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Soluble products from *Eikenella corrodens* induce cell proliferation and expression of interleukin-8 and adhesion molecules in endothelial cells via mitogen-activated protein kinase pathways

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Yumoto H, Yamada M, Shinohara C, Nakae H, Takahashi K, Azakami H, Ebisu S, Matsuo T. Soluble products from Eikenella corrodens *induce cell proliferation and expression of interleukin-8 and adhesion molecules in endothelial cells via mitogen-activated protein kinase pathways.*
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The periodontal vasculature is profoundly affected during the progression of periodontitis, and several specific bacteria are believed to be involved in this inflammatory disease. *Eikenella corrodens* is one of the common bacteria detected in periodontitis diseased lesions; however, the function of this organism in periodontitis is not well understood. In this study, we investigated the *E. corrodens*-induced endothelial cell alteration and inflammation process that leads to leukocyte infiltration in inflamed regions. Soluble products from *E. corrodens* (EcSP) induced the gene expression and protein production of vascular endothelial growth factor in oral epithelial cells and human umbilical vein endothelial cells (HUVEC). Direct stimulation by EcSP also activated endothelial cell proliferation. Moreover, EcSP induced ERK1/2 (p44/42) and p38 mitogen-activated protein kinase (MAPK) phosphorylation within 10–30 min in HUVEC, as demonstrated by Western blot analysis and up-regulated intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin and interleukin-8 (IL-8) production demonstrated by reverse transcription–polymerase chain reaction and enzyme-linked immunosorbent assay. The specific p38 MAPK inhibitor SB203580 reduced the expression of ICAM-1, VCAM-1 and IL-8, whereas the blockade of p44/42 by MAPK kinase (MEK1) inhibitor, PD98059, inhibited only IL-8 expression. Our results indicate that *E. corrodens* can trigger a cascade of events that induce inflammatory responses in periodontal tissue via the MAPK cascade and may promote chronic periodontitis without bacteria–cell contact.

Key words: cellular adhesion molecule; *Eikenella corrodens*; endothelial cells; interleukin-8; signal pathway; vascular endothelial growth factor

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Periodontitis is an inflammatory response in gingival and connective tissue elicited by bacterial colonization in periodontal pockets, and this inflammatory disease is accompanied by heavy neovascularization, swelling and edema. The periodontal vasculature is profoundly affected during the progression of periodontal diseases (17). Early in this progression, the perivascular connective tissues become disrupted, creating spaces within the tissues that are quickly filled by inflammatory cells and loose connective tissue. Capillaries then proliferate into the spaces created in the loose connective tissue by a process termed angiogenesis. Angiogenesis can also contribute to the severity of the inflammation as a result of the ability of new blood vessels to transport pro-inflammatory cells to the lesion. Vascular endothelial growth factor (VEGF) is a multifunctional angiogenic cytokine of importance in inflammation and wound healing. It stimulates endothelial cell proliferation, secretion of proteolytic enzymes, chemotaxis and migration, all of which are necessary for angiogenesis to proceed (14). It was reported that VEGF concentrations are higher within diseased gingiva than normal gingiva and VEGF is detected within vascular endothelial cells, neutrophils, plasma cells and junctional, pocket and gingival epithelia (5, 17). Another recent study suggested that VEGF may be associated with the etiology of periodontitis in its early stages according to neovascularization stimulated by periodontal pathogens causing swelling and edema (35).

Furthermore, the adhesion of circulating leukocytes and monocytes/macrophages to endothelial cells is an early step in inflammatory reactions. Multiple receptor–ligand pairs, including intercellular adhesion molecule 1 (ICAM-1) and integrins β_2 [cluster of differentiation (CD) 11a/CD18, CD11b/CD18], vascular cell adhesion molecule 1 (VCAM-1) and very late antigen-4 (VLA4), E-selectin and E-selectin ligand-1, as well as P-selectin and P-selectin-glycoprotein ligand-1 (9, 23, 26, 38), act sequentially and in an overlapping manner to effect the initial attachment, rolling, firm adhesion, and finally transmigration of leukocytes (1, 8, 9).

In addition, mitogen-activated protein kinases (MAPK) are key elements in the regulation of cellular responses to external inflammatory and proliferative signals and can be activated by a wide variety of extracellular stimuli and transmit signals from the cell surface to the nucleus to regulate gene expression. Three main groups of MAPK have been characterized to date: extracellular signal-regulated kinases (ERK1/2, p44/42), c-jun N-terminal kinase (JNK) and p38 MAPK (15, 20, 21). These MAPK subfamilies from three parallel cascades can be activated simultaneously or independently.

We previously reported that *Eikenella corrodens*, a facultative gram-negative anaerobic rod and periodontitis-related bacterium, induces oral epithelial cells to express various inflammatory mediators [interleukin-6 (IL-6), IL-8, tumor necrosis factor-α (TNF-α), prostaglandin E2, cyclooxygenase-2] and the direct contact of *E. corrodens* with oral epithelial cells is not necessarily required for the stimulation of these inflammatory mediators (39, 40). Therefore, it is suggested that the soluble components from *E. corrodens* may induce the expression of various inflammatory mediators; however, little is known about *E. corrodens*-induced endothelial cell alterations and interactions involved in the process of the inflammation reaction. In this study, we investigated the biological activity of soluble products from *E. corrodens* (EcSP) in the VEGF expression of oral epithelial cells. In addition, the cell proliferation rate, and expression of adhesion molecules and IL-8 in endothelial cells were determined, and the phosphorylation of the MAPK family in response to EcSP was investigated to elucidate the correlation between inflammatory response of the cells and signal transduction pathways.

Materials and methods

Cell culture

Human umbilical vascular endothelial cells (HUVEC) were obtained from Clonetics and maintained in complete-endothelial cell basal medium (EBM)-2 (Clonetics, Walkersville, MD) supplemented with EGM-2 SingleQuots (Clonetics), which contained human recombinant epidermal growth factor (rEGF), human fibroblast growth factor-basic, VEGF, ascorbic acid, hydrocortisone, human recombinant insulin-like growth factor, heparin, fetal bovine serum, gentamicin and amphotericin.

The KB cell line (derived from a human oral epidermoid carcinoma) was provided by Dr T. Okamoto of Hiroshima University School of Dentistry, Hiroshima, Japan. The KB cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 2 mM L-glutamine, 10% (volume/volume; V/V) fetal bovine serum (JRH Biosciences, Lenexa, KA), and 50 IU/ml penicillin, 50 μg/ml streptomycin at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2.

Human gingival epithelial cells (HGEC) were prepared from explants of normal gingival tissues from periodontally and generally healthy donors with their informed consent. The explants were washed three times with DMEM supplemented with 1000 IU/ml penicillin, 1000 μg/ml streptomycin and 50 μg/ml amphotericin B, and cut into pieces, and cultured in collagen type I-coated tissue culture dishes (Iwaki glass, Tokyo, Japan) in DMEM supplemented with 10% (V/V) fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 57 μg/ml ascorbic acid sodium and 2 mM L-glutamine at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. When cells were grown outside tissues, the medium was changed to complete-keratinocyte-SFM (Gibco BRL) supplemented with 5 ng/ml rEGF, 50 μg/ml bovine pituitary extract, 1.2 mM calcium chloride, 50 IU/ml penicillin and 50 μg/ml streptomycin. The medium was changed every 2 days until subconfluent cell monolayers were formed. HGEC obtained in this manner were maintained in complete-keratinocyte-SFM containing 0.09 mM calcium chloride and used within three passages.

HGEC, KB cells and HUVEC were cultured in a 75-cm^2 flask at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2.

Bacteria and growth conditions

E. corrodens 1073, provided by Dr S. S. Socransky of Forsyth Dental Center, Boston, MA, was cultured at 37°C in tryptic soy broth (TSB) containing 2 mg/ml KNO_3 and 5 μg/ml hemin under anaerobic conditions (95% N_2 and 5% CO_2).

Preparation of EcSP

Supernatants including EcSP were prepared as follows: The *E. corrodens* 1073 cultured as described above were pelleted by centrifugation, washed twice in phosphate-buffered saline (PBS), and suspended in culture medium devoid of antibiotics at a concentration of 1×10^8 to 2×10^8 colony-forming units/ml. The microbial suspensions were then incubated at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. Following 12-h incubation, the supernatants (conditioned media) were obtained by centrifugation at 3000 ***g*** for 15 min at 4°C and then sterilized by filtration (0.2-μm pore size). These solutions were used as EcSP for the

following experiments. All possible traces of lipopolysaccharide (LPS) contamination were determined by the Limulus Amoebocyte lysate assay using Endospecy and Toxicolor Set (Seikagakukogyo, Tokyo, Japan). This was performed with samples of EcSP in each culture medium and compared with standard LPS solution, which was *Limulus* amebocyte-lysate-positive at 0.094 EU/ml (39.1 pg/ml) (Toxicolor Et-1 set; Seikagakukogyo). All EcSP samples were negative or <50.0 pg/ml in the *Limulus* amebocyte lysate assay at the concentration used in this study. We also measured the pH of EcSP and unconditioned media in all the media used in this study. No change in pH between EcSP and unconditioned media was observed.

Cell proliferation assay

HUVEC were resuspended at 3.0×10^4 cells/ml in complete-EBM-2 and 100 µl cell suspension was added per well to a 96-well sterile flat-bottom tissue culture plate, and then pre-incubated for 1 day. Then, 100 µl of various concentrations of EcSP diluted with medium were added per well to a 96-well plate and the plates were incubated. To determine proliferation activity, the TetraColor ONE Cell Proliferation Assay System (Seikagakukogyo) was used as described in the manufacturer's instructions. Briefly, 10 µl TetraColor ONE was added to each well and the plates were incubated for 2 h. The tetrazolium monosodium salt included in TetraColor ONE was reduced by mitochondrial dehydrogenase in live cells and the concentration of the formazan produced was in proportion to the number of live cells. Proliferation, reflected by the amount of formazan, was then measured using a microplate reader at 450 nm.

EcSP stimulation assay

HGEC in complete-keratinocyte-SFM, KB cells in DMEM, and HUVEC in complete-EBM-2 were incubated until confluent monolayers developed in 24- or 96-well tissue culture plates. Cell monolayers were washed three times with Hanks' balanced salt solution (Gibco BRL), stimulated with variable concentrations of EcSP or *E. corrodens* whole cell suspension in culture medium devoid of serum and antibiotics at a concentration of 1.0×10^8 to 2.0×10^8 colony-forming units/ml, and incubated at 37°C. At the end of the incubation, the culture medium was collected and centrifuged, and the supernatant was stored at −20°C until assayed. RNA was extracted immediately from the cells as described below. The RNA and culture medium were used for reverse transcription–polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. The integrity of the monolayer before assay after EcSP stimulation was assessed by visualization under a phase-contrast microscope, and cell viability after EcSP stimulation was confirmed by trypan blue exclusion. By these assessments and confirmations, no morphological changes or cytotoxic effects, including apoptosis, were observed.

Treatment of HUVEC with specific MAPK inhibitors

In some experiments, HUVEC were treated with a specific p38 MAPK inhibitor SB203580 (10 µM; Calbiochem-Novabiochem, La Jolla, CA) for 30 min before exposure to EcSP and during the incubation period as described previously (2). A similar protocol was used for experiments using the MAPK inhibitor PD98059 (25 µM; Calbiochem-Novabiochem). PD98059 blocks ERK1/2 activation by specifically inhibiting MEK1 (MAPK kinase), a kinase that catalyses ERK1/2 phosphorylation (13).

RNA extraction and RT-PCR procedure

Total RNA was extracted from HUVEC, HGEC and KB cells, prepared as described above, with a Catrimox-14 (Iowa Biotechnology, Coralville, IA) according to the directions supplied by the manufacturer. RT-PCR was performed as described previously (39). The oligonucleotide primer sequences, the number of PCR cycles, annealing temperatures used in amplification reactions and sizes of PCR fragments are shown in Table 1. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene transcript was used as the control. A sample (10 µl) of each amplified product was subjected to electrophoresis in a 1.5% agarose gel (TaKaRa, Shiga, Japan), stained with ethidium bromide, and visualized by UV illumination. For negative controls, the Moloney murine leukemia virus reverse transcriptase was omitted from the cDNA synthesis mixture to ensure amplification from genomic DNA. The amounts of target mRNAs, compared with GAPDH mRNA in the controls, were semi-quantified by scanning densitometry of the gel using NIH IMAGE 1.62, as previously reported (10).

IL-8 ELISA

The concentration of IL-8 in cell culture supernatants was determined by ELISA. Commercially available ELISA kits (Biosource, Rochville, MD) for the quantification of IL-8 were used as described in the manufacturer's instructions.

Cell surface ELISA (Cell-ELISA) for ICAM-1, VCAM-1 and E-selectin

The expression of different adhesion molecules on the monolayer of HUVEC preincubated with EcSP was determined by cell-surface ELISA (Cell-ELISA), which was a modification of the procedures previously reported (19). Briefly, confluent pretreated HUVEC (monolayer) in 96-well flat-bottom microtiter plates were washed and finally fixed with 4% paraformaldehyde for 1 h. Bovine serum albumin was used to reduce non-specific binding and primary antibodies were added for 1 h. Anti-human CD54 (ICAM-1) mouse monoclonal antibody (Ancell, Baport, MN), anti-human VCAM-1 mouse monoclonal antibody (Upstate Biotechnology, Lake Placid, NY), and anti-human CD62E (E-selectin) mouse monoclonal antibody (Chemicon, Temecula, CA) were used as primary

Table 1. Oligonucleotide sequences of the 5′ and 3′ primers of the target genes

mRNA species	Primer	Sequence	Size of PCR fragment (bp)	No. of PCR cycles	Annealing temperature (°C)
GAPDH	5′	5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′	985	36	60
	3′	5′-CATGTGGGCCATGAGGTCCACCAC-3′			
VEGF	5′	5′-CTGCTGTCTTGGGTGCATTG-3′	368	36	55
	3′	5′-TTCACATTTGTTGTGCTGTAG-3′			
ICAM-1	5′	5′-CGTGCCGCACTGAACTGGAC-3′	447	36	60
	3′	5′-CCTCACACTTCACTGTCACCT-3′			
VCAM-1	5′	5′-ATTGGGAAAAACAGAAAAGAG-3′	642	30	56
	3′	5′-GGCAACATTGACATAAAGT-3′			
IL-8	5′	5′-ATGACTTCCAAGCTGGCCGTGGCT-3′	294	36	60
	3′	5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′			

antibodies. Thereafter, cells were washed three times and reacted with biotin-conjugated anti-mouse immunoglobulin (Dako Japan, Kyoto, Japan) for 45 min. After washing, cells were exposed to streptavidin peroxidase conjugate (Bio-Rad) for 30 min. After final washing, TMD peroxidase conjugate (Bio-Rad, Hercules, CA) was added and the development reaction was stopped by adding 1 N H_2SO_4. The developed color was then measured using a microplate reader at 450 nm.

Western blot analysis of phospho-specific MAPK activation

The phosphorylation of threonin or tyrosine on MAP kinases is an accurate indicator of their activation (29, 41). Phosphorylated p38 and p44/42 (MAP) were detected as follows. After HUVEC and EcSP were incubated for various time intervals in 24-well plates, monolayers were lysed with lysis buffer containing 62.5 mM Tris–HCl (pH 6.8), 0.5 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), and 1 mM phenylmethylsulfonyl fluoride (PMSF) at the end of the experiment. The protein content in each sample was determined with DC Protein assay (Bio-Rad) using albumin as a standard, and samples were then heated to 100°C for 10 min. The protein (6 μg) was loaded onto a 5–20% SDS–polyacrylamide gel electrophoresis (PAGE) gel. After running the gel, the proteins were transferred onto PVDF membranes (Millipore, Bellerica, MA). The membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (TBST) supplemented with 5% bovine serum albumin for 1 h. This was followed by overnight incubation at 4°C with 1/2000-diluted primary phospho-specific p44/42 MAPK monoclonal antibody (New England Biolabs) or 1/1000-diluted phospho-specific p38 MAP kinase antibody (New England Biolabs, Ipswich, MA). These antibodies recognize Thr 202- and Tyr 204-phosphorylated p44/42 MAPK and Thr 180- and Tyr 182-phosphorylated p38 MAPK, respectively. The membranes were then washed three times with TBST and incubated at room temperature for 45 min with biotinylated anti-mouse and anti-rabbit immunoglobulin (DAKO) at a dilution of 1/50 in TBST. After three washes with TBST, membranes were incubated at room temperature for 70 min with streptavidin–alkaline phosphatase conjugate (Bio-Rad) at a dilution of 1/10,000 in PBS. Alkaline phosphatase conjugate substrate (Bio-Rad) was used for detection. Controls for these Western blot experiments consisted of HUVEC harvested at the zero time-point for kinetic experiments or cultured in the absence of EcSP.

Toll-like receptor (TLR) blocking assay

For blocking of TLR2 or TLR4, KB cell monolayers were treated with 10 μg/ml mouse anti-human TLR2, TLR4 or an isotype-matched control immunoglobulin G for 1 h before EcSP, *Escherichia coli* 0111:B4 LPS (ultrapure grade; a TLR4 ligand, InvivoGen, San Diego, CA) or Pam3CSK4 (a TLR2 ligand; InvivoGen) stimulation. EcSP, LPS or Pam3CSK4 was then added to KB cells in the presence of antibody, and KB cells were incubated for 24 h. The culture supernatant fluids were collected and stored at −20°C until IL-8 ELISA.

Statistical analysis

All statistical analyses were performed using unpaired Student's *t*-test. Differences were considered significant when the probability value was less than 5% ($P < 0.05$).

Results

EcSP increases VEGF expression and production in human oral epithelial cells

As periodontal lesions progress, various inflammatory mediators produced by oral epithelial cells in response to virulent factors of periodontopathogens affect connective tissue, including the vasculature beneath the epithelial layer, to change inflammatory status. We therefore investigated VEGF expression and production from oral epithelial cells in response to EcSP, because VEGF is a multifunctional angiogenic cytokine, which enhances blood vessel growth and permeability, and is important in inflammation and wound healing. The levels of VEGF-specific mRNA from EcSP-stimulated KB cells and HGEC were significantly increased compared with those from non-stimulated control cells (Fig. 1A,B). By ELISA, the VEGF level in EcSP-stimulated HGEC was 4.7-fold higher than in the non-stimulated controls, and this ELISA finding was consistent with the RT-PCR findings (Fig. 1C). These results suggest that EcSP may act indirectly on endothelial cells via VEGF production secreted from stimulated oral epithelial cells.

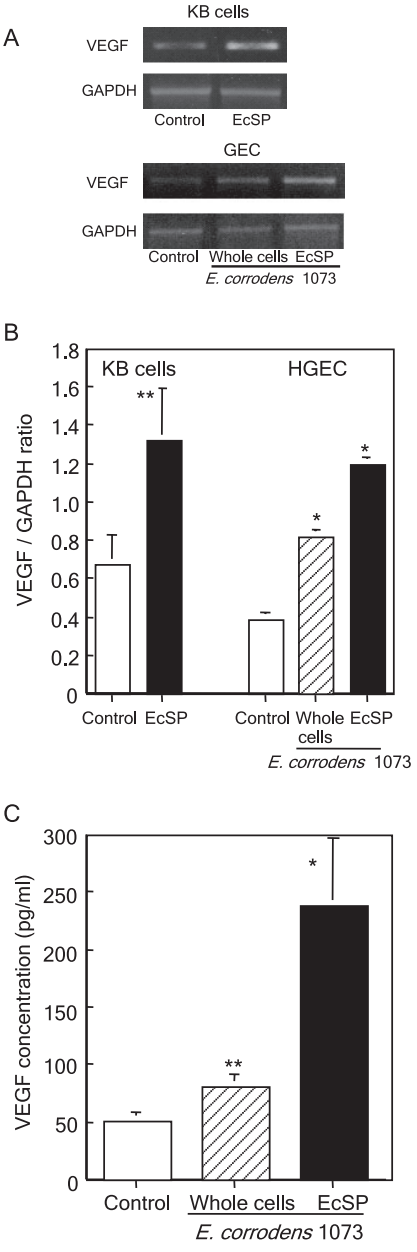

Fig. 1. VEGF gene expression (A, B) and production (C) by oral epithelial cells after stimulation with EcSP. (A) RNA was isolated from KB cells after 4-h EcSP stimulation or HGEC after 12-h EcSP stimulation (100% per well). The cDNA synthesis and RT-PCR were performed as described in the Materials and methods. The *GAPDH* housekeeping gene was used as a control. (B) The amounts of VEGF mRNAs, compared with that of GAPDH mRNA in the control, were semi-quantified by scanning densitometry of the gel with NIH IMAGE 1.62. Results are shown as a ratio of VEGF : GAPDH mRNA band density, and the means and SDs of the ratio from three different experiments. Asterisks indicate significant differences (*$P < 0.01$; **$P < 0.05$) vs. control levels. (C) Supernatants were tested after 12 h of 100% EcSP-stimulated HGEC culture, and the VEGF level was determined by ELISA. The control consisted of a monolayer in the medium alone. Values are the means and SDs of triplicate determinations from three different experiments. Asterisks indicate significant differences (*$P < 0.01$; **$P < 0.05$) vs. control levels.

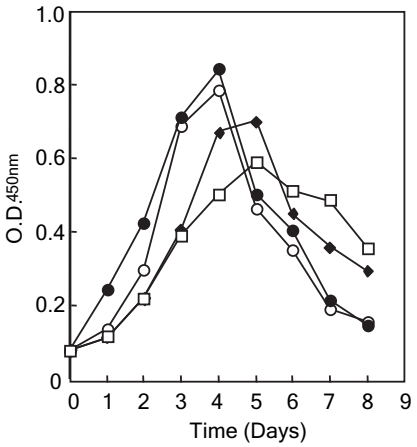

Fig. 2. Proliferation of HUVEC in response to EcSP. First, 100 µl HUVEC (3.0×10^3/well) was preincubated in 96-well plates for 1 day. Then, 100 µl of various concentrations of EcSP were added per well and the plates were incubated. Proliferation was measured daily as described in the Materials and methods. □, 0% (medium alone); ◆, 25% EcSP; ●, 50% EcSP; ○, 75% EcSP. These experiments were performed on two occasions, and one representative finding is expressed as the mean ($n = 8$).

EcSP activates human endothelial cell proliferation

To assess the ability of EcSP to directly activate endothelial cell proliferation, cell proliferation assays were performed with HUVEC after EcSP stimulation at various concentrations diluted with medium (Fig. 2). Increased proliferation activity was observed at between 25 and 75% EcSP per well, and maximal proliferation activity was detected with 50% EcSP per well. These results suggest that EcSP contains activating factors to HUVEC proliferation. In contrast, HUVEC proliferation was inhibited at 100% EcSP per well (data not shown). From this inhibition result, it is suggested that the high concentration of EcSP has a direct inhibiting effect on HUVEC proliferation, and EcSP may contain suppressors against HUVEC proliferation at low concentrations.

EcSP increases VEGF, ICAM-1, VCAM-1 and IL-8 expression in endothelial cells

To assess the levels of inflammatory mediators in endothelial cells, RT-PCR was performed with RNA isolated from HUVEC after EcSP stimulation. The levels of VEGF-, ICAM-1- and VCAM-1-specific mRNAs from HUVEC after 50% EcSP or *E. corrodens* whole cell stimulation for 12 h were significantly increased compared with those from unstimulated control cells (Fig. 3A,C). In addition, the levels of IL-8-specific mRNA from HUVEC after 100% EcSP stimulation for 15 or 24 h were significantly increased compared with those from unstimulated control cells (Fig. 3B,D).

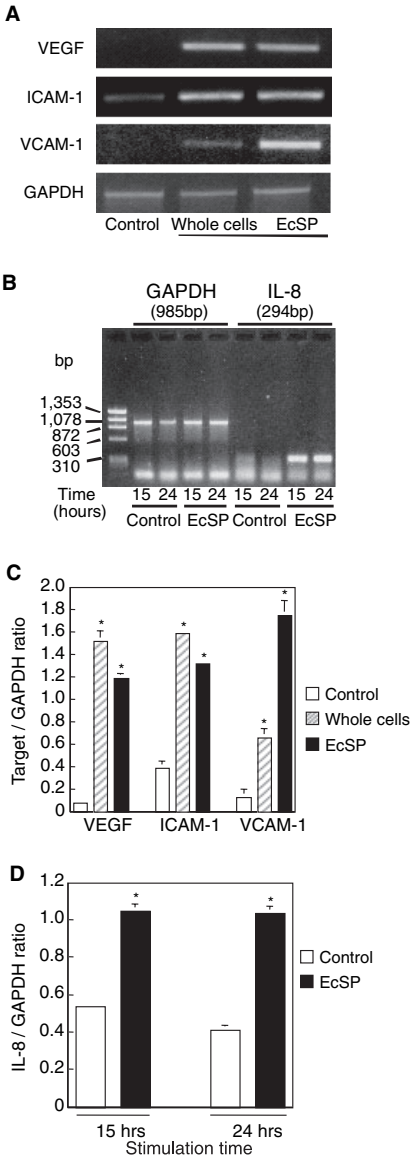

Fig. 3. VEGF, ICAM-1, VCAM-1 (A, C) and IL-8 (B, D) gene expression induced from HUVEC by EcSP stimulation. (A, B) RNA was isolated from HUVEC after 12 h (for VEGF, ICAM-1, VCAM-1; A), or 15 or 24 h (for IL-8; B) of exposure to 50% (for VEGF, ICAM-1, VCAM-1) or 100% (for IL-8) EcSP per well. Synthesis of cDNA and RT-PCR were performed as described in the Materials and methods. The control consisted of monolayers incubated in medium alone. The GAPDH housekeeping gene was used as a control. (C, D) The amounts of VEGF, ICAM-1, VCAM-1 and IL-8 mRNAs, compared with that of GAPDH mRNA in the control, were semi-quantified by scanning densitometry of the gel with NIH IMAGE 1.62. Results are shown as a ratio of target gene : GAPDH mRNA band density, and the means and SDs of the ratio from three different experiments. Asterisks indicate significant differences (*$P < 0.01$) vs. control levels.

EcSP stimulates ICAM-1, VCAM-1 and E-selectin surface expression, and IL-8 secretion in endothelial cells

For further characterization of EcSP-stimulated endothelial cell–leukocyte interaction and pro-inflammatory cytokine secretion, the surface expression of adhesion molecules and secretion of IL-8 in EcSP-stimulated endothelial cells were determined by (Cell-) ELISA. ICAM-1, VCAM-1 and E-selectin surface expression in 100% EcSP-stimulated HUVEC significantly increased after 15 h of stimulation (data not shown). IL-8 secretion in 100% EcSP-stimulated HUVEC significantly increased after 15 and 24 h of stimulation and the IL-8 protein levels were 16.7- and 13.6-fold greater than the control levels, respectively (data not shown). We also determined the surface expression of adhesion molecules and IL-8 secretion in HUVEC after EcSP stimulation at various concentrations. At EcSP concentrations from 25% to 100% per well, ICAM-1, VCAM-1 and E-selectin surface expressions, and IL-8 secretion in EcSP-stimulated HUVEC were significantly increased (Fig. 4A,B). Even the lowest EcSP concentration tested (25% per well) was able to induce significant ICAM-1, VCAM-1, E-selectin and IL-8 responses; however, these patterns in HUVEC after EcSP stimulation at various concentrations varied with the molecules tested. Stimulation of HUVEC with EcSP increased ICAM-1 surface expression concentration-dependently, but the E-selectin surface expression pattern was slightly decreased in inverse proportion. VCAM-1 surface expression and IL-8 secretion in 50% EcSP-stimulated HUVEC were maximal.

EcSP induces rapid p44/42 MAPK and p38 MAPK phosphorylation in endothelial cells

In the major MAPK families, the activation of p44/42 MAPK isoforms involved in mitogen signaling pathways is an early step in cell activation and intracellular signaling. Signal transduction through p38 MAPK is suggested to be more important in stress signaling by heat stress and osmotic stress. These MAPKs have been

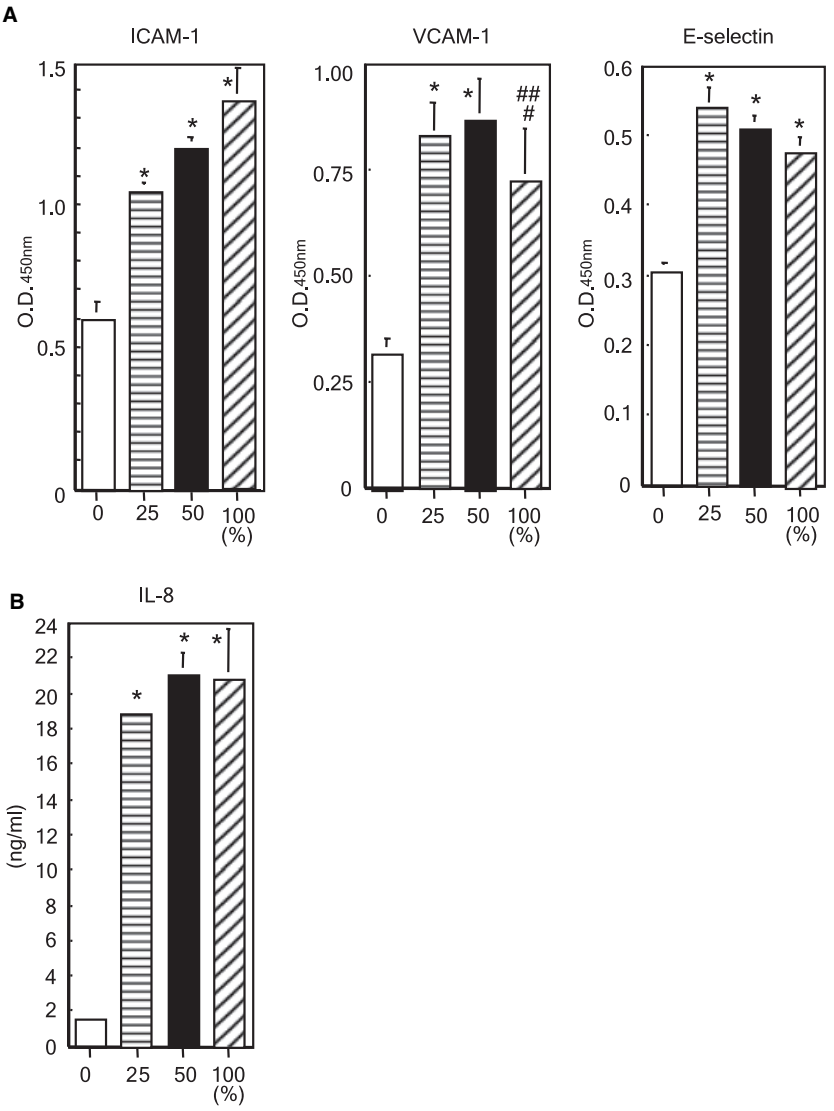

Fig. 4. Enhanced ICAM-1, VCAM-1 and E-selectin expression (A) and IL-8 protein secretion (B) in HUVEC stimulated by various concentrations of EcSP. Cells were tested at 15 h (for ICAM-1, VCAM-1 and E-selectin; A) or 25 h (for IL-8; B) of various concentrations of EcSP stimulated culture. The ICAM-1, VCAM-1 and E-selectin levels were determined by Cell-ELISA, and the IL-8 level was determined by ELISA. Values are the means and SDs of four separate experiments. Asterisks (*) indicate significant differences ($P < 0.01$) vs. 0% EcSP (medium alone) values. Single (#) and double (##) sharps indicate significant differences vs. 25% ($P < 0.05$) and 50% ($P < 0.01$) EcSP values, respectively.

implicated as key participants in inflammatory signaling pathways, leading to increased cytokine production. In our preliminary immunohistochemical studies with inflamed gingival sections from periodontitis patients, the phosphorylations of p44/42 MAPK and p38 MAPK were observed in the oral gingival sulcular epithelium and the vascular endothelial cells of extended blood vessels subjacent to the epithelium (data not shown); therefore, to confirm that EcSP induces the phosphorylation of p44/42 MAPK and p38 MAPK in HUVEC, we performed Western blotting. Western blot analysis showed the time-dependently-enhanced phosphorylation of p44 and p42 MAPK isoforms and p38 MAPK in HUVEC (Fig. 5), which peaked at 10–30 min.

Effects of p44/42 MAPK or/and p38 MAPK inhibition on cellular adhesion molecule surface expressions and IL-8 secretion in endothelial cells

EcSP stimulation up-regulated the surface expression of cellular adhesion molecules and IL-8 production in HUVEC (Figs 3 and 4), and induced p44/42 MAPK and p38 MAPK phosphorylation (Fig. 5). Both kinases regulate upstream signaling events that control cytokine and adhesion molecule transcriptions. Next, we determined whether MAPK phosphorylation is involved in adhesion molecule and IL-8 up-regulation by EcSP-stimulated endothelial cells. In these experiments, specific MAPK inhibitors, PD98059 and SB203580, which prevent p44/42 MAPK phosphorylation through blocking MEK1 kinase activity and p38 MAPK activity, respectively, were used. HUVEC was pretreated for 30 min with MAPK inhibitors used singularly or in combination. HUVEC monolayers were then stimulated with EcSP. Cellular adhesion molecule surface expressions were measured by Cell-ELISA, and IL-8 levels were measured in conditioned medium harvested after 24 h by ELISA. Neither MEK1 nor p38 MAPK inhibitors specifically reduced IL-8, ICAM-1 and VCAM-1 expression in the control group. The p38 inhibitor SB203580 reduced ICAM-1 expression by 52% ($P < 0.001$) and VCAM-1 expression by 72% ($P < 0.005$), but did not reduce E-selectin expression in EcSP-stimulated HUVEC. The MEK1 inhibitor PD98059 did not reduce cellular adhesion molecule expressions tested in EcSP-stimulated HUVEC (Fig. 6A). The MEK1 inhibitor reduced IL-8 production by 28% ($P < 0.05$), the p38 inhibitor reduced IL-8 production by 53% ($P < 0.01$), and a combination of the two inhibitors reduced IL-8 production by 72% ($P < 0.001$) in EcSP-stimulated HUVEC (Fig. 6B).

EcSP signaling through TLR2- and TLR4-independent mechanisms

The innate immune response is not only responsible for first-line microbial clearance but also plays an instructive role in the adaptive immune response through the release of inflammatory cytokines. Once bacteria are accidentally introduced into host tissues through the epithelial or endothelial layer, the innate immune response is triggered to help the host to localize the site of infection and eliminate the bacteria. In particular, TLR play an important role in innate immune signaling in response to the functional pattern recognition of microorganisms such as lipopeptide (a TLR2 ligand) and LPS (a TLR4 ligand). Next, we determined whether EcSP signaling occurs through TLR2 and/or TLR4 pathways using blocking assay. Treatment with either anti-TLR antibody did not inhibit IL-8 production from EcSP-stimulated KB cells (Fig. 7).

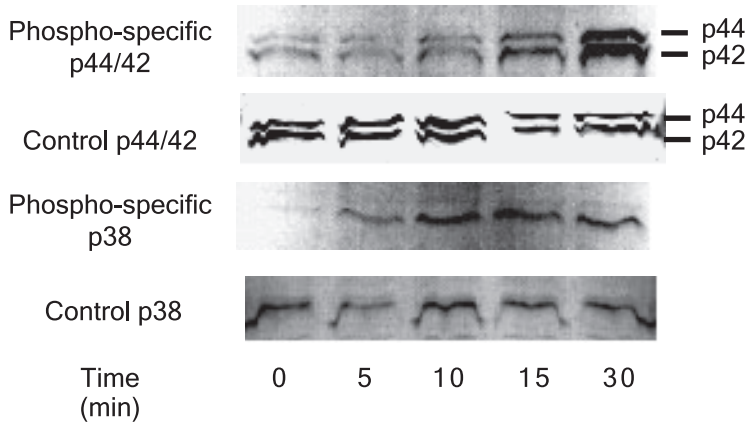

Fig. 5. EcSP activated p44/42 MAPK and p38 MAPK in HUVEC. HUVEC monolayers were stimulated with 100% EcSP. At the time points indicated, cell lysates were prepared and subjected to Western blot analysis using phospho-specific antibodies against p44 (ERK1, upper band) and p42 (ERK2, lower band), and p38 MAPKs (upper panels). Western blots performed using control antibodies that recognize ERK or p38 regardless of their phosphorylation states are shown (lower panels). Representative data of three experiments are shown.

As reported previously (39), LPS (ultrapure grade from *E. coli*) did not significantly secrete IL-8 from KB cells. Moreover, Pam3CSK4 (a TLR2 ligand) did not induce IL-8 production from the same cells. Taken together, these results indicate that EcSP does not include the components reactive to TLR2 and TLR4 and signals through another pathways, except for TLR2 and TLR4 pathways.

Discussion

Histologically, vascular dilatation and proliferation at the level of the gingival margin are evident, and the vascular proliferation occurs in the apical part of the vascular plexus beneath the gingival epithelium during the early periodontitis stage (24). VEGF is detectable in periodontal tissues within vascular endothelial cells, plasma cells, macrophages and in junctional, sulcular and gingival epithelium (5). Suthin et al. observed the enhanced expression of VEGF stimulated with bacterial components from periodontal pathogens in gingival fibroblasts (35). These results (Figs 1–3) are in agreement with this previous immunohistochemical finding and cell culture experimental result. In primary human bronchial epithelial cells, the levels of excreted VEGF are increased by transforming growth factor-β1, and HUVEC exposed to conditioned medium from primary bronchial epithelial cell cultures show an increased growth rate that is inhibited in the presence of a specific neutralizing antibody to VEGF (6). They suggested that lung epithelial cells participate in the endothelial repair and angiogenesis that follow lung injury through the synthesis of VEGF. These results also suggest that EcSP contributes to the angiogenesis that follows periodontal inflammation through the synthesis of VEGF in oral epithelium and endothelial cell proliferation.

These findings (Fig. 2) are consistent with the previous findings in which live *Bartonella henselae* enhanced the proliferation of HUVEC without direct bacteria–cell contact and non-piliated strain *B. henselae*, which is unable to attach to endothelial cells, and the culture supernatants of *B. henselae* induced HUVEC proliferation, suggesting that the stimulation of HUVEC proliferation by *B. henselae* is mediated by soluble factors secreted from bacteria (25). In contrast, HUVEC proliferation was inhibited at 100% EcSP per well (data not shown). From this inhibition, it is suggested that the high concentration of EcSP has a direct inhibiting effect on HUVEC proliferation, and EcSP may contain suppressors against HUVEC proliferation at low concentrations. Recently, it has been reported that lysine decarboxylase in cell-free saline-soluble extracts of *E. corrodens* ATCC 23834 inhibits HeLa (adenocarcinoma epithelial cells) and HL60 (promyelocytic cell line) cell growth by depleting lysine (22). Another report showed that the release of heat-labile and heat-stable virulence factors by *Haemophilus somnus* induces bovine endothelial cell apoptosis after 6 h of treatment (36). However, it remains unclear whether EcSP induces HUVEC apoptosis and includes a product with lysine decarboxylase activity; this possibility is currently under investigation.

In an inflammatory reaction, the adhesion of circulating leukocytes to endothelial cells is an early step regulated by complex communication between the cell types involved. In the next step, the trafficking of leukocytes from the vascular compartment to extravascular tissues occurs sequentially (33). Leukocyte trafficking to surrounding tissue is initiated by an inflammatory stimulus, which induces the expression of adhesion molecules on the surface of the endothelial cells. This study demonstrated that EcSP to HUVEC induced the expression of ICAM-1, VCAM-1, E-selectin and IL-8 (Figs 3 and 4), which is a CXC chemokine (3) and a potent neutrophil chemotactic factor (31, 32). These observations clarify new properties of *E. corrodens* regarding its ability to initiate a cascade of events leading to endothelial cell activation and inflammation. They are consistent with those reported by other studies (10, 11, 18, 19), which demonstrated that some pathogenic bacteria induce the up-regulation of endothelial adhesion molecules to cultured human endothelial cells.

In addition, these findings showed that the direct contact of *E. corrodens* with endothelial cells is not necessarily required for endothelial cell activation. Recently, two groups (12, 16) reported that *E. corrodens* ATCC 23834 appears to be weakly invasive or non-invasive to human gingival epithelial cells and does not actively invade human coronary artery endothelial cells. However, the most obvious cause of periodontitis is the large number of gram-negative periodontopathic bacteria present in the subgingival plaque (30), and these bacteria do not invade the periodontium in any appreciable number. In this respect, it is suggested that the solubilized components from bacteria, which can diffuse into the gingiva and associated periodontal tissues, including the epithelium and subepithelial blood vessels, activate the epithelial and endothelial initial response leading to periodontitis.

LPS, a major cell wall component of gram-negative organisms, triggers inflammatory responses, including increased cellular adhesion molecules, ICAM-1, VCAM-1 and E-selectin and the release of IL-8 in endothelial cells (4, 7). It has also been reported that LPS obtained from *E. coli* and *Psuedomonas aeruginosa*, but not that from *Porphyromonas gingivalis*, *Bacteroides forsythus* and *Helicobacter pylori*, induces E-selectin expression in HUVEC (11). However, Krull et al. reported that living *Chlamydia*, but not chlamydial LPS, may be required for endothelial cell activation by treatment with polymyxin B, an LPS inactivator (27,

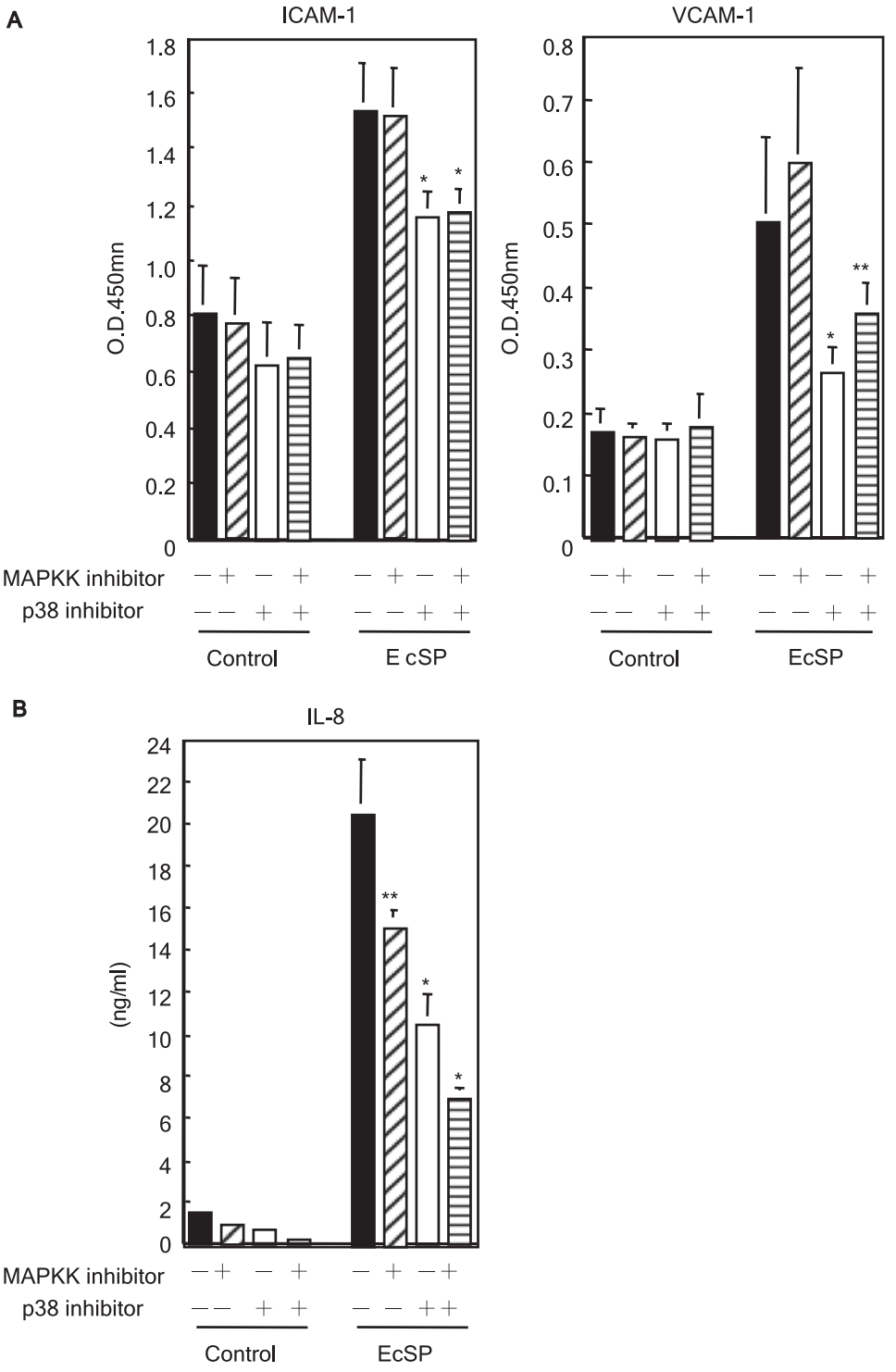

Fig. 6. MAPK activation was required for ICAM-1, VCAM-1 (A) and IL-8 (B) up-regulation in EcSP-stimulated HUVEC. The HUVEC monolayers were stimulated with 100% EcSP with or without treatment with the p44/42 MAPKK (MEK1) inhibitor PD98059 (25 μM) and/or the p38 MAPK inhibitor SB203580 (10 μM) for 15 h (A) or 24 h (B). ICAM-1 and VCAM-1 levels were determined by Cell-ELISA, and IL-8 levels were determined by ELISA. The controls consisted of monolayers incubated in medium alone. Values are the means and SDs of six (A) or three (B) separate experiments. Asterisks indicate significant differences (*$P < 0.01$; **$P < 0.05$) vs. similar (EcSP-stimulated) group values without MAPK inhibitor treatment.

34), and heat-killed bacteria (18). These results also showed that EcSP pretreated with polymyxin B (50–5000 ng/ml) was still able to stimulate and not reduce HUVEC to induce ICAM-1, VCAM-1 and E-selectin surface expression by Cell-ELISA (data not shown). These results are consistent with those of a previous chlamydial study and the result of our *Limulus* amebocyte lysate assay. Moreover, we confirmed that EcSP does not contain the ligands for TLR2 and TLR4 such as LPS (Fig. 7).

After SDS–PAGE of EcSP and unconditioned media in all media, we performed

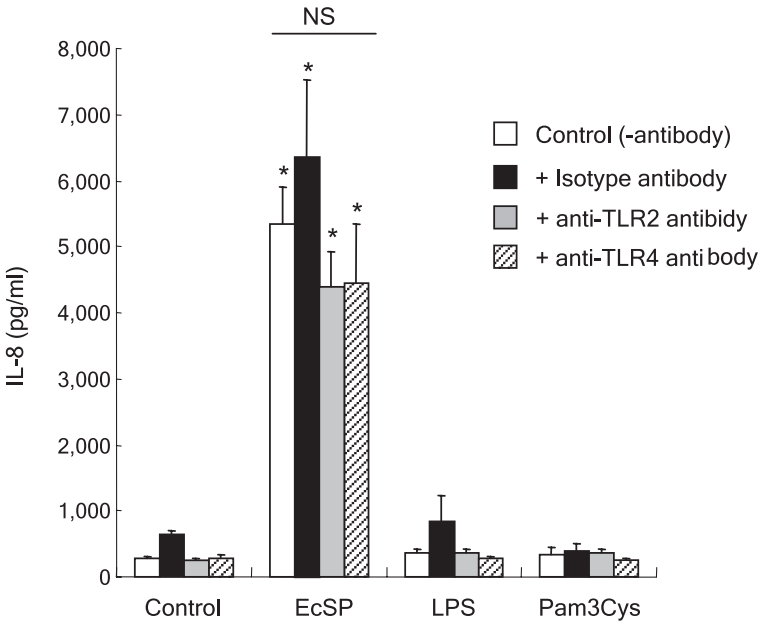

Fig. 7. EcSP did not signal through TLR2 and TLR4 pathways. KB cells were preincubated with anti-human TLR antibody (TLR2 or TLR4) or isotype-matched control antibody (10 μg/ml) for 1 h. KB cells were then stimulated with 100% EcSP for 24 h. The controls consisted of monolayers incubated in medium alone. Values are the means and SDs of four separate experiments. Asterisks indicate significant differences (*$P < 0.01$) vs. the control group. NS, no significant difference in the group.

Coomassie Brilliant Blue and silver staining of the gels. The staining patterns of EcSP in all media were similar to those in unconditioned media (data not shown); therefore, we consider that the active components in EcSP may not be protein, but may be small molecular (short chain) peptides. In our recent preliminary experiments, we prepared the EcSP from *E. corrodens* culture in PBS and the small molecule (mass-to-charge ratio: $m/z = 272.874$) in EcSP was specifically detected by time-of-flight mass spectrometry (TOF-MS) analysis performed with VOYGER™ DE/PROJ apparatus (PerSeptive Biosystems). The TOF-MS pattern of EcSP suggests that this small molecule may be a candidate active product in EcSP. We are now trying to purify this molecule and determine its activity.

Very little is known about the signal transduction pathways activating target cells with *E. corrodens* infection. We showed that EcSP rapidly phosphorylated ERK1/2 and p38 MAP kinases in HUVEC, without bacteria–cell contact. These results (Fig. 6) support the evidence that MAPK may be important for the expression of endothelial adhesion molecules including VCAM-1 and ICAM-1 (28, 37). Thus, the phosphorylation of p38 and MEK-ERK1/2 pathways is required for the maximal endothelial cell response to *E. corrodens.* When both pathways were blocked by combining the two inhibitors, EcSP-induced IL-8 and cellular adhesion molecules were incompletely abolished. This suggests that another pathway regulates IL-8 and cellular adhesion molecule production in endothelial cells following *E. corrodens* infection.

In summary, this study presented evidence that EcSP could induce endothelial cell proliferation and activate different signal transduction pathways, p44/42 and p38 MAPK stimulation, within minutes. Within hours, increased mRNA and surface expression of ICAM-1, VCAM-1 and E-selectin were detected, which in turn resulted in enhanced leukocyte–endothelial cell interaction. These findings raise the possibility that EcSP triggers a cascade of events that lead to chronic periodontal inflammation.

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