Mechanisms of Induction of Endothelial Cell E-selectin Expression by Smooth Muscle Cells and Its Inhibition by Shear Stress

by

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Short title: Shear modulation of SMC-induced E-selectin in EC

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ABSTRACT

E-selectin is a major adhesion molecule expressed by endothelial cells (ECs), which are exposed to shear stress and neighboring smooth muscle cells (SMCs). We investigated the mechanisms underlying the modulation of EC E-selectin expression by SMCs and shear stress. SMC-co-culture induced rapid and sustained increases in expression of E-selectin and phosphorylation of interleukin (IL)-1 receptor-associated kinase and glycoprotein-130, as well as the downstream mitogen-activated protein kinases (MAPKs) and Akt. By using specific inhibitors, dominant-negative mutants, and small interfering RNA, we demonstrated that activations of c-Jun-NH2-terminal kinase (JNK) and p38 of the MAPK pathways are critical for the co-culture-induced E-selectin expression. Gel shifting and chromatin immunoprecipitation assays showed that SMC-co-culture increased the nuclear factor-κB (NF-κB)-promoter binding activity in ECs; inhibition of NF-κB activation by p65-antisense, lactacystin, and N-acetyl-cysteine blocked the co-culture-induced E-selectin promoter activity. Protein arrays and blocking assays using neutralizing antibodies demonstrated that IL-1β and IL-6 produced by EC/SMC co-cultures are major contributors to the co-culture-induction of EC signaling and E-selectin expression. Pre-shearing of ECs at 12 dynes/cm² inhibited the co-culture-induced EC signaling and E-selectin expression. Our findings have elucidated the molecular mechanisms underlying the SMC-induction of EC E-selectin expression and the shear stress-protection against this SMC-induction.

Keywords: Cellular interaction; Endothelial cell; Shear stress; Signal transduction; Smooth muscle cell
INTRODUCTION

During the development of atherosclerotic lesions, vascular smooth muscle cells (SMCs) change from their physiologic contractile phenotype to the pathophysiologic synthetic phenotype and migrate into the intima, where they release pro-inflammatory cytokines and interact with vascular endothelial cells (ECs) to regulate their gene expression and function, including the modulation of leukocyte recruitment [1-3]. ECs are constantly subjected to blood flow-induced shear stress, which can modulate leukocyte-EC interaction and the subsequent leukocyte extravasation into inflamed tissue, mainly by modulating EC surface expression of adhesion molecules [3, 4].

E-selectin is a major EC adhesion molecule that regulates binding and extravasation of leukocytes from bloodstream to sites of inflammation. The effects of cytokines and shear stress on EC E-selectin expression have been extensively studied. The E-selectin gene is rapidly expressed by ECs in response to pro-inflammatory cytokines, and it is more responsive to disturbed and oscillatory flows [5, 6] than to laminar shear stress [7]. Most studies on effects of shear stress on EC gene expression have been performed on EC monolayers, which may not reflect the in vivo environment of ECs, which exist in close proximity to SMCs. By using our newly developed EC/SMC co-culture flow system [8] in which ECs and SMCs are grown on opposite sides of a porous membrane, we demonstrated that co-culture with SMCs induced E-selectin expression in ECs under static condition and that this co-culture-induced E-selectin expression was inhibited by application of shear stress (12 dynes/cm²) to ECs [8]. These results suggest a protective role of shear stress in vascular homeostasis by inhibiting the pro-inflammatory gene expression in ECs located in close proximity to SMCs. The aim of this investigation was to elucidate the mediator(s) and signaling pathway(s) that regulate the SMC-induced E-selectin expression in ECs and the mechanism of its inhibition by shear stress.
To gain insights into the mechanisms by which SMCs and shear stress regulate EC E-selectin expression, we used a cytokine protein array that contains antibodies against 120 cytokines and other proteins to analyze the pro-inflammatory factors produced by EC/SMC co-culture. We found that the cytokines interleukin-1β (IL-1β) and IL-6 produced by EC/SMC can exert paracrine effects on ECs to elevate their E-selectin expression. The E-selectin expression induced by the IL-1β and IL-6 produced by EC/SMC is mediated through the receptor-interacting molecules IL-1 receptor-associated kinase (IRAK) and glycoprotein-130 (gp130), the intracellular signaling cascades c-Jun-NH2-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), and the transcription factor nuclear factor-κB (NF-κB). Before co-culture with SMCs, pre-exposure of ECs to a high shear stress at 12 dynes/cm², but not a low shear stress at 0.5 dynes/cm², inhibits the co-culture-induced signaling and E-selectin expression. Our findings provide a molecular basis for the mechanisms by which (a) SMCs induce E-selectin expression in ECs in close proximity, and (b) high shear stress inhibits this SMC-induced E-selectin expression to effect its protective role in vascular homeostasis.
MATERIALS AND METHODS

Approval was obtained from the Institutional Review Board of National Health Research Institutes of Taiwan for these studies.

Materials. Mouse monoclonal antibodies against extracellular signal-regulated kinase 2 (ERK2; sc-1647), JNK1 (sc-7345), IκBα (sc-1643), p50 (sc-8414), p65 (sc-8008), and p-Tyr (sc-508), mouse monoclonal Phospho-ERK (sc-7383), and Phospho-JNK (sc-6254) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against p38 and Akt, mouse monoclonal Phospho-p38 antibody, and rabbit polyclonal Phospho-Akt antibody were purchased from Cell Signaling Technology (Beverly, MA). The monoclonal E-selectin antibody and neutralizing antibodies against IL-1β, IL-6, basic fibroblast growth factor (bFGF), monocyte chemotactic protein-1 (MCP-1), growth-related oncogene (GRO), regulated-on-activation normal T cell expressed and secreted chemokine (RANTES), stromal cell-derived factor (SDF-1), interferon-inducible T-cell-α chemoattractant (I-TAC), and IL-4 were obtained from R & D systems (Minneapolis, MN). The rabbit polyclonal antibodies against gp130 and IRAK were obtained from Upstate (Lake Placid, NY). The E-selectin promoter construct was a gift from Dr. P.E. DiCorleto (Case Western Reserve University School of Medicine). The catalytically inactive mutant of ERK2 (mERK), RasN17, Raf310, JNK(K-R), and RacN17 were previously described [9]. The ERK-, JNK-, p38-, Akt-, IRAK-, and gp130-specific small interfering RNA (siRNA) and control siRNA were purchased from Invitrogen (Carlsbad, CA). All other chemicals of reagent grade were obtained from Sigma (St Louis, MO).

Cell culture. ECs were isolated from fresh human umbilical cords by collagenase perfusion [10] and grown in Petri dishes in medium 199 (M199; Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS; Gibco) for 3 days. Secondary cultures were used in all experiments. Third-passage human umbilical artery SMCs were obtained
commercially (Clonetics, Palo Alto, CA) and maintained in F12K medium (Gibco) supplemented with 10% FBS. Cells between passages 4 to 6 were used.

**Shear stress experiment.** ECs were seeded onto the outer side of a 10-μm-thick membrane containing 0.4-μm pores ($5 \times 10^5$ cells/cm$^2$; Falcon cell culture inserts; Becton Dickinson, Lincoln Park, NJ) (pre-coated with fibronectin at 30 μg/cm$^2$) [8]. After incubation in M199 containing 2% FBS for 24 h, the membrane with ECs was incorporated into a parallel-plate flow chamber containing a polycarbonate insert holder [8] and connected to a perfusion loop system for the application of shear stress at a high (HSS, 12 dynes/cm$^2$) or low level (LSS, 0.5 dynes/cm$^2$) for 4 or 24 h.

**Co-culture of ECs and SMCs.** After the completion of EC shearing, the opposite (inner) side of the membrane was seeded with SMCs ($2 \times 10^5$ cells/cm$^2$) under static condition, thus forming an EC/SMC co-culture system (the adjacent-bilayer model; Figure 1A) [8]. Prior to seeding onto the membrane, the SMCs collected after trypsinization were incubated in an Ultra Low Attachment Microplate (Costar 3471, Corning Inc., NY) for 2 h to eliminate the effect of trypsinization. Controls had ECs as above, but no SMCs on the opposite side of the membrane (EC/∅). To study the effect of separation of ECs and SMCs, the ECs seeded on the outer side of the membrane were separated from the SMCs plated on the bottom surface of the outer chamber by 1 mm (EC/M/SMC; the media-separation model; Figure 1B). ECs and SMCs were maintained in the shared culture medium containing 2% FBS.

The procedures of (1) RNA isolation, (2) Northern blots, (3) reverse-transcription polymerase chain reaction (RT-PCR), (4) real-time PCR, (5) Western blots, (6) flow cytometric analysis, (7) electrophoretic mobility shift assay (EMSA), (8) immunoprecipitation, (9) chromatin immunoprecipitation (ChIP) assay, (10) reporter gene construct, DNA plasmids, siRNA, transfection, and luciferase assays, (11) protein array assay
for detecting cytokines in conditioned media, (12) antisense oligonucleotides, and (13) statistic analysis are provided in the online supplemental document.
RESULTS

Pre-exposure of ECs to HSS, but not LSS, for 24 h inhibits SMC-induced E-selectin expression in ECs. EC/SMC co-culture in the adjacent-bilayer model (Figure 1A) induced an increase in E-selectin mRNA expression in ECs (detectable within 1 h; Figure 1C). When ECs were separated from the co-cultured SMCs by 1 mm filled with media (media-separation model; Figure 1B), the increase in the EC E-selectin mRNA expression was much slower (detected at 4 h; Figure 1D). The increases in EC E-selectin mRNA expression in both co-culture models sustained for 24 h. Pre-exposure of ECs to HSS at 12 dynes/cm² for 24 h, but not 4 h, significantly inhibited the co-culture-induced E-selectin mRNA expression (Figure 1E). However, LSS at 0.5 dynes/cm² did not have such an inhibitory effect (Figure 1F). As controls, HSS and LSS per se did not alter the E-selectin mRNA expression in mono-cultured ECs in comparison to static cells (online supplemental Figure S1A). Flow cytometric analysis showed that adjacent co-culture for 24 h resulted in an increase in expression of E-selectin protein on EC surface, with a mean fluorescence intensity of 99.8, as compared with 11.3 in EC mono-cultures (Figure 1G). 24-h pre-shearing of ECs at HSS reduced the co-culture-induced E-selectin expression to a mean fluorescence intensity of 18.7.

SMC-induced EC expression of E-selectin and its inhibition by shear stress are mediated by the JNK and p38 pathways. The MAPK superfamily (i.e., ERK, JNK, and p38) and phosphatidylinositol 3-kinase (PI3K)/Akt are known to regulate gene expression and cellular functions [11, 12]. The phosphorylation of ERK, JNK, p38, and Akt in ECs increased rapidly (within 5 min) after co-culture with SMCs, reaching maximal levels at 10 min for Akt, 30 min for ERK and JNK, and 1 h for p38 (Figure 2). Following transient increases, the levels of phosphorylation decreased to nearly basal levels (ERK and p38 at 6 h) or even below (JNK at 4 h and Akt at 2 h). To determine whether the SMC-induced
E-selectin expression is mediated through the MAPKs- or PI3K/Akt-dependent pathway, ECs were incubated with the specific inhibitor for ERK (PD98059; 30 μM), JNK (SP600125; 20 μM), p38 (SB203580; 10 μM), or PI3K/Akt (LY294002; 30 μM) for 1 h before and during co-culture with SMCs. The co-culture-induced mRNA (Figure 3A) and surface protein (Figure 3B) expressions of EC E-selectin were significantly inhibited by SP600125 and SB203580, but not by PD98059 and LY294002. Treatment of ECs with SP600125 and SB203580 simultaneously did not result in additive inhibition in co-culture-induced E-selectin mRNA (Figure 3A) and surface protein (data not shown) expressions. Pre-exposure of ECs to HSS, but not LSS, for 24 h significantly inhibited the co-culture-induced JNK and p38 phosphorylation (Figure 3C), suggesting that the inhibitory effect of HSS on co-culture-induced E-selectin expression was attributable, at least in part, to its inhibition of the co-culture-induced JNK and p38 activation. As controls, HSS and LSS per se had only minor effects on the activations of ERK, JNK, p38, and Akt in ECs in comparison to static controls (online supplemental Figures S1B to S1E).

To further confirm the involvement of JNK and p38, but not ERK and PI3K/Akt, pathways in the modulation of E-selectin expression in ECs by SMC-co-culture and shear stress, we examined the effects of the dominant-negative mutants or siRNA of these signaling pathways on E-selectin expression in ECs co-cultured with SMCs in the presence or absence of pre-shearing. The co-culture-induced E-selectin mRNA expression was inhibited by transfections of ECs with JNK- or p38-specific siRNA (100 μmol/mL for each), the dominant-negative mutant of Ras (RasN17; 1 μg/mL) or Rac (RacN17; 0.5 μg/mL), or a catalytically inactive mutant of JNK [JNK(K-R); 1 μg/mL], but not by transfecting with ERK- or Akt-specific siRNA (100 μmol/mL for each), the dominant-negative mutant of Raf-1 (Raf301; 1 μg/mL), or a catalytically inactive mutant of ERK2 (mERK; 0.25 μg/mL).
(Figure 3D). The effectiveness of these treatments was validated: ERK-, JNK-, p38-, and Akt-specific siRNA (compared with control siRNA) caused a 75% reduction in ERK, JNK, p38, and Akt protein expressions, respectively (Figure 3E). The RasN17, RacN17, and JNK(K-R) inhibited co-culture-induced JNK phosphorylation (compared with empty vector control PSRα), and the Raf301 and mERK caused inhibitions in co-culture-induced ERK phosphorylation (compared with control PSRα) (Figure 3E). ECs transfected with the E-selectin-Luc showed an increase in promoter activity by the SMC-co-culture to 3.3-fold, as compared with the EC mono-cultures (Figure 3F). Pre-shearing of ECs at HSS, but not LSS, abolished this SMC-increased E-selectin promoter activity. Co-transfection of ECs with the empty vector control PSRα or control siRNA had no effect on co-culture-induced E-selectin promoter activity. Co-transfection of ECs with p38-specific siRNA, RasN17, RacN17, or JNK(K-R) resulted in a significant inhibition in the co-culture-induced E-selectin promoter activity. However, co-transfection with the Akt-specific siRNA, Raf301, or mERK had no effect on the SMC-co-culture inducibility. These results provide additional evidence that the JNK and p38 pathways play significant roles in the regulatory effects of SMCs and shear stress on E-selectin expression in ECs.

**SMC-induced EC expression of E-selectin and its inhibition by shear stress are dependent on NF-κB.** NF-κB is an important mediator for cellular responses to inflammatory stimuli [13]. We examined the effects of interference with NF-κB expression on the SMC-induction of E-selectin expression in ECs by using the antisense oligonucleotides to NF-κB subunit p65 (p65-antisense), the transcriptional inhibitor lactacystin, and the antioxidant N-acetyl-cysteine (NAC). The addition of both NAC and lactacystin to ECs transfected with the E-selectin-Luc completely abolished the co-culture-induced E-selectin promoter activity (Figure 4A). Moreover, co-transfection of
the cells with p65-antisense, but not p65-sense, oligonucleotides significantly inhibited the co-culture-induced promoter activity. The results of EMSA showed that co-culture with SMCs caused the NF-κB-DNA binding activity in the EC nucleus to increase within 10 min (Figure 4B) and remain elevated for at least 2 h. This increase in NF-κB-DNA binding activity was accompanied by a concomitant decrease in IκBα, an inhibitory protein that prevents translocation of NF-κB dimers into the nucleus (Figure 4C). Pre-shearing of ECs at HSS (but not LSS) significantly inhibited the increase in NF-κB-DNA binding activity (Figure 4D) and the decrease in IκBα protein expression induced by SMC-co-culture (Figure 4F). Pre-shearing at HSS for 24 h had no effects on NF-κB-DNA binding activity in ECs in comparison to static controls; in contrast, pre-shearing at LSS resulted in an increase in NF-κB-DNA binding activity in ECs (online supplemental Figure S1F). SP600125 and SB203580 did not have significant effects on the co-culture-mediated changes in NF-κB-DNA binding activity (Figure 4E) and IκBα protein expression (Figure 4F). The formation of the NF-κB-DNA complex required the presence of wild-type NF-κB-binding site, as demonstrated by lack of direct binding to or competition by the mutant oligonucleotide, whereas excess unlabeled wild-type oligonucleotide was able to effectively compete with 32P-labeled oligonucleotide for NF-κB binding (Figure 4G). The specificity of this binding for NF-κB was further substantiated by the supershifting in gel mobility of the NF-κB-DNA complex after pre-incubation of nuclear proteins with an antibody to p65.

To further assess the in vivo regulation of NF-κB binding to the promoter regions of E-selectin gene in ECs co-cultured with SMCs in the presence or absence of pre-shearing, we performed ChIP assays in these ECs by using an antibody against p65 and the promoter-specific primers. ECs co-cultured with SMCs increased the in vivo NF-κB binding to their E-selectin promoter as early as 10 min after the co-culture, reaching maximal
levels (~4.8-fold in comparison to mono-cultured ECs) within 30 min (Figure 4H). The levels of NF-κB-promoter binding declined after 2 h of co-culture with SMCs, but still remained higher than mono-cultured ECs. This co-culture-induced in vivo NF-κB binding to the E-selectin promoter was abolished by pre-shearing of ECs at HSS (but not LSS), and it was not blocked by pre-treatment of ECs with SP600125 and/or SB203580 (Figure 4I).

**IL-1β and IL-6 produced by EC/SMC are the major factors contributing to the SMC-induced signaling and E-selectin expression in ECs.** The increases in E-selectin expression in ECs by SMC-co-culture with the media-separation model suggest that the co-culture caused the release of certain mediator(s) to exert paracrine effects on ECs to induce their E-selectin expression. To address this possible paracrine effect, we examined the expression levels of cytokines in the conditioned media of EC/SMC and EC/EC by using a human cytokine array system containing antibodies against 120 cytokines and other proteins (online supplemental Figure S2 and Table S1). Using this array, we identified the proteins whose expression was significantly different between the media bathing the EC/SMC vs. EC/EC (i.e., for \( P \leq 0.05 \) and the mean co-culture/mono-culture ratio \( \geq 2 \) or \( \leq 0.5 \)). Applying these criteria to analyze the results on 120 cytokines present on the array, we identified IL-1β and IL-6 as proteins that were released at significantly higher levels from EC/SMC than EC/EC (Figure 5A and online supplemental Table S1), with the protein ratios of 4.16 ± 0.12 and 4.13 ± 0.19, respectively. Incubation of ECs with a neutralizing antibody against IL-6 or IL-1β (or in combination) significantly inhibited the co-culture-induced E-selectin mRNA expression (Figure 5B), as well as the co-culture-induced increases in JNK and p38 phosphorylation (Figure 5C) and NF-κB-DNA binding activity (Figure 5D), and decreases in IκBα protein levels (Figure 5E). In concert with these results, ChIP assays revealed that incubation of ECs with antibodies against IL-1β and/or IL-6 blocked the
co-culture-induced *in vivo* binding of NF-κB to the E-selectin promoter in ECs (Figure 5F). As controls, incubation of ECs with a neutralizing antibody against bFGF, MCP-1, GRO, RANTES, or SDF-1 (whose expression was elevated in the EC/SMC vs. EC/EC), I-TAC (whose expression was not changed), or IL-4 (whose expression was reduced) did not inhibit the co-culture-induced E-selectin mRNA expression (online supplemental Figure S3).

**IRAK and gp130 are involved in regulatory effects of SMC-co-culture and shear stress on EC E-selectin expression.** Given our findings that IL-6 and IL-1β produced by EC/SMC co-cultures are the major factors contributing to the SMC-induced EC signaling and E-selectin expression and that pre-shearing (at HSS) of ECs inhibited these SMC-induced changes, we postulated that the modulation of EC signaling and gene expression by SMC-co-culture and shear stress may be mediated via the IL-6 and IL-1β receptors in ECs. To test this possibility, we used the specific siRNA against gp130, which is an IL-6 receptor, and IRAK, which forms a complex with the receptor of IL-1β upon its stimulation, to suppress the expressions of gp130 and IRAK and examined their effect on the SMC-induced E-selectin expression. Transfection of ECs with gp130- and IRAK-specific siRNA at concentrations of 100 μmol/mL reduced the expressions of gp130 and IRAK mRNAs by 80%, as compared with the cells transfected with control siRNA (Figure 6A). These reductions of gp130 (Figure 6B) and IRAK (Figure 6C) were accompanied by a decrease of the SMC-induced E-selectin expression in ECs. To further examine the effect of SMC-co-culture and shear stress on the activation of gp130 and IRAK in ECs, extracts of ECs co-cultured with SMCs in the presence or absence of pre-shearing were immunoprecipitated with an antibody against gp130 or IRAK, followed by Western blot analysis with an antibody against p-Tyr or IRAK. Co-culturing ECs with SMCs induced phosphorylation of gp130 and IRAK over the 30-min period tested (Figure 6D), and such increases in gp130 and IRAK phosphorylation were inhibited by pre-shearing ECs at HSS.
(but not LSS) for 24 h (Figure 6E). These results suggest that the effects of SMC-co-culture and shear stress on EC signaling and gene expression were mediated, at least in part, through the modulations of IL-6 and IL-1β receptor activation in ECs.
DISCUSSION

E-selectin, which plays significant roles in atherosclerosis, is a major adhesion molecule expressed by vascular ECs, which exist in close proximity to vascular SMCs and are constantly subjected to blood flow-induced shear stress. Our present study aims at elucidating the molecular mechanisms underlying the roles of SMCs and shear stress in modulating E-selectin expression in ECs. Using our newly developed EC/SMC co-culture flow system, we demonstrated that co-culture of ECs with SMCs under static condition induced rapid and sustained increases of EC E-selectin expression. This increase in E-selectin expression was attributable, at least in part, to the paracrine effects of cytokines IL-1β and IL-6 produced by the EC/SMC co-culture, acting on the ECs to activate their receptor-interacting molecules IRAK and gp130, as well as the downstream JNK/p38 and NF-κB pathways. Pre-exposure of ECs to a high level, but not a low level, of shear stress significantly inhibited such co-culture-induced signaling and E-selectin expression. Our findings provide a molecular basis for the mechanisms underlying the SMC-induction of EC E-selectin expression and the protective function of shear stress against this SMC-induction.

The paracrine effect exerted by SMCs on ECs to induce E-selectin expression was substantiated by the increased expression of E-selectin in ECs co-cultured with SMCs in a media-separation model (Figures 1B and 1D), in which the two types of cells were separated by 1 mm of media. The time needed to induce E-selectin expression, as expected, was longer (4 h) than that in the adjacent-bilayer model (1 h; Figures 1A and 1C). This slower induction of E-selectin in the media-separation model may be attributed to the longer distance between ECs and SMCs and the lower concentration of paracrine substances reaching the ECs, in comparison to the adjacent-bilayer model. By using protein arrays to perform a systematic analysis of the expression levels of cytokines produced in the co-cultures, we
identified IL-1β and IL-6 as proteins whose expression in the conditioned media of EC/SMC was significantly higher than that of EC/EC. The higher levels of IL-1β and IL-6 in the media of EC/SMC may not due to increased productions of IL-1β and IL-6 by co-cultured ECs, since the expressions of these two cytokines in ECs were not altered by their co-culture with SMCs (data not shown). IL-1β and IL-6 have been shown to be highly expressed in human and experimental atherosclerotic lesions and are recognized as biomarkers of activated SMCs [14]. Thus, the SMCs used in the present study exhibit characteristics resembling neointimal SMCs, which display a synthetic phenotype characterized by increased cytokine expression [2, 3]. Our recent data showed that SMCs under the condition of the present experiments had lower levels of expression of contractile marker proteins (e.g., smooth muscle α-actin, myosin heavy chain, h-caldesmon, and calponin) than those cultured in the medium containing only 0.5% FBS [4]. We have found by using protein array that the SMCs used in the present study had higher levels of IL-1β and IL-6 expression than the ECs (data not shown). Treatment of mono-cultured ECs with IL-1β or IL-6, or with the supernatant of SMCs, mimicked the effect of SMC-co-culture in inducing EC E-selectin expression (data not shown). These results suggest that the SMCs in our present study were in a synthetic phenotype, which may affect the adjacent ECs to induce their pro-inflammatory gene expression and function through the paracrine release of IL-1β and IL-6.

By using neutralizing antibodies against IL-1β and IL-6, we have demonstrated that these two cytokines produced by EC/SMC are the major factors contributing to the co-culture-induced E-selectin expression in ECs. This co-culture-induced E-selectin expression was mediated by the IRAK/gp130 and downstream JNK/p38 and NF-κB pathways. Several lines of evidence support this finding. First, ECs co-cultured with
SMCs induced a rapid phosphorylation of IRAK/gp130, which have been shown to activate several intracellular signaling pathways, including MAPKs, PI3K/Akt, and NF-κB [15]. Second, co-culture with SMCs induced rapid phosphorylations of ERK, JNK, p38, and Akt in ECs; however, only specific inhibitors to JNK and p38 (i.e., SP600125 and SB203580) inhibited the co-culture-induced E-selectin expression, suggesting that the activation of JNK/p38 is critical for the co-culture-induced E-selectin expression. The involvement of JNK/p38 pathway in co-culture-induced E-selectin expression was confirmed by the inhibition of co-culture-induced E-selectin mRNA expression and promoter activity in ECs by transfecting with a dominant negative mutant of JNK, Rac, or Ras, or a specific siRNA of JNK or p38. Third, the results of EMSA and ChIP assays showed that the SMC-co-culture increased the binding activity and in vivo promoter binding of NF-κB in EC nuclei. This increase in NF-κB binding activity was accompanied by a reduction of IκBα. NF-κB inhibitors, including p65-antisense, lactacystin, and NAC, inhibited the co-culture-induced E-selectin promoter activity, suggesting the involvement of NF-κB in the co-culture-induced E-selectin expression. Finally, the inhibitory effects of neutralizing antibodies against IL-1β and IL-6 on the co-culture-induced activations of JNK/p38 and NF-κB and E-selectin expression in ECs indicate that the effects of SMC-co-culture are mediated by the binding of IL-1β and IL-6 to their corresponding receptors in ECs. Moreover, the involvement of IRAK and gp130 in co-culture-induced E-selectin expression was confirmed by the reduction of this E-selectin expression in ECs by transfecting with a specific siRNA of IRAK or gp130.

It has been demonstrated that IL-6 exerts its biological activity through binding to its specific receptor (IL-6R) and gp130, which serves as a signal-transducing unit [16]. However, some studies have suggested that ECs do not express IL-6R and require exogenous soluble IL-6R (sIL-6R) to trigger gp130-signaling [17], while others have reported that ECs
do express IL-6R and that IL-6 can directly activate EC signaling and gene expression [18, 19]. These differences in results could be due to the functional and molecular heterogeneity that exists among different types of ECs [20]. Since direct addition of IL-6 (2.5 ng/mL, Sigma) to EC mono-cultures without exogenous addition of sIL-6R can mimic the effects of SMC-co-culture in inducing EC activation of gp130, MAPKs, and NF-κB, and expression of E-selectin (data not shown), the results suggest that IL-6 may be able to exert direct effects on these signaling and gene expression in ECs. Using RT-PCR and ELISA we have shown that the ECs used in the present study have constitutive expressions of IL-6R and sIL-6R, whereas the expressions of IL-6R and sIL-6R by SMCs were not detectable (online supplemental Figure S4). It is probable that the constitutive expressions of IL-6R and sIL-6R by ECs can contribute to the SMC/IL-6-signaling in ECs.

Read et al. [21] have identified NF-κB and positive domain II (PDII), which contains a cAMP-responsive element/activating transcription factor (ATF)-like binding site, in the E-selectin promoter as responsive elements for expression of this gene induced by tumor necrosis factor-α (TNF-α). They showed that a heterodimer of transcription factors ATF-2 and c-JUN is constitutively bound to the PDII site, and that TNF-α stimulation of ECs induces marked activation of the JNK and p38 and their associations with c-JUN and ATF-2. Using immunoprecipitation assay, we have shown that co-culture of ECs with SMCs for 30 min induces an increase in JNK/c-JUN and p38/ATF-2 associations (online supplemental Figure S5), suggesting that the c-JUN and ATF-2 are downstream of JNK and p38 and may also be involved in SMC-induced E-selectin expression in ECs. Our results of EMSA using double-stranded oligonucleotides containing the PDII site (5’-GTACAATGATGTCAGAAA CTCTGTC-3’) [21] did not show specific DNA bindings of c-JUN/ATF-2 in the nucleus of ECs co-cultured with SMCs. Thus, the c-JUN/ATF-2 responsive element in the E-selectin promoter in ECs in response to co-culture with SMCs remains to be determined. In our
present study, the specific inhibitors of JNK and p38 (i.e., SP600125 and SB203580) did not inhibit the co-culture-mediated increases in NF-κB-DNA binding activity and in vivo NF-κB-promoter binding, nor the decrease in the protein level of IκBα. This is in agreement with the findings by Read et al. [21] that NF-κB and JNK/p38 represent two separate signaling pathways, both of which are required for inflammatory cytokine responsiveness of E-selectin. It is possible that these two pathways are rapidly activated and converge on the E-selectin promoter to result in full activation of this gene in ECs by co-culture with SMCs.

Our results indicate that the ‘pre-conditioning’ of ECs by different levels of shear stress (HSS vs. LSS) differentially modulates the response of EC gene expression to SMC-co-culture. The way in which HSS inhibits the EC E-selectin expression in co-cultures is likely to be multifactorial. Blocking assays using antibodies against αvβ3 and β1 integrins, which are well-recognized mechanosensors in ECs in response to shear stress [22], did not eliminate the inhibitory effect of shear stress on SMC-induced E-selectin expression (online supplemental Figure S6), suggesting that integrins may not be involved in the effect of shear stress on E-selectin expression in co-cultured ECs. Since our results showed that HSS-pre-shearing can inhibit the co-culture-mediated activation of IRAK/gp130, JNK/p38, and NF-κB and reduction of IκBα, it is likely that the shear-mediated inhibition in co-culture-induced E-selectin expression is attributable to the shear-mediated inhibition of the activation of these signaling pathways in co-cultured ECs. In addition, our previous study showed that the SMC-induction of pro-inflammatory genes in ECs was associated with their attenuation of endothelial nitric oxide synthase (eNOS) expression [8]. Given that HSS can induce up-regulation of eNOS and production of nitric oxide (NO) [23-26], the inhibitory effect of HSS on SMC-induced E-selectin expression in ECs could also be associated with
the increased levels of NO in pre-sheared ECs. Moreover, krüppel-like factor 2 (KLF2) has been shown to be an important regulator of EC activation in response to pro-inflammatory stimuli and shear stress [27-30]. Given that (1) the KLF2 expression in ECs can be inhibited by IL-1β and induced by shear stress and (2) overexpression of KLF2 can inhibit the IL-1β-induction of E-selectin in ECs [28], it is likely that the KLF2 expression in ECs could be down-regulated by their co-culture with SMCs and the inhibitory effect of pre-shearing on SMC-induced EC E-selectin expression could be due to, at least in part, the increase in the KLF2 expression in pre-sheared ECs. Thus, KLF2 may serve as an additional mediator of the effects of SMCs and shear stress on E-selectin expression in ECs.

In summary, our present study has characterized the mechanisms by which (a) SMCs in close adjacency to ECs induce EC E-selectin expression and (b) shear stress inhibits this SMC-induced E-selectin expression (summarized in Figure 7). The cytokines IL-1β and IL-6 produced by EC/SMC co-culture may interact with their corresponding receptors in ECs to induce their coupling with and activation of receptor-associated proteins IRAK and gp130 and the consequent activation of both JNK/p38 and NF-κB signaling pathways, and ultimately E-selectin expression. Shear stress may inhibit the SMC-induced E-selectin expression via the inhibition in SMC-activation of IRAK/gp130, JNK/p38 and NF-κB. Our findings provide insights into the mechanisms underlying the interplays of SMCs with ECs and the protective homeostatic function of shear stress in modulating EC signaling and gene expression.
REFERENCES


9. Wung BS, Cheng JJ, Chao YJ, Hsieh HJ, Wang DL. Modulation of Ras/Raf/extracellular...


FIGURE LEGENDS

**Figure 1.** Pre-shearing of ECs at HSS, but not LSS, inhibits SMC-induced EC E-selectin expression. ECs were kept as controls (EC/∅) or co-cultured with SMCs in an adjacent-bilayer model (EC/SMC) (A, C, and E-G) or a media-separation model (EC/M/SMC) (B and D) for 4 h (E and F), 24 h (G), or the times indicated (C and D). The E-selectin mRNA (C-F) and surface protein (G) expressions of these ECs were determined by using the Northern blot and flow cytometric analyses, respectively. In some experiments, ECs were pre-sheared at HSS (12 dynes/cm²) for 4 h (HS4) or 24 h (HS24) or LSS (0.5 dynes/cm²) for 24 h (LS24) before SMC-co-culture (E-G). Control ECs were co-cultured with SMCs without pre-shearing (CL) (E and F). Data in (C-F) are presented as percentage changes in band density from control EC/∅ normalized to GAPDH RNA level and are shown as mean±SEM from three independent experiments. *p < 0.05 vs. control EC/∅. #p < 0.05 vs. control EC/SMC. Results of flow cytometric analysis (G) are representative of triplicate experiments with similar results. ECs incubated with FITC-conjugated control IgG or FITC-conjugated antibody alone were used as IgG or negative controls (i.e., Blanks: B). Numbers are mean±SEM of mean fluorescent intensity for all experiments determined by comparison with corresponding negative controls.

**Figure 2.** Co-culture with SMCs induces ECs to increase their phosphorylation of ERK (A), JNK (B), p38 (C), and Akt (D). ECs were kept as controls (EC/∅) or co-cultured with SMCs in the adjacent-bilayer model (EC/SMC) for the times indicated, and the phosphorylations of their ERK, JNK, p38, and Akt were determined by using Western blot analysis. The amounts of phosphorylated ERK, JNK, p38, and Akt proteins in EC/SMC are presented as band densities (normalized to the total protein levels) relative to those in control EC/∅. The results are mean±SEM from three independent experiments.
*p < 0.05 vs. control EC/∅.

**Figure 3.** **Induction of EC E-selectin expression by SMC-co-culture and its inhibition by shear stress are mediated by JNK and p38 pathways.** ECs were kept as controls (EC/∅) or co-cultured with SMCs in the adjacent-bilayer model (EC/SMC) for 30 min (C and E), 4 h (A and D), or 24 h (B and F). Before kept as controls or co-culture with SMCs, ECs were (1) pre-treated with PD98059 (PD; 30 μM), SP600125 (SP; 20 μM), SB203580 (SB; 10 μM), or LY294002 (LY; 30 μM) individually or SP600125 and SB203580 simultaneously (CB) for 1 h (A and B), (2) pre-sheared at HSS (HS) or LSS (LS) for 24 h (C), or (3) transfected with control siRNA or a specific siRNA of ERK, JNK, p38, or Akt (100 μmol/mL for each), or empty vector control PSRα (1 μg/mL), RasN17 (1 μg/mL), RacN17 (0.5 μg/mL), JNK(K-R) (1 μg/mL), Raf301 (1 μg/mL), or mERK (0.25 μg/mL) for 48 h (D-F). Control ECs (CL) were co-cultured with SMCs without any pre-treatment (A) or pre-shearing (C). In some experiments, ECs were transfected with different siRNA and chimera at various concentrations for 48 h (E). In (F), ECs were co-transfected with chimera (2 μg) containing 540 bp of E-selectin promoter region and the reporter gene luciferase. The mRNA (A and D) or surface protein (B) expression or promoter activity (F) of E-selectin or the expression or phosphorylation (C and E) of different MAPKs or Akt in these ECs was determined by using Northern blot (A), flow cytometric (B), Western blot (C, E), real-time PCR (D), or luciferase assay analysis (F), respectively, as described in Materials and Methods. The results in (A, C, D, and F) are shown as mean±SEM from three or four separate experiments, and the results in (B and E) are representative of triplicate experiments with similar results. Data in (A and C) are presented as percentage changes in band density from control EC/∅ normalized to 18S RNA (A) or JNK or p38 protein level (C). In (B), ECs incubated with FITC-conjugated control IgG or FITC-conjugated antibody alone were
used as IgG controls or negative controls (i.e., Blanks: B). Numbers are mean±SEM of mean fluorescent intensity for all experiments determined by comparison with corresponding negative controls. *p < 0.05 vs. control EC/∅.  #p < 0.05 vs. control EC/SMC.  $p < 0.05 vs. cells transfected with control siRNA or empty vector control PSRα.

**Figure 4.** NF-κB is involved in SMC- and shear stress-modulations of E-selectin expression in ECs.  ECs were kept as controls (EC/∅) or co-cultured with SMCs in the adjacent-bilayer model (EC/SMC) for 30 min (D-G and I), 24 h (A), or indicated times (B, C, and H).  Before kept as controls or co-culture with SMCs, ECs were (1) transfected with E-selectin-Luc for 48 h and/or pre-treated with antisense (p65-a; 1 μg/mL) or sense (p65-s; 1 μg/mL) oligonucleotides to the NF-κB subunit p65 for 24 h or with lactacystin (Lac; 20 μM) or N-acetyl-cysteine (NAC; 20 mM) for 1 h (A), (2) pre-sheared at HSS (HS) or LSS (LS) for 24 h (D, F, and I), or (3) pre-treated with SP600125 (SP; 20 μM) or SB203580 (SB; 10 μM) individually or in combination (CB) for 1 h (E, F, and I).  Control ECs (CL) were co-cultured with SMCs without any pre-shearing (D, F, and I) or pre-treatment (D, F, and I).  The E-selectin promoter activity (A), NF-κB-DNA binding activity (B, D, E, and G), IκBα protein expression (C and F), and in vivo NF-κB-promoter binding (H and I) in these ECs were determined by using luciferase assay (A), EMSA (B, D, E, and G), Western blot analysis (C and F), and ChIP assay (H and I), respectively, as described in Materials and Methods.  In some experiments (G), EMSA was performed using total nuclear extracts and 32P-labeled oligonucleotides containing wild-type (CL) or mutant (Mut) human E-selectin NF-κB binding sites.  The specificity of the retarded complexes (NF-κB) was assessed by pre-incubating the nuclear extracts either with 20-fold excess unlabeled oligonucleotides (wild-type or mutant) as a competitor or with p50 and/or p65 antibodies (1 μg).  Nuclear extracts pre-incubated with the p65 antibody show a super shift band (SH) (G).  The results
in (B-G) are representative of two or three independent experiments with similar results. Data in (A, H, and I) are represented as mean±SEM from three to five independent experiments. *p < 0.05 vs. control EC/∅. #p < 0.05 vs. control EC/SMC.

**Figure 5.** IL-1β and IL-6 produced by EC/SMC are the major factors contributing to the SMC-induced signaling and E-selectin expression in ECs. (A) Detection of protein levels of cytokines in conditioned media of EC/EC or EC/SMC co-culture. The membranes spotted with antibodies against 120 different cytokines and other proteins (online supplemental Figure S2) were incubated with twofold-diluted conditioned media of EC/EC or EC/SMC co-culture, and then incubated with a mixture of biotin-labeled antibodies, as described in Materials and Methods. Signal detection by ECL shows that the expression levels of IL-1β (spots in solid boxes) and IL-6 (spots in dash boxes) produced by EC/SMC were significantly higher than that of EC/EC. Results are representative of four independent experiments with similar results. (B-F) ECs were kept as controls (EC/∅) or co-cultured with SMCs in the adjacent-bilayer model (EC/SMC) for 30 min (C-F) or 4 h (B). In parallel experiments, ECs were pre-incubated with a neutralizing antibody against IL-1β or IL-6 (5 μg/mL for each) or their combination for 1 h, and then co-cultured with SMCs in the presence of the antibodies. Control ECs were co-cultured with SMCs in the presence of control IgG (CL). The E-selectin mRNA expression (B), JNK and p38 phosphorylations (C), NF-κB-DNA binding activity (D), IκBα protein expression (E), and *in vivo* NF-κB-promoter binding (F) were determined by using Northern blot analysis, Western blot analysis, EMSA, and ChIP assay, respectively, as described in Materials and Methods. Data in (B, C, and E) are presented as percentage changes in band densities from control EC/∅ normalized to GAPDH RNA (B), JNK or p38 protein (C), or actin protein (E). The results shown are mean±SEM from three to four independent experiments. *p < 0.05 vs. control EC/∅. #p <
0.05 vs. control EC/SMC.

**Figure 6.** IRAK and gp130 contribute to the modulation of EC signaling and E-selectin expression by SMC-co-culture and shear stress. (A) ECs were kept as controls or transfected with control siRNA (siCL) or a specific siRNA of gp130 (sigp130) or IRAK (siIRAK) at indicated concentrations for 48 h, and their gp130 or IRAK mRNA expression was determined by RT-PCR analysis, as described in Materials and Methods. (B-E) ECs were kept as controls (EC/∅) or co-cultured with SMCs in the adjacent-bilayer model (EC/SMC) for 10 min (E), 4 h (B and C), or indicated times (D). Prior to co-culture with SMCs, ECs were transfected with control siRNA or a specific siRNA of gp130 (B) or IRAK (C) (100 μmol/mL for each) for 48 h, or were pre-sheared at HSS (HS) or LSS (LS) for 24 h (E). Control ECs (CL) were co-cultured with SMCs without transfection (B and C) or pre-shearing (E). The mRNA expressions of E-selectin and gp130 (B) or IRAK (C), or the phosphorylations of gp130 and IRAK (D and E) in these ECs were determined by using RT-PCR analysis or immunoprecipitation assay and Western blot analysis, respectively. Results in (A-E) are representative of triplicate experiments with similar results.

**Figure 7.** Schematic representation of the signaling pathways regulating SMC-induced E-selectin expression in ECs and its inhibition by shear stress.
Figure 1 (-continue-)

(A) Schematic diagram of the experimental setup showing ECs and SMCs separated by a 0.4 μm pore insert. The distance between ECs and SMCs is 10 μm.

(B) Schematic diagram of the experimental setup showing ECs and SMCs separated by a 1 mm gap.

(C) Western blot analysis of E-selectin and GAPDH mRNA levels in EC/∅ and EC/SMC treated with various time points (1, 2, 4, 6, 24 h).

(D) Western blot analysis of E-selectin and GAPDH mRNA levels in EC/M/SMC treated with various time points (1, 2, 4, 6, 24 h).

* indicates statistical significance compared to EC/∅.
Figure 1

(E) mRNA levels of E-selectin and GAPDH in EC/∅, EC/SMC, CL, HS4, and HS24 conditions. Bars represent mean ± SEM, *p < 0.05, #p < 0.01.

(F) mRNA levels of E-selectin and GAPDH in EC/∅, EC/SMC, CL, HS24, and LS24 conditions. Bars represent mean ± SEM, *p < 0.05, #p < 0.01.

(G) Flow cytometry analysis of IgG, EC/∅, EC/SMC, HS24+EC/SMC, and LS24+EC/SMC. Bars represent mean ± SEM, *p < 0.05, #p < 0.01.
Figure 2
Figure 3 (continue)

(A) mRNA Level (% of EC/∅)

(B) Level of Phosphorylation (% of EC/∅)

(C) Level of Phosphorylation (% of EC/∅)
Figure 3 (continue-)
Figure 3
Figure 4 (-continue-)

(A) E-selectin-Luc

Fold of Induction (Coculture/Monoculture)

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(B) EC/∅ EC/SMC

10’   30’  1h  2h

NF-κB

free probe

(D) EC/∅ EC/SMC

CL    HS    LS

NF-κB

free probe
Figure 4 (-continue-)
Figure 4
Figure 5 (continue)
(D) EC/∅ CL IL6 IL1 IL6+IL1

NF-κB

free probe

(E) EC/∅ CL IL6 IL1 IL6+IL1

kBα Protein Level (% of EC/∅)

(F) EC/∅ CL IL6 IL1 IL6+IL1

NF-κB Binding Level (% of EC/∅)

Figure 5
Figure 6
Shear stress

Endothelial cell

JNK, p38

IκBα

NF-κB

c-JUN/ATF-2

E-selectin

IL-6

IL-1β

Smooth muscle cell

Nucleus

Figure 7
Mechanisms of induction of endothelial cell E-selectin expression by smooth muscle cells and its inhibition by shear stress

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