E-selectin Gene Expression Is Induced Synergistically with the Coexistence of Activated Classic Protein Kinase C and Signals Elicited by Interleukin-1β but Not Tumor Necrosis Factor-α*

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Masahiro Tamaru† and Shosaku Narumi‡§¶
From the †Preventive Medicine, School of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan and the ‡Department of Molecular Preventive Medicine, School of Medicine, University of Tokyo, Tokyo 113-0033, Japan

We have examined the effect of protein kinase C (PKC) on the expression of the E-selectin and intercellular adhesion molecule-1 (ICAM-1) mRNAs in human umbilical vein endothelial cells. The lower classic PKC activity on pretreatment with phorbol ester (phorbol 12-myristate 13-acetate (PMA)) for 24 h markedly decreased IL-1β-induced E-selectin mRNA expression in the presence of fetal calf serum and basic fibroblast growth factor, although the induction of ICAM-1 mRNA expression was only influenced a little by the PKC down-regulation. On the other hand, tumor necrosis factor-α (TNFα)-induced gene expression of these adhesion molecules was unaffected by such PKC modulation. The intracellular signals generated by interleukin (IL)-1β and TNFα themselves are not mediated through classic PKC activation, because the response to neither stimulant was inhibited by the PKC down-regulation. Because the response to IL-1β was inhibited by the PKC down-regulation, the intracellular signals generated by IL-1β and TNFα are not mediated through classic PKC activation, because the response to neither stimulant was inhibited by the PKC down-regulation. The lower classic PKC activity on pretreatment with PMA for 24 h markedly decreased IL-1β-induced E-selectin mRNA expression in the presence of fetal calf serum and basic fibroblast growth factor. Simultaneous treatment with IL-1β and PMA synergistically induced E-selectin gene expression but not when TNFα was substituted for IL-1β. ICAM-1 mRNA expression was only additively induced on the cotreatment. The synergistic effect on E-selectin mRNA induction was independent of de novo protein synthesis and mediated by elevated transcriptional activity. Promoter analysis of E-selectin indicated that the NF-ELAM1/activating transcription factor element is critical for the synergistic effect of the cotreatment with IL-1β and PMA.

Adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular adhesion molecule-1 (VCAM-1) contribute to the recruitment of leukocytes not only during the inflammatory or immune response, but also during the injury following ischemia-reperfusion (1–5) and the development of atherosclerosis (6, 7). Indeed, there has been an explosion of interest in these topics, which has been fueled by remarkable advances in new therapies directed at several adhesion molecules (reviewed in Ref. 8). Most of these molecules are inducible and regulated through gene expression. Regulation of adhesion molecule expression has been largely studied in vitro using human umbilical vein endothelial cells (HUVEC), in which two monokines, IL-1 and TNFα, can induce these adhesion molecules in the same manner through NF-κB activation (9–11). However, a study involving dermal microvascular endothelial cells has shown that ICAM-1 is induced by either IL-1 or TNFα, whereas VCAM-1 is induced only by TNFα (12), suggesting that the signals generated by IL-1 and TNFα for adhesion molecule induction are different in part.

We have examined whether or not there is such tissue-specific regulatory control of the gene expression of adhesion molecules in vivo, since the expression of chemokine mRNAs following systemic treatment with proinflammatory cytokines was induced in a tissue-specific manner in mice (13, 14). Recently, we found that E-selectin mRNA expression following systemic treatment with IL-1β was induced in the heart in a tissue-specific manner and that IL-1β was a much stronger inducer of E-selectin mRNA than TNFα, while ICAM-1 and VCAM-1 were induced similarly by either IL-1β or TNFα (15). We assumed the presence of heart-specific microenvironments as one reason. There are many stimuli that affect cells in various tissues, including localized cytokines, growth factors, and specific extracellular matrices. Although the intracellular signals elicited by them remain to be elucidated, some of them would induce the activation of protein kinase C (PKC) (16–21). Since E-selectin mRNA expression is also induced by treatments that activate PKC (22), we have examined the cooperative effect of PKC activation on IL-1β- or TNFα-induced gene expression of E-selectin.

Our results indicate that although the intracellular signals generated by IL-1β and TNFα themselves are not mediated through classic PKC activation, the coexistence of activated classic PKC and signals elicited by IL-1β, but not TNFα, synergistically induces the expression of E-selectin mRNA via increased transcription. Furthermore, promoter analysis of the E-selectin gene suggests that NF-ELAM1/activating transcription factor (ATF) element is indispensable for the synergistic transcription of E-selectin induced by the combined treatment with IL-1β and PMA.

EXPERIMENTAL PROCEDURES

Reagents—Dulbecco’s phosphate-buffered saline, medium 199, and phorbol-12-myristate-13-acetate (PMA) were purchased from Life Technologies, Inc. Fetal calf serum (FCS) was purchased from Equitech-Bio (Ingram, TX). The penicillin/streptomycin solution, agarose, diethyl pyrocarbonate (DEP), dextran sulfate, and MOPS were from Sigma. Nick translation kits and proteinase K were purchased from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Formamide and 50× Denhardt’s solution were obtained from Wako Pure Chemical Industries.
Inc. (Osaka, Japan). RNase-free DNase was obtained from Promega (Madison, WI). Restriction endonucleases and T4 polynucleotide kinase were products of Takara Shuzo Co. (Otsu, Japan). NEN Life Science Products was the source of \( \alpha-32P \)dCTP, \( \gamma-32P \)ATP, and \( \alpha-32P \)UTP.

Recombinant human TNF-\( \alpha \) (9 \( \times \) 10^7 units/mg) and recombinant human IL-1\( \beta \) (2.8 \( \times \) 10^8 units/mg) were from Genzyme Corp. (Cambridge, MA). Human recombinant basic fibroblast growth factor (bFGF) was obtained from Progen Biotechnik GmbH (Heidelberg, Germany). Collagen type 4 (Cellmatrix type 4) was from Nitta Gelatin Inc. (Osaka, Japan). Polyclonal antibodies against ATF-2, ATF-3, c-Jun, cAMP-responsive element-binding protein (CREB), NF-\( \kappa B \) p50, p52, Rel-B, and c-Rel were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals were obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Cell Culture—HUVEC were isolated from human umbilical cords using a perfusate obtained with trypsin (Life Technologies). HUVEC were serially passaged (in a 1:5 split ratio) and maintained in medium 199 containing 10% FCS, antibiotics (50 units/ml penicillin and 50 mg/ml streptomycin) and 10 ng/ml human recombinant bFGF. HUVEC were usually precultured in medium 199 supplemented with 0.5% FCS and antibiotics for 24 h before experiments. Tissue culture dishes were precoated with collagen type 4. HUVEC at the sixth to eighth passage were used for experiments.

Preparation of Plasmid DNA and Oligonucleotide Probes—The plasmids encoding the genes for human ICAM-1 and E-selectin were purchased from British Biotechnology Ltd. (Oxford, United Kingdom). For Northern hybridization, plasmid DNA (1 mg) was radiolabeled by nick translation with \([\gamma-32P]dCTP\) to a specific activity of approximately 10^8 cpm/mg and was used at 7 \( \times \) 10^6 cpm/blot. Double-stranded oligonucleotides containing the NF-ELAM1/ATF cis-element (5'-GAGACAGAGTTTCTGACATCATTGTAATTTTAAGCATC-3'), the oligonucleotides that introduced the mutations in the NF-ELAM1/ATF cis-element (5'-GAGACAGAGTTTCTGACATCATTGTAATTTTAAGCATC-3'), the distal two \( \kappa B \) consensus sequences (5'-GCCATTGGGGATTTCCTCTTTACTGG-3'), or the proximal \( \kappa B \) site (5'-GCCATTGGGGATTTCCTCTTTACTGG-3') in the E-selectin promoter region were synthesized. The oligonucleotides (10 pmol) were radiolabeled with T4 polynucleotide kinase and \( \gamma-32P \) ATP.

![Fig. 1. Different effects of PKC down-regulation on E-selectin gene expression induced by IL-1\( \beta \) or TNF\( \alpha \) treatment in the presence or absence of FCS and bFGF. A, HUVEC were pretreated with PMA (1 \( \mu \)g/ml) for 24 h in the presence or absence of FCS and bFGF and then washed and stimulated with IL-1\( \beta \) (400 pg/ml) or TNF\( \alpha \) (500 pg/ml) for 3 h. At the end of the treatment, the cells were collected and subjected to Northern hybridization. B, the resulting autoradiographs were quantified by videodensitometry. The data are presented as the ratios of the optical density values to those of \( \beta \)-actin.](image-url)
Northern hybridization was performed with RNA from cells untreated or treated for 3 h with IL-1β (400 pg/ml) or TNFα (500 pg/ml) either alone or in combination with PMA (100 ng/ml). The resulting autoradiographs were quantified by videodensitometry as described in the legend to Fig. 1B.

**DNA Transfection and Luciferase Assay**—HUVEC were plated onto six-well collagen-coated culture dishes (2 × 10³ cells/well) 24 h prior to transfection. Transfection was performed with Lipofectin (Life Technologies) according to the manufacturer’s recommendations. Briefly, reporter DNA (20 μg) was mixed with 2 μl of Lipofectin and up to 200 μl of serum-free medium (Opti-MEM; Life Technologies). After a 30-min incubation, further Opti-MEM (800 μl) was added, and the mixture was applied to cells that had been washed twice with Opti-MEM. Four hours later, the medium was changed to medium 199 containing 10% FCS and 10 ng/ml bFGF. After a 24-h culture, the medium was changed to medium 199 containing only 0.5% FCS, followed by culture for a further 24 h. After the indicated treatment for 6 h, the cells were harvested. The cells were lysed in 150 μl of lysis buffer (Toyo Ink), and 20 μl of the resultant extract was used for the luciferase assay (Toyo Ink). The luciferase activities were normalized relative to total protein concentrations of the cell extracts.

**Preparation of Nuclear Extracts**—Nuclear extracts were prepared by a modification of the procedure of Dignam et al. (29). Cells were washed three times with phosphate-buffered saline, scraped off, and then harvested by centrifugation. The cells were resuspended and then incubated on ice for 15 min in hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and then vortexed for 10 s with 0.6% Nonidet P-40. Nuclei were separated from the cytosol by centrifugation at 12,000 × g for 90 s and then resuspended in buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and shaken for 30 min at 4 °C. Nuclear extracts were obtained by centrifugation at 12,000 × g. Protein concentrations were measured by means of the Bradford assay (Bio-Rad).

**Electrophoretic Mobility Shift Assay (EMSA)**—For the binding reac-
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RESULTS

Induction of E-selectin mRNA Expression Is Synergized by the Combined Treatment with IL-1β/PMA, but Not with TNFα/PMA, through Elevated Transcriptional Activity—Initially, we wished to determine if classic PKC activation was involved in the intracellular signaling pathway through which IL-1β or TNFα induces E-selectin mRNA in HUVEC, since the PKC dependence of IL-1β or TNFα-induced E-selectin mRNA expression has not been well defined (31). One reason for the discrepancy is that available PKC inhibitors such as H7 are not strictly specific for that kinase (32), and another reason is whether or not the culture medium during the period of HUVEC stimulation includes FCS and bFGF, which can activate classic PKC. Therefore, we examined the effect of PKC down-regulation, due to pretreatment with PMA, on the gene expression of E-selectin and ICAM-1 induced by IL-1β or TNFα in the presence or absence of FCS and bFGF. HUVEC were preincubated for 24 h with PMA (1 μg/ml) in medium 199 containing 10% FCS and 10 ng/ml of bFGF or containing only 0.5% FCS. Then the HUVEC were washed three times with phosphate-buffered saline and stimulated with IL-1β (400 pg/ml) or TNFα (500 pg/ml) for 3 h. Total RNA was isolated and analyzed by Northern hybridization as to the specific mRNA content using radiolabeled probes for the E-selectin, ICAM-1, and β-actin genes (Fig. 1). In the presence of FCS/bFGF, IL-1β-induced E-selectin mRNA expression was strongly decreased after a 24-h pretreatment with PMA, whereas ICAM-1 mRNA induction was decreased only a little by such PKC down-regulation. Interestingly, TNFα-induced gene expression of E-selectin and ICAM-1 was unaffected by the classic PKC down-regulation. In contrast, in the absence of FCS/bFGF, pretreatment with PMA did not affect the inducibility of these genes upon treatment with either IL-1β or TNFα. These data demonstrate that PKC-mediated pathways are not involved in the intracellular signaling pathways through which IL-1β or TNFα induces gene expression of E-selectin and ICAM-1 and that classic PKC activation by FCS/bFGF and IL-1β (but not TNFα) cooperatively affects the selective induction of E-selectin mRNA expression in HUVEC.

To determine whether or not activated PKC synergizes with IL-1β, but not with TNFα, to induce E-selectin mRNA expression, HUVEC were treated with IL-1β or TNFα, either alone or in combination with PMA (100 ng/ml), in medium 199 containing only 0.5% FCS, without bFGF. Following autoradiography, the expression levels of E-selectin and ICAM-1 mRNA were normalized relative to that of β-actin (Fig. 2). Treatment with IL-1β, TNFα, or PMA stimulated equivalent gene expression of E-selectin and ICAM-1. The combined treatment with IL-1β and PMA synergistically induced E-selectin mRNA expression, although the cotreatment had only an additive effect on the induction of ICAM-1 mRNA. In contrast, when TNFα was substituted for IL-1β, the cotreatment with PMA had no synergistic effect on the gene expression of E-selectin or ICAM-1. These data demonstrate that classic PKC activation and the IL-1β-induced intracellular signaling pathway, which differs from the TNFα-induced one, are necessary for full induction of E-selectin mRNA.

To confirm the markedly different effects of IL-1β and TNFα on E-selectin mRNA expression in the presence of PMA, dose-response profiles for IL-1β and TNFα were examined. HUVEC were treated with increasing doses of IL-1β, ranging from 16 to 400 pg/ml, or TNFα, from 20 to 500 pg/ml, in the presence or

**Fig. 3.** IL-1β cooperatively increases E-selectin mRNA expression in a dose-dependent manner in the presence of PMA. HUVEC were treated with increasing amounts of IL-1β (0, 16, 80, and 400 pg/ml) or TNFα (0, 20, 100, and 500 pg/ml) with or without PMA (100 ng/ml) for 3 h. At the end of the treatment, the cells were collected and subjected to Northern hybridization. The resulting autoradiographs were quantified as described in the legend to Fig. 1B.
absence of PMA (100 ng/ml) (Fig. 3). The dose curve for IL-1β-mediated induction of E-selectin mRNA revealed that in the presence of PMA, E-selectin was cooperatively induced by IL-1β in a dose-dependent manner. In contrast, E-selectin mRNA expression was only slightly increased by treatment with increasing doses of TNFα in the presence or absence of PMA.

The synergistic effect of combined treatment with IL-1β and PMA on E-selectin mRNA induction is independent of de novo protein synthesis, because it was potentiated and not inhibited by the presence of cycloheximide at the concentration (10 μg/ml) sufficient to inhibit protein synthesis by 95% (33) (data not shown).

To determine if the effects of IL-1β, TNFα, and PMA on the gene expression of adhesion molecules are mediated by increased transcriptional activity of the genes, nuclear run-on studies were performed. HUVEC cultures were treated with IL-1β and TNFα, either alone or in combination with PMA for 2 h; nuclei were isolated; and the transcription that had started in intact cells was allowed to reach completion in the presence of [32P]UTP. The radiolabeled RNA transcripts were subsequently hybridized to slot-blotted plasmid DNA fragments encoding E-selectin, ICAM-1, and β-actin. The transcript levels for E-selectin and ICAM-1 were quantified by videodensitometry and normalized relative to those for β-actin. The value for each gene in untreated cells was taken as 1.

The NF-ELAM1/ATF Site in the 5' Promoter Region of the E-selectin Gene Is Required for the Synergistic Expression In-
duced by Cotreatment with IL-1β and PMA—To identify the cis-regulatory sequences required for the synergistic effect of IL-1β and PMA on induction of E-selectin mRNA expression, HUVEC were transiently transfected with a series of 5'-flanking deletion mutants inserted into a firefly luciferase expression vector. Twenty-four hours after transfection, FCS in the medium was reduced to 0.5%, and the HUVEC were cultured further for 24 h. Then the cells were treated for 6 h with IL-1β or TNFα, either alone or in combination with PMA (100 ng/ml), and harvested for the luciferase assay. A transient transfection study on HUVEC with a deletion mutant with 800 bp upstream of the transcription start site demonstrated that simultaneous treatment with IL-1β and PMA induced the luciferase activity synergistically, while cotreatment with TNFα and PMA caused only additive induction (Fig. 5B). This result demonstrates the precise element(s) within the −166 to −116 bp region required for the synergism between IL-1β and PMA, which was recently identified (11, 36, 37). To further delineate the promoter region involved in the synergistic E-selectin gene expression on cotreatment with IL-1β and PMA lies within the −166 to −116 bp region. As shown in Fig. 5A, this region contains one NF-ELAM1/ATF site (34, 35) and one NF-κB site, which was recently identified (11, 36, 37). To further confirm that the synergistic effect of IL-1β and PMA is mediated through the NF-ELAM1/ATF site, a reporter construct with mNF-ELAM1/ATF was introduced into whose NF-ELAM1/ATF site a mutation was introduced. It has been reported that the dATP residues at −151 and −146 bp are important for the binding of ATF proteins to the NF-ELAM1/ATF site of the E-selectin promoter (34). So we introduced the same mutations at the NF-ELAM1/ATF site (mNF-ELAM1/ATF) and assayed the luciferase activity (Fig. 6B). As expected, the reporter construct with mNF-ELAM1/ATF did not cause synergistic induction of the luciferase gene in response to cotreatment with IL-1β and PMA.
bined treatment with IL-1β and PMA. These results clearly demonstrate that the NF-ELAM1/ATF site is indispensable for the synergistic transcription of E-selectin induced by simultaneous treatment with IL-1β and PMA. Next, to clarify that only the NF-ELAM1/ATF site is necessary and sufficient for the synergism between IL-1β and PMA, we made a reporter construct driven by a 4 times repeated NF-ELAM1/ATF site (4× NF-ELAM1/ATF) and assayed the luciferase activity (Fig. 6C). Although the activity was induced a little by each treatment, synergistic induction in response to combined treatment with IL-1β and PMA was never observed. This indicates that the NF-ELAM1/ATF site is necessary but not sufficient for the synergistic transcription of E-selectin induced by simultaneous treatment with IL-1β and PMA.

Synergistic Transcription Induced by Combined Treatment with IL-1β and PMA Does Not Occur through the Changed DNA Binding Activity of NF-ELAM1 and NF-κB—The NF-ELAM1/ATF site is recognized by members of the ATF family of transcription factors, including ATFa, ATF2, and ATF3 (35). It has been reported that homo- or heterodimers consisting of not only these members of the ATF family but also c-Jun and CREB bind to the ATF site of the E-selectin promoter (35, 38). Furthermore, Kaszubska et al. have demonstrated that each of the three ATF members has a different effect on E-selectin promoter activity (35). Therefore, we examined if the nuclear proteins that bind to the NF-ELAM1/ATF site of the E-selectin promoter differed quantitatively or qualitatively, depending on the stimulant, such as IL-1β, TNFα, and PMA, either alone or in combination. Nuclear extracts were prepared from HUVEC treated for 2 h with IL-1β or TNFα, either alone or in combination with PMA (100 ng/ml), and then the EMSA was performed using a oligonucleotide probe including the NF-ELAM1/ATF site at –166 to –129 bp of the E-selectin promoter (Fig. 6B). As reported previously (38), three distinct complexes (C1–C3) were detected constitutively. Although the presence of PMA decreased the amount of the C1 complex, there was neither quantitative nor qualitative difference in the binding of NF-ELAM1 to the specific DNA sequence between the combined treatments with IL-1β/PMA and TNFα/PMA. Because the reporter construct with mNF-ELAM1/ATF sequence did not cause synergistic induction of the luciferase gene in response to the cotreatment with IL-1β and PMA (Fig. 6B), we next examined the effect of mNF-ELAM1/ATF sequence on the EMSA using the nuclear extracts from HUVEC treated with IL-1β/PMA and TNFα/PMA (Fig. 7C). Only the faint binding activity to the mNF-ELAM1/ATF oligonucleotides was seen using both nuclear proteins, and the binding activities of both nuclear proteins to the NF-ELAM1/ATF site was slightly inhibited by the mNF-ELAM1/ATF sequence to the same extent, while they were completely inhibited by the NF-ELAM1/ATF oligonucleotides. These results demonstrate that the binding of NF-ELAM1 to the corresponding cis-element is indispensable for the synergistic transcriptional activity of E-selectin induced by the cotreatment with IL-1β/PMA and that the binding affinities of both the NF-ELAM1 activated by the treatment with IL-1β/PMA and TNFα/PMA were the same.

To identify the protein complexes binding to the NF-ELAM1/ATF site in the nuclear extracts of HUVEC treated with IL-1β and TNFα in combination with PMA, we performed supershift analysis with polyclonal antibodies against ATF2, ATF3, c-Jun, and CREB. In HUVEC treated with IL-1β/PMA, the antibodies against ATF2 and ATF3 caused shifts in the mobilities of the

The value obtained from untreated cells was taken as 1. The results shown in A, B, and C are representative of three identical experiments performed. The results are the means ± S.E.
C2 and C3 complex in part, respectively, and anti-c-Jun antibody caused shifts in both the C2 and C3 complexes, while the anti-CREB antibody caused no shift (Fig. 7D). With the nuclear extracts of HUVEC stimulated with TNFα/PMA, the antibodies had the same effects on the mobilities of the C2 and C3 complexes as mentioned above (data not shown), suggesting that...
the composition of the proteins binding to the NF-ELAM1/ATF site was not different between HUVEC treated with IL-1β and TNFα in combination with PMA. Furthermore, UV cross-linking experiments on the C2 and C3 complexes from HUVEC treated with IL-1β/PMA and TNFα/PMA showed that the C2 and C3 complexes contained four proteins and two proteins, respectively, whose protein compositions on both treatments appeared to be identical on the basis of SDS-PAGE mobility (Fig. 7E).

Direct protein-protein associations are important mechanisms by which transcription factors synergistically cooperate. Since ATF family members and c-Jun have been shown to physically interact with NF-κB, a quantitative or qualitative difference in NF-κB may be responsible for the NF-ELAM1/ATF-dependent synergistic transcription of the E-selectin gene induced by IL-1β/PMA but not by TNFα/PMA treatment. EMSA involving an oligonucleotide probe containing two distal κB sites (−128 to −101 bp) or the other proximal one (−100 to −75 bp) of the E-selectin promoter showed that while two complexes (C1 and Cc2) were induced on treatment with IL-1β, TNFα, or PMA in the same manner, combined treatment with IL-1β/PMA or TNFα/PMA did not cause synergistically quantitative or qualitative change in NF-κB activation (Fig. 8A and B). Supershift analyses involving the antibodies against p50, p52, p65, Rel-B, and c-Rel with nuclear extracts of HUVEC stimulated with IL-1β/PMA (Fig. 8C) and TNFα/PMA (data not shown) at the proximal κB site revealed that Cc1 was a p50/p65 heterodimer and Cc2 was a p50 homodimer. In addition, UV cross-linking experiments on the Cc1 and Cc2 complexes showed that there was no difference between nuclear extracts from HUVEC treated with IL-1β/PMA and TNFα/PMA (data not shown).

**DISCUSSION**

While phorbol ester PMA principally activates classic protein kinase C (31, 39, 40) and is capable of inducing the expression of E-selectin as well as other adhesion molecules (22, 31), whether or not IL-1β and TNFα induce the expression through PKC activation has not been well defined (31). In this study, we have clarified that the IL-1β- or TNFα-provoked intracellular signaling pathway that induces gene expression of E-selectin and ICAM-1 is not through classic PKC activation (Fig. 1). To further confirm this, we also assayed PKC activation, which was assessed as PKC translocation in HUVEC following treatment with IL-1β, TNFα, or PMA. Although PMA caused a rapid decrease in PKC activity in the cytoplasmic fraction and a proportional increase in the membrane fraction, such PKC activation was not detected upon treatment with IL-1β or TNFα (data not shown).

However, our results demonstrated that PKC activation, which occurred in the presence of FCS and bFGF as well as PMA, induced E-selectin gene expression synergistically with IL-1β but not with TNFα, while such a synergistic effect was not observed on the expression of ICAM-1 (Figs. 1–3). Since HUVEC are usually cultured in a medium containing FCS and FGF, the synergistic effect of activated PKC and signals elic-
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Initiated by IL-1β is one reason why the PKC dependence of E-selectin mRNA expression induced by proinflammatory cytokines has been misunderstood. Although it has been reported that IL-1 and TNFα induce NF-κB activation, which is indispensable for the expression of adhesion molecules (reviewed in Ref. 41) through ceramide formation (42), some studies have suggested that signals generated by both proinflammatory cytokines differ, at least in part (12, 43). Swerlick et al. have shown that ICAM-1 was induced on cultured dermal microvesSEL endothelium by either IL-1 or TNFα, whereas VCAM-1 was induced only by TNFα (12). Treatment of HUVEC with the combination of IFNγ and TNFα induced the surface expression of ICAM-1 and E-selectin, whereas the combination of IL-1β and IFNγ had a minimal effect (43). Furthermore, our recent study demonstrated that IL-1β is a much stronger inducer of E-selectin mRNA than TNFα in the heart, although both proinflammatory cytokines induce ICAM-1 and VCAM-1 mRNAs to similar extents (15). From these observations, there appear to be both common (NF-κB activation) and specific pathways of endothelial adhesion molecule expression induced by IL-1β and TNFα, although what kinds of signals generated by both cytokines result in stimulus-specific induction of adhesion molecules has never been clarified. Since the promoter regions of endothelial adhesion molecule genes contain κB elements in common and specific regulatory elements such as the NF-ELAM1/ATF site in E-selectin, the Sp1 and interferon regulatory factor binding sites in VCAM-1 and the CCAAT enhancer-binding protein element and γ-activated sequences in ICAM-1 (reviewed in Ref. 41), the different effects of IL-1β and TNFα on the full expression of adhesion molecules may result from the different modulation of nuclear factors that can bind to these specific regulatory elements.

Indeed, our results (Fig. 6) demonstrated that the NF-ELAM1/ATF site of the E-selectin promoter is required for the synergistic expression induced by the cotreatment with IL-1β and PMA and that the individual transcriptional activities of NF-κB and NF-ELAM1 are not involved in the synergistic effect of treatment with IL-1β/PMA.

Various publications on E-selectin reporter constructs containing multiple point mutations or deletions that abrogate NF-ELAM1 binding differ in the effects described (11, 34–38). The results range from unchanged to reduced basal reporter activity or to severely decreased induction by IL-1β or TNFα. Variations in transfection protocols as well as reporter genes, leading to differences in the detection limits of the respective reporter assays, and whether or not the NF-ELAM1/ATF site was upstream of the wild-type E-selectin promoter or of a minimal heterologous promoter (SV40) might explain the divergent results and interpretations. Although the effects of the two proinflammatory stimulants were never compared in these publications, a survey comparing the effects of IL-1β and TNFα under similar experimental conditions, mentioned in the publications, led us to the suggestion that the NF-ELAM1/ATF site is much more necessary for full transcription of the E-selectin gene when IL-1β is used as the stimulant than when TNFα is used.

Since it has been reported that cAMP inhibits TNF-induced E-selectin promoter activity through alteration of the composition of nuclear factors that bind to the NF-ELAM1/ATF element (38), we have considered that the synergistic effect of combined treatment with IL-1β and PMA might occur through the same mechanism. However, EMSAs together with supershift experiments and UV cross-linking studies involving HUVEC nuclear extracts showed no differences in the binding activity and the protein composition of NF-ELAM1 between combined treatment with IL-1β/PMA and TNFα/PMA (Fig. 7, A–C), demonstrating that the synergistic effect of IL-1β and PMA on the induction of E-selectin mRNA expression is not dependent on the binding activity or protein composition of NF-ELAM1. Since direct physical interaction between the proteins (ATFα, ATF2, ATF3, and c-Jun) bound to the NF-ELAM1/ATF element and NF-κB subunits (p50 and p65) has been demonstrated and reported to be the mechanism underlying the cooperativity yielding maximal levels of E-selectin gene transcription (34, 35), the binding activity and protein composition of NF-κB activated by combined treatment with IL-1β and PMA have been examined in comparison with NF-κB induced by cotreatment with TNFα/PMA (Fig. 8). The results showed no differences in the binding activity or protein composition of NF-κB activated by both combined treatments and the direct transcriptional activity mentioned above. Therefore, increased transactivation of NF-ELAM1 and/or NF-κB in conjunction with each other, which cannot be detected on EMSA, is most likely the mechanism through which E-selectin expression is induced synergistically upon combined treatment with IL-1β and PMA but not with TNFα/PMA.

Recently, it was reported that ATF2 is phosphorylated as well as c-Jun by proinflammatory cytokines or ultraviolet radiation through the c-Jun NH2-terminal protein kinase cascade and that the transcriptional activity of the phosphorylated ATF2 increases without changes in its DNA binding activity (44). In the same report, it was suggested that ATFα is also a substrate of c-Jun NH2-terminal protein kinase. Although IL-1β and TNFα, as well as phorbol ester, were all reported to be potent activators of c-Jun NH2-terminal protein kinase in several cells (45–48), the effects of the two proinflammatory cytokines alone or in combination with PMA have not been compared. Another subgroup of mitogen-activated protein (MAP) kinases, the p38 family, also strongly phosphorylate ATF2 but phosphorylate c-Jun very weakly (49–51). Interestingly, at least two members of the p38 family (p38 and p38-2) have been shown to be activated more strongly by IL-1β than TNFα or PMA in COS cells (50), corresponding to the finding in this study that IL-1β is a more potent stimulant in combination with PMA for E-selectin transcription than TNFα in vascular endothelial cells. This is also consistent with our finding in a recent in vivo study that IL-1β is a much stronger inducer of E-selectin in the heart (15). Furthermore, p38 MAP kinases such as p38, p38-2, and p38β have been shown to be expressed at very high levels in the heart in a tissue-specific manner (49, 50), in accord with our observation of E-selectin induction in vivo (15). Therefore, it is suggested that p38 MAP kinases play key roles in tissue- and stimulus-specific expression of E-selectin through phosphorylation and increased transactivational ability of ATF2 and/or its family. However, since other groups have reported that p38 MAP kinases are activated to similar extents by IL-1β and TNFα in HeLa cells (49, 51) or that both p38 and c-Jun NH2-terminal protein kinase are activated with different time courses by the two proinflammatory cytokines (52), it is necessary to study, using vascular endothelial cells, the effects of IL-1β and TNFα in combination with PMA not only on the activation of MAP kinases (p38 and c-Jun NH2-terminal protein kinase) but also on the phosphorylation and transactivational abilities of ATF2 and its family as well as c-Jun.

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