RNA isolation and Northern blot analysis. Total RNA was isolated from the co-cultured ECs by the guanidium isothiocyanate/phenochloroform method. The RNA (10 μg/lane) was separated by electrophoresis on a 1% agarose formaldehyde gel and transferred onto a nylon membrane (Nytran, Schleicher & Schuell Inc., Germany) by using a vacuum blotting system (VacuGene XL, Pharmacia, Piscataway, NJ). The E-selectin cDNA probe was a product of reverse transcriptase-polymerase chain reaction (RT-PCR) [sense primer: 5′-GTGAACCCA ACAATAGGCAA-3′; antisense primer: 5′-CAGGTGAAGTTGCAGGATGA-3′; product length, 705 base pairs (bp)]. After hybridization with the ^{32}P-labeled cDNA probe, the membrane was washed with 1× saline-sodium citrate (SSC) containing 1% sodium dodecylsulfate (SDS) at room temperature for 30 min and then exposed to X-ray film (Kodak X-Omat-AR, Rochester, NY) at -70°C. Autoradiographic films were scanned, and the results were analyzed by using a densitometer (Computing Densitometer 300S, Molecular Dynamics, Sunnyvale, CA).

Reverse transcriptase-polymerase chain reaction (RT-PCR). 5 to 7 μg total RNA of the EC monolayer (approximately 2.5×10^6 cells) in co-culture was isolated by the guanidium isothiocyanate/phenochloroform method and then converted to cDNA using the Superscript II reverse transcriptase system and oligo-dT primers (Life Technologies, Rockville, MD). Briefly, total RNA [2 μg, in diethyl pyrocarbonate (DEPC) water] was incubated with 50 U/μL Superscript II RNase H reverse transcriptase in buffer containing 20 mM Tris-HCl, pH 8.4, 2.5 mM MgCl2, 0.5 mM deoxynucleoside triphosphate (dNTP) mix, 10 mM dithiothreitol (DTT) and oligo-dT12-18 (0.5 μg/mL) for 50 min at 42°C. Reactions were terminated with *Escherichia coli* RNase H (2 U/μL). The cDNA was diluted 1:20 before the performance of PCR by using 1 μL cDNA in 20 mM Tris-HCl, pH 8.4, 3 mM MgCl2, 0.2 mM dNTP mix, 0.5 μM sense and antisense primers, and Taq polymerase (2 U/μL; Takara Shuzo, Shiga, Japan). Primer sequences were designed as E-selectin (sense: 5′-GTGAACC CAACAATAGGCAA-3′; antisense: 5′-CAGGTGAAGTTGCAGGATGA-3′; product length, 705 bp); gp130 (sense: 5′-GGACCAAAGATGCCTCAACTTG-3′; antisense: 5′-CGGTACC ATCTTCTTGTTGCCA-3′; product length, 645 bp); IRAK (sense: 5′-AAAGGAGGCCTCC TATGACC-3′; antisense: 5′-ATGATGCAGAGCTGCCAAG-3′; product length, 441 bp). Amplification of the cDNA was performed in parallel samples using human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers (sense: 5′-CCACCCATGGCAAATTC CATGGCA-3′; antisense: 5′-TCTAGACGGCAGGTCAGGTCCACC-3′; product length, 599 bp). The samples were amplified using primers for 25 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min, using a GeneAmp System 9700 (PE Biosystems, Foster City, CA). Amplification was linear with respect to the cDNA concentration by optimizing the primer concentration, amplification cycles, and MgCl2 concentration for each PCR reaction. The amplified cDNAs were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. Band intensities were quantified directly from the stained agarose gels using video imaging and a densitometry software system (GDS-8000 ImagingWorkstation; UVP, Upland, CA).

Quantitative real-time PCR. Total RNA of ECs in co-culture was extracted and cDNA was synthesized using standard methodologies as described above. cDNA was amplified through PCR on a LightCycler (Roche Diagnostics, East Sussex, United Kingdom) using LightCycler FastStart DNA MasterPlus SYBR Green I (Roche Diagnostics) with 0.5 μM each E-selectin primer (sense: 5′-CTGAAAGATGGACGCTCAAT-3′; antisense: 5′-CGTTT CAGAAGCCAGAAGAG-3′;

product length, 181 bp). PCR was performed in triplicate at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 5 sec, extension at 72°C for 8 sec, and single signal acquisition for 10 sec. β-actin gene expression was used as an internal control (sense primer: 5′-AAATCGTCCGTGACATCAA G-3′; antisense primer: 5′-GGAAGGAAGGCTGGAAGAGA-3′; product length, 180 bp). The PCR conditions were optimized to obtain a PCR product with a single peak on melting curve analysis on the LightCycler. PCR product was also run on a 1.0% agarose gel and stained with ethidium bromide to obtain a single band of the expected size. Raw data collected from the LightCycler were analyzed using LightCycler Software Version 3.5 (Roche Diagnostics). The E-selectin gene expression levels were normalized with β-actin gene expression levels in the same sample.

Western blot analysis. ECs in co-culture were collected by scraping and lysed with a buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). The total cell lysate (100 μg of protein) was separated by SDS-PAGE (12% running, 4% stacking) and transferred onto a polyvinylidene fluoride membrane (Immobilon P, 0.45-μm pore size). The membrane was then incubated with the designated antibodies. Immunodetection was performed by using the Western-Light chemiluminescent detection system (Applied Biosystems, Foster City, CA).

Immunofluorescence with flow cytometry. ECs co-cultured with SMCs were washed with M199 three times, detached with Versene buffer containing EDTA, and centrifuged. Each sample (4×10^5 cells) was washed with PBS containing 0.5% BSA and resuspended in 0.2 mL PBS containing an E-selectin antibody (20 μg/mL). After incubation at 4°C for 30 min, the cells were centrifuged at 1500 rpm for 5 min and washed twice with PBS to remove the unbound antibody. The cells were then incubated with anti-mouse IgG (Cappel, West Chester, Pennsylvania) conjugated with FITC for 30 min at 4°C. After two final washes in PBS, the cells were resuspended in 0.5 mL PBS containing 10% FBS and assayed within 1 h. Fluorescein-labeled cells ($\sim1.0\times10^4$ cells/sample) were analyzed with the flow cytofluorometer (FACScan, Becton Dickinson). Cells incubated with FITC-conjugated control IgG or FITC-conjugated antibody alone were used as IgG or negative controls.

Reporter gene construct, DNA plasmids, siRNA, transfection, and luciferase assay. The E-selectin promoter construct (E-selectin-Luc) contains 540 bp of E-selectin 5′-flanking DNA linked to the firefly luciferase reporter gene of plasmid pGL2 (Promega Inc.) [1]. This fragment of E-selectin promoter contains NF-κB binding sites. The dominant-negative mutants of Ras (RasN17), Rac (RacN17), and Raf (Raf301), and the catalytically inactive mutants of JNK [JNK(K-R)] and ERK2 (mERK) were previously described [2]. DNA plasmids at various concentrations (0.1~1 μg/mL) were transfected into bovine aortic ECs at 60% confluence by using lipofectamine (Gibco). The pSV-β-galactosidase plasmid was co-transfected to normalize the transfection efficiency. The transfected ECs were seeded onto the outer side of the membrane and incubated overnight before co-culture and shear stress experiments. For siRNA transfection, ECs at 70-80% confluence were transfected with the designated siRNA at various concentrations (60, 100, and 200 μmol/mL) using oligofectamine (Invitrogen, Carlsbad, CA).

Electrophoretic mobility shift assay (EMSA). ECs in co-culture were collected by scraping in PBS. After centrifugation of the cell suspension at 2000 rpm, the cell pellets were resuspended in

cold buffer A (containing, in mmol/L, KCl 10, ethylenediamine tetraacetate [EDTA] 0.1, dithiothreitol [DTT] 1, and phenyl methylsulfonyl fluoride [PMSF] 1) for 15 min. The cells were lysed by adding 10% NP-40 and then centrifuged at 6000 rpm to obtain pellets of nuclei. The nuclear pellets were resuspended in cold buffer B (containing, in mmol/L, 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid [HEPES] 20, EDTA 1, DTT 1, PMSF 1, and NaCl 400), vigorously agitated, and then centrifuged. The supernatant containing the nuclear proteins was used for the EMSA or stored at -70°C until used. A 25-bp oligonucleotides containing the human E-selectin NF-κB site was synthesized (5′-GCCATTGGGGATTTCCTCTTTACTG-3′ and complement [3], κB site underlined). Mutant human E-selectin NF-κB probe containing the sequence 5′-GCCATTG CTCATTTCCTCTTTACTG-3′ and complement [3] were also synthesized (mutant base pair underlined). The oligonucleotides were end-labeled with [γ-^{32}P]ATP. The extracted nuclear proteins (10 μg) were incubated with 0.1 ng ^{32}P-labeled DNA for 15 min at room temperature in 25 μL binding buffer containing 1 μg poly(dI-dC). In the antibody supershift assay, antibodies to NF-κB subunits p50 and p65 (1 μg each) were incubated with the mixture for 10 min at room temperature, followed by the addition of the labeled probe. The mixtures were electrophoresed on 5% nondenaturing polyacrylamide gels. The gels were dried and imaged by autoradiography.

Chromatin immunoprecipitation (ChIP) assays. ECs co-cultured with SMCs were collected by scraping and lysed in L1 buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% NP-40, 10% glycerol). Nuclei were pelleted at 3,000 rpm and resuspended in L2 buffer (50 mM Tris, pH 8.0, 1% SDS, 10 mM EDTA). Chromatin was sheared by sonication and immunoprecipitated with an antibody against p65 [4]. Input and immunoprecipitated chromatin were incubated at 67°C overnight to reverse crosslinks. DNA was extracted with phenol/chloroform, precipitated with ethanol, and analyzed by quantitative real-time PCR using the LightCycler Sequence Detection System (Roche Diagnostics) with LightCycler FastStart DNA MasterPlus SYBR Green I (Roche Diagnostics) and the E-selectin promoter-specific primers (sense: 5′-GGGAAAGTTTTTGGATGCCATT-3′; antisense: 5′-TGTCCACATCCAGTAAAGAGGAAAT-3′; product length, 51 bp) [5].

Protein array assay for detecting cytokines in conditioned media. The array membranes (Human Cytokine Antibody Array C Series 1000, Raybiotech, Inc., Technology Parkway, Norcross, GA) were blocked with 5% BSA/Tris buffered saline (TBS) (0.01 M Tris-HCl pH 7.6/0.15 M NaCl) for 30 min and then incubated with 1 mL of twofold-diluted conditioned media of EC/EC or EC/SMC for 2 h. After extensive washing with TBS/0.1% Tween 20 to remove unbounded proteins, the membranes were incubated with biotin-conjugated anti-cytokine antibodies. Following extensive washing, signals were detected by incubation with HRP-conjugated streptavidin (2.5 pg/mL) coupled with an ECL system (Applied Biosystems).

Immunoprecipitation. ECs in co-culture were scraped and lysed with buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). The cells were disrupted on ice by repeated aspiration through a 21-gauge needle. The same amount of protein from each sample was incubated with the designated antibody for 2 h at 4°C with gentle shaking. The immune complex was then incubated with protein A/G agarose for 1 h and collected by centrifugation. These agarose-bound immunoprecipitates were washed and incubated with boiling sample buffer containing 62 mM Tris-HCl, pH 6.7, 1.25% (w/v) SDS, 10% (v/v) glycerol, 3.75% (v/v) mercaptoethanol, and 0.05% (w/v)

bromphenol blue. The samples were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

Antisense oligonucleotides. Phosphorothio analogue oligonucleotides for the human NF-κB p65 subunit were synthesized and purified by high-performance liquid chromatography (Greiner, Japan). The sequences of oligonucleotides used in this study were as follows: antisense, 5′-GGGGAACAGTTCGTCCATGGC-3′; sense, 5′-GCCATGGA CGAACTGTTCCCC-3′. These oligonucleotides were added to the culture medium of ECs for 24 h before co-culture or shear stress experiments. In parallel experiments, ECs were treated with a proteasome inhibitor, lactacystin (20 μM), or an antioxidant, N-acetyl-cysteine (NAC, 20 mM), for 1 h before the experiments.

Statistical analysis. The results are expressed as mean ± SEM. For protein array results, the expression ratios for each protein from four independent sets of experiments were used to perform paired Student's t-test to assess the significance of the difference of the mean ratio from unity. For other experiments, statistical analysis was performed by using analysis of variance (ANOVA) followed by Scheffe's test for multiple comparisons. $P < 0.05$ was considered statistically significant.

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