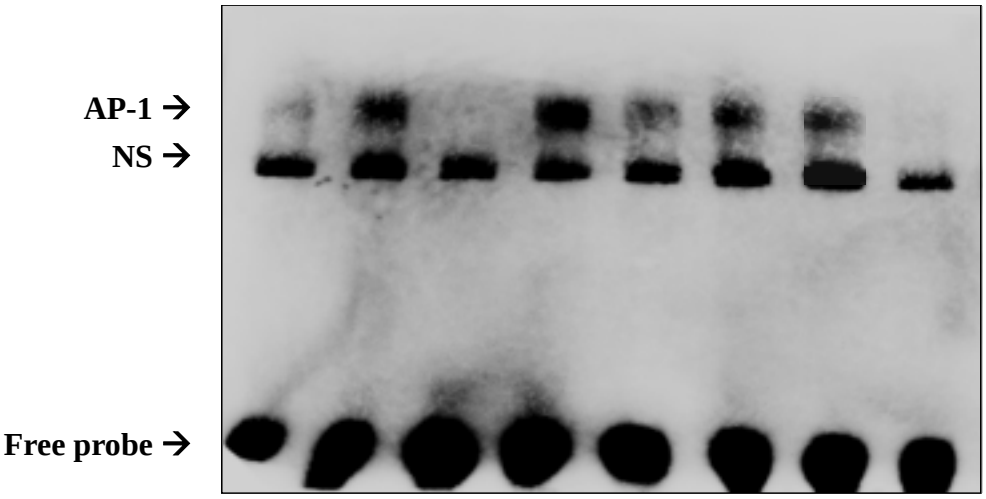
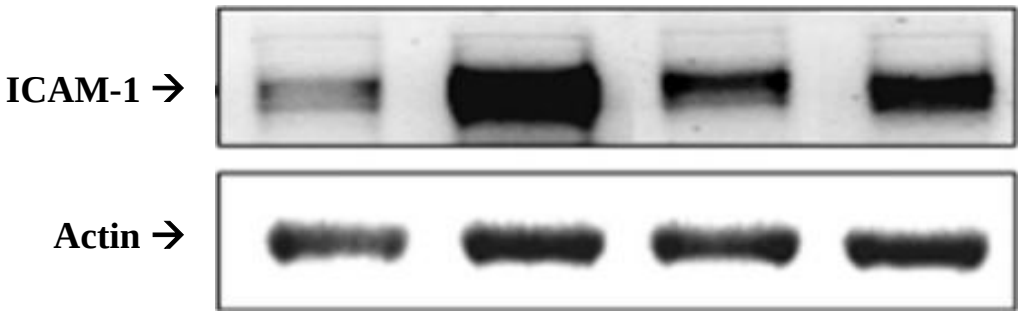


Figure 2

(A)

TNF-α (ng/ml)	**0**	**20**	**0**	**0**
CSE (%)	**0**	**0**	**1**	**5**
Time (hr)	**0**	**2**	**6**	**6**



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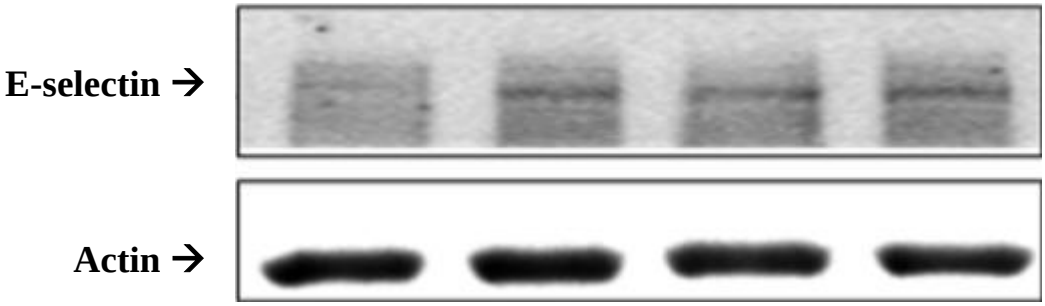
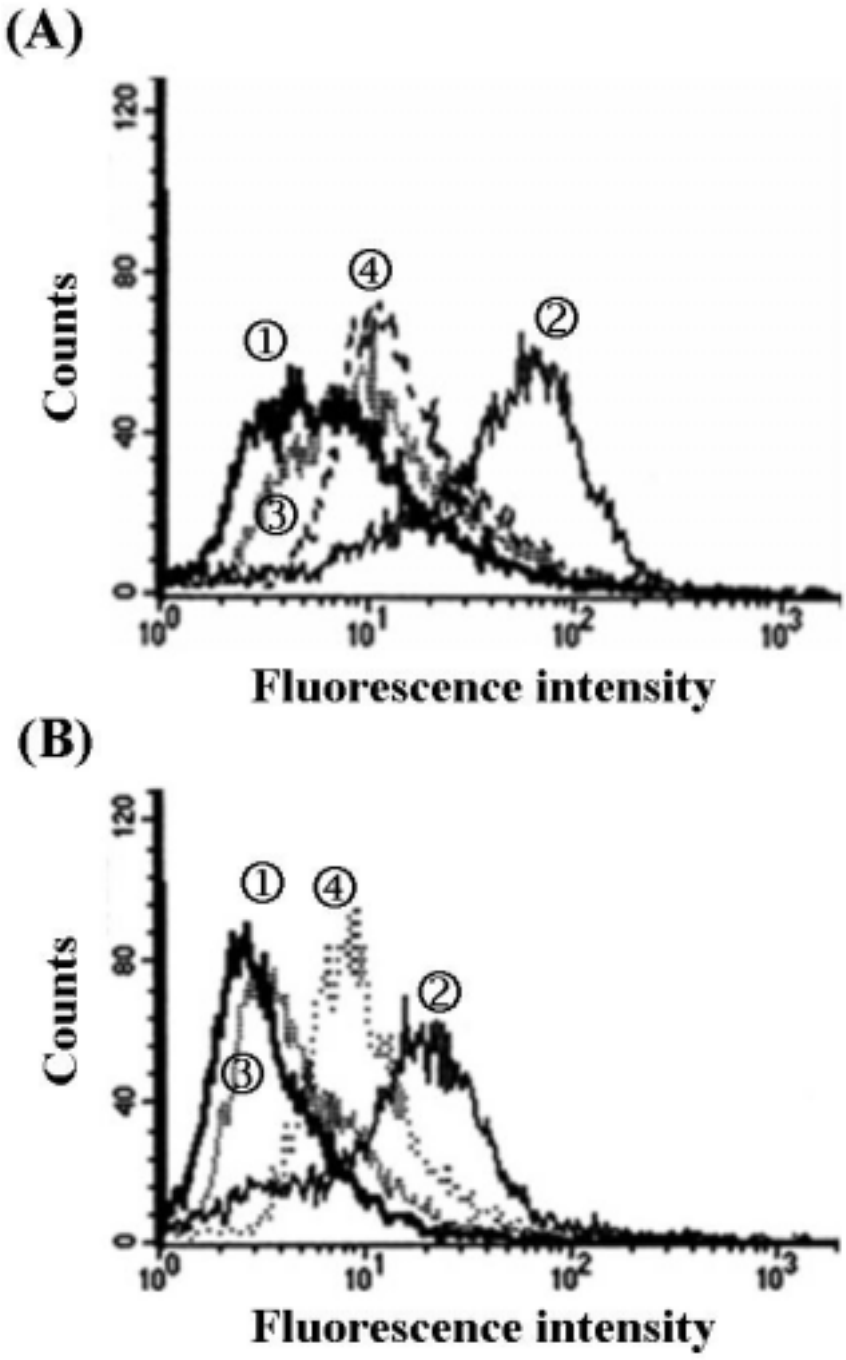


Figure 1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Fluorescence intensity
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Fluorescence intensity

determined by flow cytometry. Control ①; CSE 5% 6hr ②; cytochalasin D 50 nM plus CSE 5% 6hr③; cytochalasin D 100 nM plus CSE 5% 6 hr ④.

Table 1. Western blot analyses of VCAM-1 and ICAM-1 protein in CSE-treated HUVECs..

Treatment	Fold increase relative to untreated control	
	ICAM-1	E-selectin
Untreated	1^{a}	1^{a}
TNF-α (20 ng/ml), 2h	5.6 ± 0.2^{c}	3.3 ± 0.3^{b}
CSE (1%), 6h	2.9 ± 0.3^{b}	3.2 ± 0.6^{b}
CSE (5%), 6h	4.0 ± 0.3^{c}	3.2 ± 0.3^{b}

Values with the same alphabet along the same column are not significantly different from Fischer's protected LSD ($p < 0.05$). # positive control.

Figure Legends

Figure 1. Flow cytometry analysis of the effect of CSE on ICAM-1 and E-selectin expression in HUVECs. The HUVECs were treated with (1% or 5%) or without CSE for 6 hr. The treatment of 20 ng/ml TNF-α for 2 h was used as positive control. After treatment, the cells were collected and the surface expression level of ICAM-1 (A) and E-selectin (B) on HUVECs was determined by flow cytometry. ① control; ②TNF-α 20 ng/ml 2 hr; ③ CSE 1% 6 hr; ④ CSE 5% 6 hr.

Figure 2. Western blot analysis of the effect of CSE on ICAM-1 and E-selectin expression in HUVECs. The HUVECs were treated with (1% or 5%) or without CSE for 6 hr. The whole cells lysates were prepared and the expression level of ICAM-1 and E-selectin was determined by Western blot. The treatment of 20 ng/ml TNF-α for 2 h was used as positive control.

Figure 3. Time course response of ERK1/2 (A), JNK (B), and p38 (C) phosphorylation in HUVECs treated with CSE. Cells were exposed to 1% CSE for various amount of time (5-60 min). After treatment, the cells were lysed, and equivalent amounts of cell lysates were subjected to SDSPAGE followed by western blot analysis using antibodies against phospho-MAP kinases (upper panel) or MAP kinase (lower panel). Figures shown represent data obtained in three independent experiments. The treatment of 20 ng/ml TNF-α for 2 h was used as a positive control.

Figure 4. Densitometric analysis of ERK1/2 (A), JNK (B), and p38 (C) phosphorylation level in HUVECs treated with CSE. The results represent as the mean ± SE of the three distinct experiments.

Figure 5. Time course responses of AP-1 activation induced by CSE in HUVECs. Cells were treated for the indicated time with 1% CSE and nuclear extracts obtained as described in “Materials and Methods”, then AP-1 activation was measured by EMSA using a radiolabelled consensus probe for AP-1. The specificity of the shifted band was determined by comparison with a 50-fold excess unlabeled cold and radiolabeled mutant probes. TNF-α (20 ng/ml) treated HUVECs was used as a positive control. NS: non-specific band

Figure 6. The effect of cytochlasin D on CSE induced surface expression of ICAM-1(A) and E-selectin (B) in HUVECs. The HUVECs were pretreated with or without cytochalasin D (50 nM or 100 nM) for 30 min and then washed with PBS. Washed cells were incubated with 5% CSE for another 6 hr. After treatment, the cells were collected and the surface expression level of ICAM-1 (A) and E-selectin (B) on HUVECs were

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Interestingly, we used an actin filament disruptor, cytochalasin D 30-min pretreatment could partially reduce 5% CSE-induced up-regulation of ICAM-1 and E-selectin surface expression in HUVECs (Figure 5). However under same treatment condition, both ERK and JNK inhibitors could not reduce 5% CSE-induced up-regulation of ICAM-1 and E-selectin in HUVECs (data not shown). Previous report has been shown that actin networks inhibitors can attenuate TNF- -induced ICAM-1 surface expression but these compounds are ineffective in altering E-selectin surface expression in human aortic endothelial cells and epidermoid cells (Norgauer et al., 1995; VanderBerg et al., 2004). Taken together, these results suggest that actin reorganization play an important role at least in CSE-induced ICAM-1 surface expression in endothelial cells. The molecular mechanism(s) of how actin networks modulate stimuli-induced cell adhesion molecules expression in endothelial cells is still unclear. The possible mechanisms could be explained by actin network that may directly affect the activation of transcription factor required for transcription control of ICAM-1 and E-selectin regulation. A regulatory role of actin cytoskeleton in transcription and cell surface expression of ICAM-1 has been found (Norgauer et al., 1995; VandenBerg et al., 2004). Recent evidence confirms that actin cytoskeleton engaged a stimulus-specific manner to facilitate RelA/p65 nuclear import and thereby ICAM-1 expression in endothelial cells (Fazal et al., 2007). However, it is unlikely that actin cytoskeleton-dependent facilitates RelA/p65 nuclear import to play in this study, because CSE exposure did not activate NF-κB in HUVECS. On the contrary, a known AP-1 co-activator, Jab1 is a possible candidate for CSE exposure to activate AP-1 in HUVECs through a MAPK-independent pathway in this study. Jab1 can be tyrosine phosphorylated by Src, and this event allows JAB1 translocation to the nucleus, where it can stabilize c-Jun and increase AP-1 activity, leading to an increase in gene transcription of specific genes (Claret et al., 1996). Although the relationship between cytoskeleton and Jab1 nuclear translocation is not been establish, the cytoskeleton may play a pivotal role in its nuclear translocation. In fact, the role of the cytoskeleton in regulating the intracellular localization of cellular organelles, viruses, and individual proteins has been the subject of much investigation in recent years (Campbell & Hope, 2003).

Besides of actin-cytoskeleton, microtubule-cytoskeleton has been shown to modulate stimuli-induced ICAM-1 and E-selectin expression in endothelial and non-endothelial cells (Kaatz et al., 2006; Cuschieri et al., 2003; Cronstein et al., 1995). Our and others' findings implies that actin and/or microtubule cytoskeleton reorganization might be a key mediator for cigarette smoke extract-induced up-regulation of ICAM-1 and E-selectin surface expression. Previous studies show that cigarette smoke exposure induced alternations in fibroblasts and endothelial cells cytoskeleton (Bernhard et al., 2005; Poggi et al., 2002; Rota et al., 2001) ,and the evidence of cigarette smoke metal-catalyzed protein oxidation leads to vascular endothelial cell contraction by de-polymerization of microtubules (Bernhard et al., 2005) strongly support the above postulation. Taken together, we propose a novel working hypothesis for CSE up-regulate ICAM-1 and E-selectin in HUVECs that includes four sequential steps: (1) CSE-induced cytoskeleton protein oxidation, (2) trigger Jab1 nuclear translocation, (3) activate AP-1 phosphorylation, (4) up-regulate ICAM-1 & E-selectin expression. Therefore, the further studies are necessary to clarify the modulation role of both actin and microtubule cytoskeleton reorganization on CSE-induced ICAM-1 and E-selectin up-regulation in HUVECs.

expression of E-selectin (Westra et al., 2005). (4) Stimulation of endothelial cells with TNF- -induced rapid increases in the phosphorylation of their MAPKs; the inhibitor for JNK (SP600125), but not those for ERK (PD98059) and p38 MAPK (SB203580), attenuated this TNF- -induced E-selectin expression. Surprisingly, pre-treatment with MAPKs specific inhibitors (SP600125 and PD98059) did not decrease CSE-induced up-regulation of ICAM-1 and E-selectin surface expression on HUVECs in this study. In fact, even co-treatment with specific MAPK inhibitors did not reduce CSE-induced surface expression of ICAM-1 and E-selectin in HUVECs (data not shown). These inconsistent responses suggest that MAPKs (ERKs and JNK) phosphorylation activation may not mediate CSE-induced surface expression of ICAM-1 and E-selectin in HUVECs. A recent report has shown that ERK, JNK and JNK activation did not involve in TNF-α-stimulated adhesion molecules ICAM-1 and VCAM-1 expression in endothelial cells (Zhou et al., 2007), which supports our findings in this study. Our finding in this study and other reports also suggest a possible role of MAPKs involving in adhesion molecules expression that may vary depending on the nature of stimulis and/or cell types. These results also indicate that nicotine may not be the component in CSE to induced ICAM-1 and E-selectin in HUVECs. Furthermore, our findings also suggest that nicotine may not be the major component mediated CSE-induced up-regulation of ICAM-1 and E-selectin surface expression in HUVECs.

The regulatory region of the ICAM-1 and E-selectin gene has binding sequences for AP-1 and NFκB (Stade et al., 1990; Voraberger et al., 1991; Schindler et al., 1994; Jensen & Whitehead, 2003). In this study, we found that CSE treatment significantly enhanced DNA binding activity of AP-1 rather than NFκB. This finding suggested that CSE-induced up-regulation of ICAM-1 and E-selectin surface expression may result from an AP-1 dependent and NFκB -independent pathway. Recent report of Verna et al. (2006) shows that *in vivo* LDL-induced ICAM-1 and VCAM-1 surface expression is correlated with AP-1 but not with NF- B that supports our above finding. Nevertheless, whether AP-1 activation acts exclusively on the CSE-induced up-regulation of ICAM-1 and E-selectin surface expression in HUVECs needs further study. To examine the effect of AP-1 inhibitor on the up-regulation of CSE-induced surface expression of ICAM-1 and E-selectin is undergoing on our laboratory.

In addition to the activation of AP-1 results in up-regulation of ICAM-1 expression in endothelial cells, the report of Gorgoulis et al. (2003) has shown that p53 directly activates the expression of ICAM-1 in a NF-κ B-independent manner. They also identified two p53 functional responsible elements within the intronic sequences of ICAM-1 gene. Our previous report showed that CSE could induce DNA damage and may activate p53 to directly up-regulate ICAM-1 surface expression in HUVECs. However pretreatment with antioxidants completely inhibited the CSE-induced DNA damage but not the CSE-induced up-regulation of ICAM-1 and E-selectin surface expression in HUVECs (Chen et al., 2004). And these findings did not support the possibility of CSE induced a p53-dependent up-regulation of ICAM-1 and E-selectin surface expression in HUVECs. In addition, there is no evidence that the regulatory region of human E-selectin gene has any possible p53 responsible element.

Cytoskeleton and CSE-induced ICAM-1 and E-selectin expression

Discussion

The findings of this study demonstrate that CSE significantly induced E-selectin and ICAM-1 surface expression and total expression, but not VCAM-1 in HUVECs. Western blot analysis showed that CSE phosphorylated ERKs and JNK, however, neither ERKs nor JNK inhibitor 30 min pre-treatments affected surface expression of ICAM-1 and E-selectin in HUVECs. On the contrary, pretreatment with cytoskeleton inhibitor cytochalasin D could partially suppress CSE-induced surface expression of ICAM-1 and E-selectin. These results indicate that CSE-induced surface expression of ICAM-1 and E-selectin is at least partially controlled by actin cytoskeleton reorganization.

CSE and endothelial cells activation

Activation of endothelial cells by pro-inflammatory stimulis results in increased migration of leukocytes across the endothelium, which contributes to the progression of atherosclerosis. Cigarette smoke is a major risk factor for many human diseases that also include atherosclerosis, although the underlying mechanisms are not clearly understood. Cigarette smoke up-regulated the expression of E-selectin, ICAM-1, and VCAM-1 is a possible mechanism. Our previous and this study showed that aqueous cigarette smoke extract could up-regulate the surface expression of ICAM-1 and E-selection on HUVECs (Chen et al., 2004). Besides aqueous cigarette smoke extract, the cigarette smoke condensate, the particulate fraction of cigarette smoke also induced ICAM-1 and E-selectin surface expression on HUVECs (Shen et al., 1996; Kalra et al., 1994). Our recent study also found that gas fraction was stronger inducer of cigarette smoke than those of the particulate fraction of cigarette smoke (data not shown). These results suggest that both gas and particulate phase of cigarette smoke extracts could significantly induce cell adhesion molecules at least ICAM-1 and E-selectin surface expression on endothelial cells, and this up-regulation may link to cigarette smoke-induced cardiovascular diseases.

MAPKs activation and CSE-induced cell adhesion molecules expression

The activation of signal cascade of MAPKs is important to the stress-induced genes expression, including cigarette smoke, lipopolysaccharide, and TNF- (Rao, 2001; Mossman et al., 2006). These stimuli could up-regulate E-selectin, ICAM-1, or VCAM-1 expression and enhance leukocyte adhesion with activated endothelial cells (Zhang et al., 2006; Chen et al., 2004; Sakai, 1996; Lehr, 1993). Several evidence show that MAPKs specific inhibitor could block the up-regulation of E-selectin, ICAM-1 or VCAM-1 on endothelial cells under different stimulis. They include (1) Treatment of endothelial cells with the JNK-specific inhibitors (SP600125 or JNK inhibitory peptide 1) resulted in a significant decrease in thrombin-induced ICAM-1 expression as demonstrated by Western blot analysis (Miho et al., 2005), (2) Treatment of HUVECs with the ERKs specific inhibitor (PD098059) and p38 inhibitor (SB203580) could inhibit the nicotine-induced surface and soluble VCAM-1, E-selectin up-regulation (Wang et al., 2006). (3) Pretreatment of endothelial cells with the p38 MAPK inhibitor (RWJ 67657) reduced TNF-α and IL-1βinduced mRNA and membrane

likely to modify AP-1 activity. To examine whether the presence of CSE alter AP-1 DNA binding activity, we investigated the binding activity of AP-1 in CSE-treated HUVECs by EMSA (electro-mobility shift assay). A significant increase in AP-1 DNA binding activity was detected in nuclear extracts of 5 to 30 min 1% CSE-treated HUVECs, but the activation of AP-1 DNA binding activity diminished completely after 60 min (Figure 4). The positive control of 20 ng/ml TNF-α also showed a significant increase in the AP-1 DNA binding activity after 30 min exposure (Figure 4).

MAPKs activation and CSE-induced ICAM-1 and E-selectin expression

To elucidate the possible involvement of ERKs and JNK activation pathways in CSE-induced surface expression of ICAM-1 and E-selectin, we examined the effects of MAPK specific inhibitors on CSE-induced ICAM-1 and E-selectin expression in HUVECs by flow cytometry. Although pretreatment with 20M ERK inhibitor PD098059 or JNK inhibitor SP600125 for 30 min could significantly reduce CSE-induced phosphorylation of ERKs and JNK, both MAPKs specific inhibitors were not attenuate 5% CSE-induced surface expression of E-selectin and ICAM-1 in HUVECs (data not shown). In contrast to MAPKs specific inhibitors, pretreatment of 50 nm cytochalasin D (a actin polymerization inhibitor) for 30 min and washed with PBS before 5% CSE was added could partially inhibit 5% CSE-induced surface expression of E-selectin and ICAM-1 in HUVECs (Figure 5). The inhibition percentages of cytochalasin D on CSE-induced surface expression of E-selectin and ICAM-1 were 48.2% and 35.8%, respectively. Furthermore, a higher dose of cytochalasin D (100 nm) could not enhance the inhibition extent of CSE-induced surface expression of E-selectin and ICAM-1.

Results

CSE Induced Expression of Cell Adhesion Molecules

Western blotting and flow cytometry analysis were used to investigate the effect of CSE on total and surface ICAM-1, E-selectin protein expression in human umbilical vein endothelial cells (HUVECs), respectively. The total ICAM-1 protein expression was increased with a dose-dependent manner, and the fold induction values of 1% and 5% CSE treatment were 2.9 $\pm$ 0.3 and 4.0 $\pm$ 0.3 (Figure 1A and table1), respectively. Induction of total expression of E-selectin was also significantly increased at CSE treatment and not dose-dependent in contrast to the effect on the total ICAM-1 protein expression. The fold induction values of 1% and 5% CSE treatment in HUVECS were 3.2 $\pm$ 0.6 and 3.2 $\pm$ 0.3 (Figure 1B and table1). A positive control TNF-α-stimulated HUVECs showed a 5.6- and 3.3-fold induction of the total ICAM-1 and E-selectin protein expression after a 2 h 20 ng/ml treatment as compared to the untreated control. In this study, neither CSE nor TNF-α significantly induced the total VCAM-1 protein expression in HUVECs (data not shown). The results of CSE-induced ICAM-1 and E-selectin expression in HUVECs analyzed by flow cytometry were consistent with those obtained by Western blotting (Figure 2). These results showed that both total protein expression and surface expression of ICAM-1 and E-selectin were upregulated in HUVECs by CSE exposure.

CSE Activates MAPKs

Since MAP kinases, including ERK, p38, and JNK, are activated in response to a variety of extracellular stimulis, we investigated whether CSE stimulated the activation of MAPKs by Western blotting using anti-phospho-ERK, -p38, and –JNK antibodies in HUVECs. The results showed that CSE at the concentration of 1% was able to increase the phosphorylation levels of ERKs and JNK at different time points within 60 min (Figure 3 and Table 2). Treatments with 1% CSE, ERKs and JNK phosphorylation reach a maximum at 30 min and the induction levels were 2.4- and 3.4-fold, respectively. At 60 min of 1% CSE stimulation, the ERKs phosphorylation was decreased significantly, but the JNK phosphorylation was remained elevated. In contrast to ERKs and JNK, 1% CSE stimulation increased only slightly in the p38 phosphorylation within 60 min with a dose-independent manner. The positive control TNF-α could significantly stimulate the ERKs, JNK and p38 phosphorylation. In addition, CSE-induced ERKs and JNK phosphorylation could be significantly prevented by their specific inhibitors PD98059 and SP600125, respectively. The inhibitory percentage of 30 min pre-treatment of 20M inhibitor on 1% CSE-induced ERK1/2 and JNK phosphorylation was about 100% and 67%, respectively (data not shown).

CSE Activates AP-1 DNA binding activity

The level and the activity of AP-1 complexes are regulated in response to a lot of extracellular stimuli. An important role in this regulation is controlled by MAPKs (Karin, 1996). CSE-activated ERKs and JNK were

even loading, membranes were stripped and probed with an actin antibody (Sigma, St. Louis, MO). Blots were quantitated by densitometry and normalized using the actin signal to correct differences in loading of the proteins from the control and experimental groups. For the densitometric analysis, the protein bands on the blot were measured by Alpha Innotech Digital Imaging System (San Leandro, CA).

Statistical Analysis

The data is expressed as the mean ± S.E. from at least three independent experiments. Standard error bars were included for all data points. However, in some cases they were smaller than the symbols used and cannot be seen. Statistical differences between test and control values were analyzed by means of Student's two-tailed unpaired t-test. Data were considered significant and indicated by "*" if $P < 0.05$ versus control.

0.5% NP-40). The cells were held on ice for 15 min, then the nuclear fraction was precipitated by centrifugation at 6000 × *g* for 15 min at 4 °C. The nuclei pellet was washed once with hypotonic buffer and lysed with 50 μl high salt buffer (10 mM Hepes, pH 7.9, 1 mM $MgCl_2$, 10% glycerol, 400 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 4 mg/ml leupeptin, 20 mg/ml aprotinin), and then incubated under continuous shaking, at 4 °C, for 30 min. The sample was centrifuged at 10,000 × *g* for 30 min at 4 °C. The nuclear extract was aliquoted and stored at −80 °C. Total nuclear protein was determined by Coomassie plus protein assay reagent kit (Pierce, Rockford, IL).

ICAM-1 and E-selectin Expression Assays

Total expression and cell-surface expression of intercellular adhesion molecules-1 (ICAM-1) and E-selectin were determined by Western blot assay and flow cytometry, respectively in HUVECs. After treatment, the cells were harvested, homogenized in lysis buffer, and centrifuged. The whole cell lysate samples were separated by 10% SDS-PAGE, transferred to PVDF membrane, incubated with anti-ICAM-1, anti-E-selectin antibodies (Santa Cruz, CA) and HRP-conjugated secondary antibodies. The results were visualized using the ECL kit (Perkin-Elmer Life Science, Boston, MA). The surface expression of endothelial cell adhesion molecules of E-selectin and ICAM-1 was measured by flow cytometry as our previous method (Chen et al., 2004).

Electromobility gel shift assay (EMSA)

EMSA was performed according to our previous study (Tsai et al., 2007). The LightShift Chemiluminescent EMSA Kit from Pierce Chemical Co. and synthetic biotin-labeled double-stranded AP-1 consensus oligonucleotides (5'-biotin-TTAAGGCTGAGTCATCAAGCG-3') were used to measure the effect of CSE on AP-1-DNA binding activity. Nuclear extract (5 μg), poly(dI-dC), and biotin-labeled double-stranded AP-1 oligonucleotides were mixed with the binding buffer (to a final volume of 20 μl) and were incubated at room temperature for 30 min. The nuclear protein-DNA complex was separated by electrophoresis on a 6% TBE-polyacrylamide gel electrophoresis and then was electrotransferred to Hybond-N^+ membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Next, the membrane was treated with streptavidin-horseradish peroxidase and the nuclear protein-DNA bands were developed using the Amersham ECL kits.

Detection of MAPK phosphorylation in the whole cell lysates

Whole cell lysate proteins were subjected to 10% SDS-PAGE and transferred to a PVDF membrane (Amersham, Arlington Heights, IL). After blocking with non-fat dry milk (5% w/v), the membrane was incubated with antibodies specific for total ERK, p-ERK, p38, p-p38 (Cell Signaling, Beverly, MA), JNK, and p-JNK (Santa Cruz Biotechnology) to detect the total and phosphorylated forms of MAPKs. To confirm

Materials and Methods

Chemicals

Gelatin, heparin, endothelial cell growth supplement, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), sodium salt of L-ascorbic acid, α-tocopherol succinate (from natural α-tocopherol), and 1,10-phenanthroline were obtained from Sigma–Aldrich (St. Louis, MO). Mouse fluorescein isothiocyanate (FITC)-conjugated antibody to human CD54 (ICAM-1) and human CD62E (E-selectin) were purchased from Serotec Ltd. (Oxford, UK).

Cell Culture

Human umbilical vein cells (CC-2157) were purchased from Clonetics (Walkersville, MD). The cells were cultured in M199 medium supplemented with 20% fetal calf serum, 2mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mg/ml heparin and 50_g/ml endothelial cell growth factor in gelatin coated 10 cm tissue culture plates at 37^0C and 5% CO_2 incubator. The cells were used in passages 3 and 4. Cell viability was determined by MTT staining.

Cigarette Smoke Extract Preparation

A none-fractionated cigarette smoke extract (NFWS-CSE) was prepared according to the procedures described by Su et al. (1998) and our previous method (Chen et al., 2004).

Whole cell lysate preparation

Cells were washed with PBS to remove residual media. Add 150 μl of 1x lysis buffer (10 mM Tris-HCl, 50 mM EDTA, 0.2 mM PMSF, and 20ug/ml apotinin) and scrape the adherent cells off of the plate. Then the mixture of cells were homogenized by sonication on ice bath for 20 seconds. After centrifugation (30 min, 4°C, 10,000x*g*), the supernatants were collected as whole cell lysate. Protein concentration in the whole cell lysates was determined by Coomassie plus protein assay reagent kit (Pierce, Rockford, IL).

Nuclear Extracts Preparation

Nuclear extracts were obtained using a modification of a previously described method (Ito et al., 2004). Briefly, the cell pellet was washed once in 1 ml phosphate-buffered saline (0.01 M, pH 7.4) and carefully resuspended in 400 μl hypotonic buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 1 mM EDTA, 1 mM MgCl2, 0.5 mM dithithreitol, 0.52 mM phenylmethylsulfonyl fluoride, 4 g/ml leupeptin, 20 g/ml aprotinin, and

Introduction

Cigarette smoking has been a major risk factor for cardiovascular disease (Bernhard & Wang, 2007; Leone, 2007). Even low-tar cigarettes and smokeless tobacco have been shown to increase the risk of cardiovascular events to smokers comparing to nonsmokers (Negri et al., 1993; Bolinder et al., 1994; Arabi, 2006). Furthermore, recent experimental data provide a deeper insight into the patho-physiological mechanisms linking second and smoke/passive smoking cardiovascular disease (Yuan et al., 2007; Raupach et al., 2006). It has became increasingly evident that inflammatory endothelial cells play a major role in the progression of atherosclerosis by their capacity to attract, bind and allow the extravasation of monocytes to sites of inflammation. (Martin et al., 2007; Tesfamariam & DeFelice, 2007). A large body of research shows that cigarette smoking can result in both morphological and biochemical disturbances to the endothelium in vivo and in cell culture systems (Kim et al., 2005; Mullick et al., 2002; Poredos et al., 1999). Thus, the evidence linking cigarette smoke exposure to cardiovascular disease is clearly present, yet the exact components of cigarette smoke and the mechanisms responsible for this association have not been clearly demonstrated.

The adhesion of leukocytes to the vascular endothelium, mediated by the endothelial cell adhesion molecules including vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, is the pivotal early event in atherogenesis. Our previous data has shown that cigarette smoke extract up-regulates E-selectin and ICAM-1 surface expression in human umbilical vein endothelial cells (Chen et al., 2004). Recent report has shown that ERK1/2 and p38 phosphorylation mediated nicotine-induced surface/soluble VCAM-1 and E-selectin expression in HUVECs. Although nicotine is the main activate component of cigarette smoke, it is still no fully described that whether cigarette smoke extract induced surface expression of adhesion molecules MAPK phosphorylation plays role directly in this signal transduction process. Therefore, our study was designed to investigate whether MAPK activation participates in the regulation of cigarette smoke extract-induced up-regulation of adhesion molecules expression in HUVECs. We also examined the possible involvement of actin fiber reorganization as a trigger mechanism for cigarette smoke extracts-induced up-regulation of adhesion molecules expression in HUVECs.

附件一

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

香煙萃物在人類臍靜脈內皮細胞造成膜上 E-selectin 及 ICAM-1 表達增加之分子機制探討

計畫類別：■ 個別型計畫　　□ 整合型計畫
計畫編號：NSC－95－2314－B－040－034
執行期間：095 年 08 月 01 日至 096 年 07 月 31 日

計畫主持人：王祖興
共同主持人：
計畫參與人員：蘇磁慧、張永聰、陳郁婷

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：中山醫學大學、生物醫學科學學系