Activation and Homologous Desensitization of Human Endothelial Cells by CD40 Ligand, Tumor Necrosis Factor, and Interleukin 1

By Karin Karmann,* Wang Min,* William C. Fanslow,‡ and Jordan S. Pober*

*From the *Molecular Cardiobiology Program, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut 06536; and ‡Immunex Research and Development Corporation, Seattle, Washington 98101*

Summary

We have reported previously that activation of human umbilical vein endothelial cells (HUVECs) through CD40, using a recombinant soluble form of trimerized CD40 ligand, leads to induction of E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1). Here, we compare the effects of CD40 ligand with those of tumor necrosis factor (TNF) and interleukin 1 (IL-1). All three ligands induce transient increases in E-selectin (peak 4 h) and VCAM-1 (peak 8–24 h), as well as sustained increases in ICAM-1 (plateau 24 h). Quantitatively, TNF is more potent than IL-1, which is much more potent than CD40 ligand. The same hierarchy is observed for transcriptional activation of an E-selectin promoter reporter gene construct in transiently transfected HUVECs. TNF and CD40 ligand each induced activation of the transcription factors NF-κB, IRF-1, and ATF-2/c-Jun, measured by electrophoretic mobility shift assays, but this response appeared quantitatively similar. All three agents transiently (peak 30 min) activated Jun NH_2-terminal kinase (JNK), which has been implicated in transcription of E-selectin through its actions on ATF-2/c-Jun. Activation of JNK again showed a hierarchy of potency (TNF> IL-1>> CD40 ligand), although the time course of induction was similar for all three agents. After 44 h of pretreatment, TNF, IL-1, and CD40 ligand each display homologous desensitization for reinduction of surface expression of E-selectin. A similar pattern of homologous desensitization for reactivation of JNK was observed. We conclude that TNF, IL-1, and CD40 ligand all activate similar responses in ECs, and that homologous desensitization of JNK may explain the inability of individual cytokines to reinduce E-selectin expression.

Endothelial cells (ECs)[1] can be activated by cytokines such as TNF and IL-1 to express various adhesion molecules that bind circulating leukocytes at sites of local inflammation (1). For example, TNF- and IL-1–treated human umbilical vein ECs (HUVECs) express E-selectin (also called CD62E, endothelial leukocyte adhesion molecule-1; 2, 3), a receptor protein that mediates the initial tethering to, and rolling on, endothelium by neutrophils and certain other leukocytes during the early phase of acute inflammation (4, 5). Cytokine-treated ECs also express vascular cell adhesion molecule-1 (VCAM-1; 6, 7), which promotes the initial attachment and subsequent firm adhesion of T lymphocytes and other specialized effector cells, but not neutrophils (8, 9). VCAM-1 expression has also been associated with chronic inflammatory processes such as atherosclerosis (10). Intercellular adhesion molecule-1 (ICAM-1), which is constitutively expressed at low levels on HUVECs, can be significantly upregulated by TNF and IL-1 (11). ICAM-1 can mediate firm adhesion of both neutrophils and T lymphocytes to endothelium (12), and it also contributes to the transmigration of both cell types through the endothelial monolayer (13, 14).

Although E-selectin, VCAM-1, and ICAM-1 are each induced by TNF and IL-1, the kinetics of their expression is different. On HUVECs, E-selectin expression begins at 1–2 h, peaks at 4 h, and declines to 20% of peak expression by 24–48 h. In contrast, VCAM-1 expression begins at 3–4 h,

[1] *Abbreviations used in this paper:* cRE, cAMP-responsive element; EC, endothelial cell; GST, glutathione S-transferase; HUVEC, human umbilical vein EC; ICAM, intercellular adhesion molecule; JNK, Jun NH_2-terminal kinase; TRAF, TNF receptor-associated factor; VCAM, vascular cell adhesion molecule.

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peaks between 8 and 24 h and is only gradually downregulated thereafter. ICAM-1 expression rises steadily between 3 and 24 h and is sustained at peak levels in the presence of cytokine for at least 72 h (11, 15). The dramatic fall in E-selectin occurs in the face of active cytokine and cytokine-mediated signaling, which is necessary to sustain ICAM-1 expression. Readdition of fresh cytokine does not reinduce E-selectin expression. Interestingly, HUVECs treated with TNF can be reinduced to express E-selectin by IL-1 and vice versa, indicating that downregulation is a form of homologous desensitization (2, 16). In vivo, the early rise in E-selectin correlates with the onset of neutrophil recruitment, whereas the later rise in VCAM-1 and ICAM-1 correlates with the onset of T cell recruitment (17–19). The decline of E-selectin expression and its resistance to reinduction may contribute to the changeover from neutrophil- to T cell-rich infiltrates characteristic of antigen-induced inflammation in humans (20, 21).

EC induction of adhesion molecule expression by TNF is transcriptionally regulated (22, 23). Transcription of E-selectin, ICAM-1, and VCAM-1 each depend on activation of NF-κB p50/p65 heterodimers (22–24). Although NF-κB is necessary for the induction of these three genes, there is a significant discrepancy between the kinetics of its activation and the transcription of E-selectin. Specifically, NF-κB is activated within 15 min of cytokine treatment and remains activated in TNF-treated cells, showing no significant evidence of decay for at least 24 h (25), whereas the decline in E-selectin has been associated with a cessation of transcription (22) that is not observed in other NF-κB–dependent responses (e.g., for class I MHC molecules; 25). The discrepancy between NF-κB activation and E-selectin expression may be explained by the involvement of other transcriptional regulator elements. Specifically, the regulation of E-selectin gene expression also depends on a cAMP-responsive element (CRE)-like binding site in the E-selectin promoter (22, 26) that is located just upstream of three tandem κB-binding sites. By electrophoretic mobility shift assay, this CRE-like site binds at least three different nuclear factors in ECs, identified by antibody supershift as ATF-2 homodimers, ATF-2/c-Jun heterodimers, and CREB proteins (26–28). ATF-2 appears essential for E-selectin expression, since E-selectin, but not VCAM-1 induction, is inhibited in ATF-2 knockout mice (29). TNF treatment increases the relative content of ATF-2/c-Jun heterodimers compared to ATF-2 homodimers that bind to the CRE-like element (28), a change that appears to depend on new synthesis of c-Jun (De Luca, L.G., and J.S. Pober, unpublished observation). Both c-Jun and E-selectin transcription in response to TNF may reflect the activation of one or more Jun-NH_2-terminal kinases (JNK), which phosphorylate the amino terminus of ATF-2 and c-Jun (30), thereby increasing transactivating potency of these factors (31). To date, however, the role of JNK activity in E-selectin transcription has not been directly established.

VCAM-1 gene expression is mediated by binding of p50/p65 heterodimers to two NF-κB like binding sites (32). In addition, an IRF-1–binding site has been identified, just downstream of the κB sites, which appears to be important for TNF-induced VCAM-1 expression. IRF-1 may cooperate with NF-κB to mediate cytokine-induced VCAM-1 expression in ECs (33), as was previously demonstrated for regulation of MHC class I genes (34). The requirement for de novo synthesis of IRF-1 in response to TNF may contribute to the delay in onset of VCAM-1 transcription compared to E-selectin.

Recently, we and others have reported that ECs express CD40, and that CD40 ligand can stimulate HUVECs to express E-selectin, VCAM-1, and ICAM-1 (35–37). The purpose of the present study was to compare CD40 ligand-induced signaling with that initiated by cytokines. Both TNF and CD40 ligand activate NF-κB, ATF-2/c-Jun, and IRF-1. Furthermore, all of the mediators activate JNK in HUVECs. Moreover, we find that JNK activation, like E-selectin induction, is rapid and transient, shows the same rank of order of potency (TNF> IL-1>> CD40 ligand) and displays homologous desensitization. These observations establish the similarity of CD40-mediated signals to those induced by cytokines and more directly implicates JNK as a regulator of E-selectin transcription.

Materials and Methods

Cytokines and CD40 Ligand Trimer. Human rTNF was a gift of Biogen (Cambridge, MA). Human rIL-1 was obtained from R & D Systems, Inc. (Minneapolis, MN). Trimeric recombinant murine or human CD40 ligand was formed from monomers tagged with a leucine zipper tail (38); such preparations of murine CD40 ligand trimer have been shown to be biologically active on human cells, delivering stimulatory signals to B cells and EC (35, 39). All mediators used in this study are free of detectable endotoxin by the Limulus assay and heat-inactivation experiments.

Monoclonal and Polyclonal Antibodies. Murine mAbs used as a specific antibody for fluorescence flow cytometric analysis of surface molecules were partly purified from ascites fluid by ammonium sulfate precipitation and chromatography on protein G columns (Pharmacia Biotech, Inc., Piscataway, NJ). Antibodies used in this study were E1/6 (anti-VCAM-1/CD106; gift from M. Bevilacqua, Amgen, Boulder, CO); K16/16 (nonbinding IgG1, gift from D. Mendrick, Brigham and Women's Hospital, Boston, MA), and H4/18 (anti-CD62E, E-selectin; IgG1 reference 2]). FITC-conjugated mAb anti-ICAM-1/CD54 (clone B-C14; IgG1), anti-HLA-class I ABC (clone B-H9), and isotype control FITC-conjugated IgG1 were obtained from Biosource International (Camarillo, CA).

Rabbit polyclonal IgG used for "supershift" in electrophoretic mobility shift assays were anti–NF-κB p50, anti–NF-κB p65, anti–c-Rel p75, anti–c-Jun/AP-1 and anti–ATF-2 (obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA), and anti-CREB (obtained from Upstate Biotechnology, Inc., Lake Placid, NY).

Cell Isolation and Culture. Human ECs were isolated from umbilical veins and cultured as previously described on gelatin-coated tissue culture plastic (Falcon, Lincoln Park, NJ) (40, 41) in Medium 199 containing 20% FBS (both from GIBCO BRL, Gaithersburg, MD), 50 μg/ml of EC growth factor (Collaborative Biomedical Products, Bedford, MA), 100 μg/ml porcine he-

parin (Sigma Immunochemicals, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL). In the experiments described, ECs were used at passage 2–4. Such cultures stain uniformly for von Willebrand factor and are free of leukocyte contamination, as judged by the absence of CD45 immunofluorescence.

Flow Cytometry. Cells were suspended for flow cytometry by washing monolayers twice with Dulbecco's PBS and incubating for 2 min with Trypsin/EDTA (GIBCO BRL). Suspended cells were washed once in PBS containing 1% BSA and incubated with either unconjugated specific first mAb, diluted 1:200 in PBS/5% goat serum, or with FITC-conjugated specific mAb, diluted according to the recommendations provided by the supplier, for 30 min at 4°C. For indirect immunofluorescence labeling with unconjugated mAb, cells were washed twice with PBS/1% BSA and incubated with a secondary FITC-conjugated $F(ab')_2$ goat anti–mouse IgG (heavy and light chain; Boehringer Mannheim, Indianapolis, IN) at a 1:50 dilution in PBS/5% goat serum for 30 min at 4°C. After indirect or direct immunofluorescence labeling, cells were washed one time in PBS/1% BSA and two times in PBS, and then fixed with 2% paraformaldehyde before being analyzed. Cells were analyzed by using a FACSort® (Becton Dickinson & Co., Mountain View, CA) running LYSIS II software.

Corrected mean fluorescence values were calculated as follows: for each treatment, the mean fluorescence values for the isotype-matched control mAb was subtracted from the mean fluorescence value for the specific mAb.

Transient Transfection of HUVECs. Transient transfection of HUVECs were performed using a DEAE-dextran protocol (42) with E-selectin promoter growth hormone reporter constructs (a gift from T. Collins, Brigham and Woman's Hospital, Boston, MA; 28). In brief, HUVECs were plated at 50–60% confluence on 100-mm tissue culture plates (Falcon) and were washed twice with PBS/1 mM Hepes. Two ml 1 mM Hepes/PBS containing 12 μg DNA and 250 μg/ml DEAE-dextran (Promega, Madison, WI) were added to each plate, and cells were incubated for 30 min at 37°C. 8 ml of HUVEC growth media containing 0.08 mM chloroquine (Sigma) were added to each plate, and cells were incubated for another 2.5 h at 37°C. The medium was removed, and the cells were incubated with Medium 199 containing 10% FBS and 10% DMSO for 2.5 min. Cells were then incubated overnight in fresh HUVEC growth media. After 24 h, transfected cells were replated on gelatin-coated 24-well plates (Falcon). 48 h after transfection, cells were treated with either TNF, IL-1, or CD40 ligand at the indicated concentrations for 24 h. Supernatants were collected and assayed for human growth hormone by radioimmunoassay (Allegro HGH assay system; Nichols Institute, San Juan Capistrano, CA).

Electrophoretic Mobility Shift Assay. Confluent HUVEC monolayers were either left untreated (control) or treated for 1.5 h with either TNF or CD40 ligand with the indicated concentrations. Nuclear extracts were prepared as described previously (43), normalized for protein concentration, and 4 μg were incubated with 100 ng poly (dI·dC) (Pharmacia) for 20 min at room temperature, and then for another 15 min with a ^{32}P-labeled probe in a final volume of 15 μl. The probes used were (5′–3′; complement sequence not shown): ELAM-1–κB binding site: GCCATTGGGGATTTCCTCTTT; ELAM-1 CRE-like binding site: GAGACAGAGTTTCTGACATCATTGTAA; and ISG15/ISRE (IRF-1 binding site): GATCCTTCAGTTTCGGTTTCCCTTTCCCG. Double-stranded oligonucleotide probe was end-labeled with [γ-^{32}P]ATP (Amersham Life Science, Arlington Heights, IL) using T4 polynucleotide kinase (New England BioLabs, Beverly, MA). Cold competitor DNA at a 100-fold excess was added where indicated. For immunochemical identification of nuclear factors (antibody "supershift" analysis), 1 μl of the indicated antibodies was incubated with nuclear extracts for 1 h at 4°C before the incubation with ^{32}P-labeled probe for 15 min at room temperature. DNA–protein complexes were resolved on a 6% nondenaturing polyacrylamide gel containing 7.5% glycerol in 0.25% Tris borate–EDTA buffer, pH 8.3 (onefold TBE = 89 mM Tris borate, 89 mM boric acid, 20 mM EDTA). Dried gels were exposed to x-ray film (Eastman Kodak Co., Rochester, NY) for autoradiography.

Jun NH_2-terminal Kinase Assay. The coding sequence of amino acids 1–80 of c-Jun was amplified from pGEM4c-Jun (gift from T. Curran, Roche Research Center, Nutley, NJ; 44) with two primers: primer 1, 5′-GCGGATCCATGACTGCAAAGATGGAA-3′; primer 2, 5′-GCAAGCTTGATCAGGCGCTCCCAGCTC-3′. The PCR product was digested with BamHI and HindIII (both from New England BioLabs) and cloned into the BamHI and HindIII sites of pGEX-kg, a GST fusion protein expression vector (45) that was used to transform into the DH5a strain of *Escherichia coli*. Protein induction by isopropyl β-D-thiogalacto-pyraniside (Sigma) and protein purification was as described (46). The amount of purified protein was estimated using the Bio Rad (Hercules, CA) protein assay.

The kinase assay was performed essentially as described by Hibi et al. (47). Briefly, ECs were cultured in 24-well plates (Falcon), as described above, and treated with cytokines as indicated. ECs were extracted with lysis buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 0.137 M NaCl, 20 mM β-glycerophosphate, 2 mM EDTA, 1 mM orthovanadate, 2 mM pyrophosphate, 10 μg/ml leupeptin, 1 mM PMSF). The extracts were mixed with 10 μl of glutathione *S*-transferase (GST)-agarose suspension (Sigma) to which 10 μg of GST–c-Jun (1-80) was added. The mixture was rotated at 4°C for 3 h in a microcentrifuge tube and pelleted by centrifugation. The beads were washed three times with lysis buffer and once with kinase buffer (20 mM Hepes, pH 7.6, 20 mM $MgCl_2$, 25 mM β-glycerophosphate, 100 μM sodium orthovanadate, 2 mM DTT, 20 μM ATP). The kinase assay was performed at 25°C for 30 min by mixing 1 μl (10 μCi) of [γ-^{32}P] ATP with 5 μl GST–c-Jun (1-80) agarose suspension in kinase buffer. The reactions were terminated by the addition of Laemmli sample buffer (48), and the products were resolved by SDS-PAGE (12.5%). The phosphorylated GST–c-Jun (1-80) was visualized and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Desensitization Protocol. Confluent monolayers of HUVECs in 12-well plates (Falcon) were either mock pretreated or pretreated for 2 d with optimal concentrations of either TNF (100 U/ml), IL-1 (250 U/ml), or CD40 ligand (10 μg/ml). Fresh medium containing cytokine was added after 24 h to ensure complete desensitization. At the end of the pretreatment period, replicate cultures from each group were then mock treated or cytokine treated (with TNF, IL-1, or CD40 ligand) for an additional 4 h for FACS® analysis of E-selectin expression or for an additional 15 min for analysis of JNK activity. E-selectin expression on cytokine-pretreated cells was compared to expression levels reached on mock-pretreated cells, and is expressed as the percent of inhibition of increase in corrected mean fluorescence intensity (cMFI), calculated by the following formula: 100 − [(cMFI of treated cells pretreated with cytokine A − cMFI of mock-treated cells pretreated with cytokine A) /cMFI of treated

cells mock-pretreated with cytokine] × 100, where cytokine A is TNF, IL-1, or CD40 ligand as indicated.

Results

Activation of Human ECs through CD40 Is Qualitatively Similar to the Activation by TNF or IL-1. We have reported previously that CD40 ligand binding to CD40 on HUVECs upregulates adhesion molecule expression (35), and similar observations have also been reported by others (36, 37). Here, we compare endothelial activation by CD40 ligand to that induced by TNF and IL-1. We find, as shown in Fig. 1, that CD40 ligand induces the same molecules as TNF or IL-1, namely E-selectin, VCAM-1, and ICAM-1, with a similar time course. Of note, CD40 ligand, like TNF and IL-1, induces only transient expression of E-selectin. We have observed a reproducible hierarchy of potency, namely that induction by TNF is stronger than by IL-1, which is much stronger than by CD40 ligand at optimally effective concentrations. No differences were noted between mouse and human CD40 ligand preparations (not shown). In the same experiments, we also examined effects on class I MHC molecules. Interestingly, CD40 ligand, like IL-1, is relatively ineffective at increasing class I MHC molecule expression, compared to TNF.

Activation of Gene Transcription in Human EC Transiently Transfected with an E-Selectin Promoter Reporter Gene Construct. IL-1 has been shown, by nuclear run off experiments to induce the transcription of E-selectin in HUVECs (22, 23); we have observed similar results with TNF (Palmer-Crocker, R., and J. S. Pober, unpublished observations). To analyze smaller effects of CD40 ligand on E-selectin transcription and to compare these effects with IL-1 and TNF, we used transient transfection of HUVECs with an E-selectin promoter human growth hormone reporter gene construct, a more sensitive assay for transcription. 48 h after transfection, cells were treated for 24 h with either TNF, IL-1, or CD40 ligand and accumulation of growth hormone was assayed in the medium. As shown in Fig. 2, transfection of a promoterless vector containing the growth hormone reporter gene did not result in transcriptional activation when the cells were treated with either TNF, IL-1, or CD40 ligand. In contrast, cells that had been transfected with the full-length E-selectin promoter reporter gene, showed strong induction (50-fold above background) of gene transcription in response to TNF and IL-1. Transcriptional activation by CD40 ligand was readily detectable, about fivefold above background, but was much weaker than the response to TNF and IL-1. These results reflect the same hierarchy of potency observed for protein expression.

Activation of the Transcription Factors NF-κB, ATF-2/c-Jun, and IRF-1. We analyzed the activation by TNF and CD40 ligand of three transcription factors implicated in the induction of E-selectin, VCAM-1, ICAM-1, and MHC class I transcription using electrophoretic mobility shift assays. We first investigated the induction of NF-κB, which is important for transcriptional activation of all four genes by TNF (22, 24, 32, 34, 49–51). We observed (Fig. 3 *A*) strong activation of NF-κB by CD40 ligand and TNF. Surprisingly, no difference in the magnitude of response at optimal me-

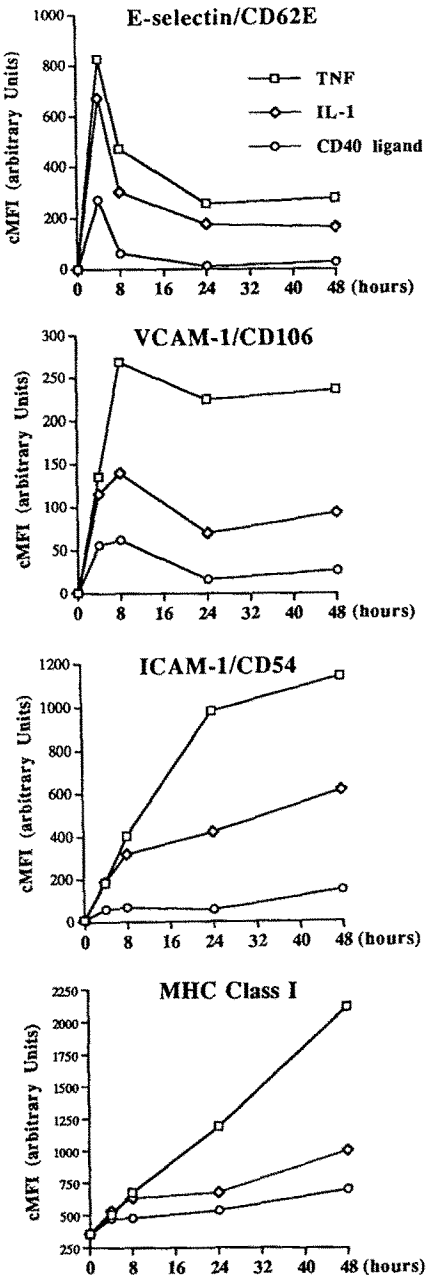

Figure 1. Time dependence of adhesion molecule expression and class I MHC molecule expression on HUVECs after treatment with TNF, IL-1, or CD40 ligand. Cells were treated for 4, 8, 24, and 48 h with either TNF (100 U/ml), IL-1 (250 U/ml), or CD40 ligand (20 μg/ml), and were analyzed by indirect fluorescence flow cytometry for surface expression of E-selectin, VCAM-1, ICAM-1, or class I MHC molecules. The data shown are the corrected mean fluorescence intensities (*cMFI*), as described in Materials and Methods. Data shown are one of four experiments with similar results.

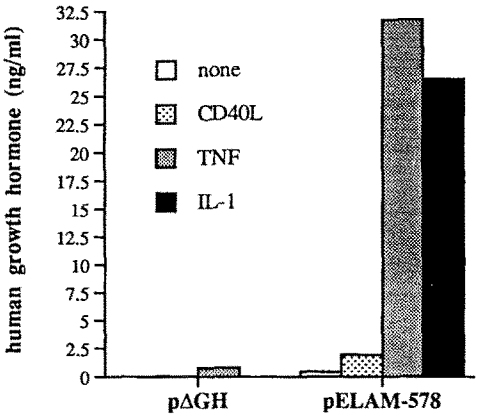

Figure 2. Transcriptional activation of a full-length E-selectin promoter reporter gene construct by CD40 ligand, TNF, or IL-1 in HUVECs. Cells were transfected with the full-length E-selectin promoter human growth hormone construct or mock transfected with the promoterless growth hormone vector, as described in Material and Methods. 48 h after transfection, cells were left untreated or treated with either CD40 ligand (20 μg/ml), TNF (100 U/ml), or IL-1 (250 U/ml) for 24 h. Accumulation of human growth hormone was assayed as described in Materials and Methods. Data shown are one of three experiments with similar results.

diator concentrations was evident. A supershift analysis of the composition of the NF-κB in the nucleus indicated that TNF and CD40 ligand each primarily activated the same p50/p65 heterodimeric form of NF-κB.

We next investigated the induction of the heterodimeric transcription factor ATF-2/c-Jun, which has been shown to be increased in response to TNF in bovine aortic ECs, and which is thought to participate in E-selectin transcription (22, 26–28). As shown in Fig. 3 *B*, both TNF and CD40 ligand increased the relative quantity of complex 2 in HUVECs, which, when analyzed with specific antibodies against c-Jun and ATF-2, is identified as an ATF-2/c-Jun heterodimer.

Finally, we investigated the induction of IRF-1, a transcription factor thought to be important for TNF-induced upregulation of VCAM-1 and class I MHC. We found (Fig. 3 *C*) that IRF-1 is induced by TNF and CD40 ligand. Again, the relative amount of IRF-1 complex induced by TNF and CD40 ligand does not appear to be quantitatively different.

Activation of JNK in HUVECs. JNK has been hypothesized to play a role in transcription of the E-selectin gene (22, 26–28) by increasing the transactivating potency of ATF-2/c-Jun heterodimers (30). As shown in Fig. 4, TNF, IL-1 and CD40 ligand all activate JNK in EC, measured by phosphorylation of c-Jun 1-80. Activation is minimal after

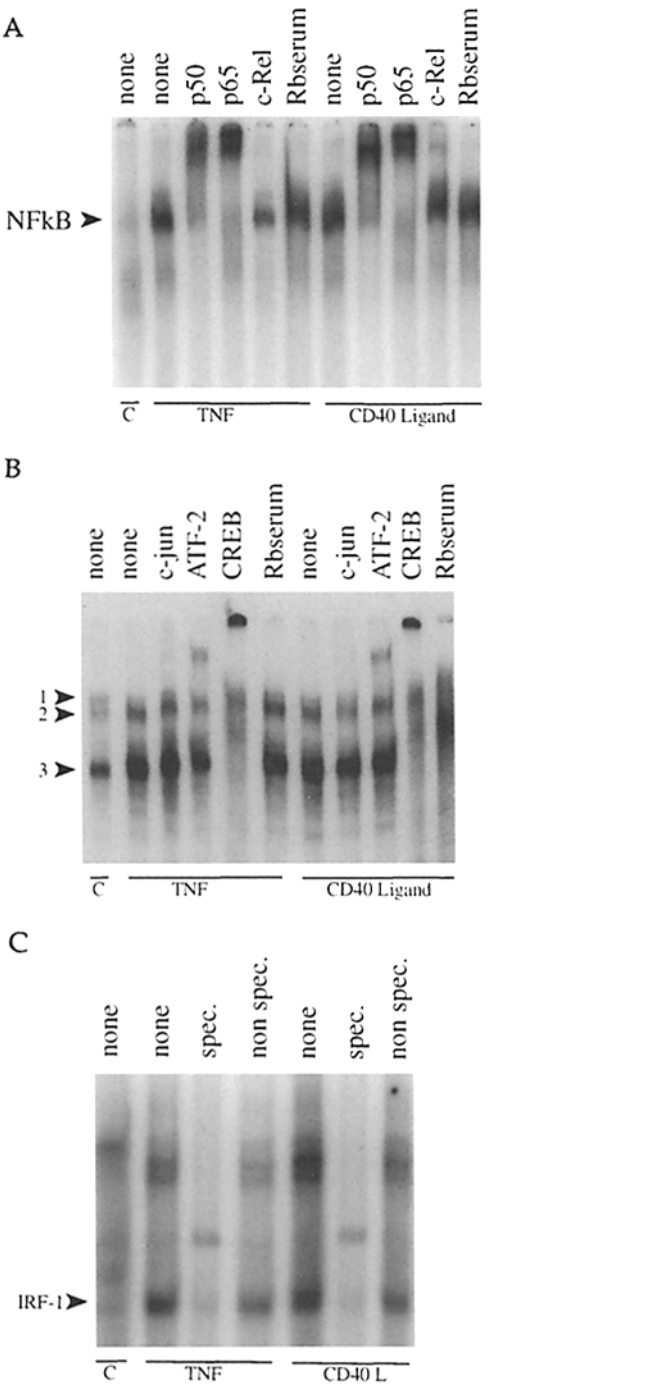

Figure 3. CD40 ligand and TNF activate transcription factors NF-κB, C-Jun/ATF-2, and IRF-1 in HUVECs. (*A*) Nuclear extracts were prepared from cells either left untreated or treated for 1 h and 30 min with either TNF (100 U/ml) or CD40 ligand (5 μg/ml). Nuclear extracts were either preincubated with specific antibodies to p50, p65, or c-rel or with polyclonal rabbit serum at 1 μg/sample for 45 min, before addition of the E-selectin NF-κB sequence for 20 min before electrophoresis. (*B*) Nuclear extracts were prepared from cells treated as described in *A* , and incubated with antibodies to c-Jun, ATF-2 and CREB, or rabbit serum for 1 h, followed by the addition of the E-selectin CRE/ATF sequence for 20 min before electrophoresis. (*C*) Nuclear extracts were prepared from cells treated as described in *A* , and either preincubated with specific cold competitor (*ISG15/ISRE*) or nonspecific cold competitor (*E-selectin NF-κB*) for 15 min were indicated, followed by the addition of the ISG15/ISRE sequence 15 min before electrophoresis. The band identified as IRF-1 was identified by supershift in two other experiments (not shown). Results shown in the figure are representative of three experiments.

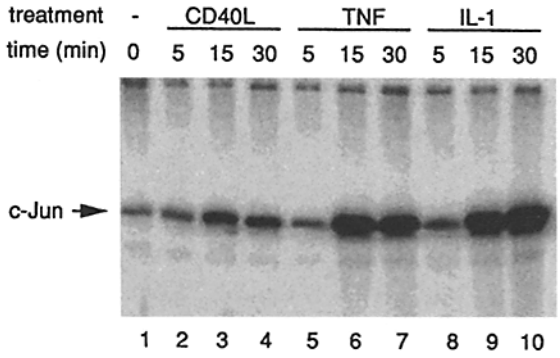

Figure 4. Time dependence of JNK activation by TNF, IL-1, and CD40 ligand. HUVECs were treated with TNF (10 U/ml), IL-1 (100 U/ml), and CD40 ligand (10 μg/ml) for 5, 15, and 30 min. HUVECs were cultured without cytokines as a control. JNK activity was measured as described in Materials and Methods. The results shown are representative of three similar experiments.

5 min of treatment. Peak levels are reached at 15 min, with a slight decrease of activity after 30 min. By 48 h, JNK activity is only 20% or less of peak levels (Fig. 5). The time course of JNK activation appears similar for all three agents, but there is a significant difference in the level of activity induced by each cytokine. The highest activity was measured with TNF, slightly lower activation levels were reached by IL-1, and JNK was activated to a much lesser degree by CD40 ligand. Measures of JNK activity obtained by quantitating phosphorylation of c-Jun(1-80) at a single time point, however, may not give an accurate measure of active enzyme. Nevertheless, the relative levels of activity are consistent with the hierarchy of potency observed for E-selectin transcription and expression.

Homologous Desensitization of E-Selectin Expression and JNK Activation. We have previously described the phenomenon of homologous desensitization in response to TNF or IL-1 in the induction of E-selectin expression on ECs (2, 16, 52). Specifically, after 24–48 h of cytokine treatment, E-selectin expression is decreased to ~20% of peak expression and retreatment of EC with the same cytokine results in only minimal reinduction of E-selectin expression. This desensitization is homologous because cells respond fully to treatment with a different cytokine. Here, we have extended this analysis to CD40 ligand. As shown in Table 1, expression of E-selectin was significantly inhibited when cells pretreated with CD40 ligand were rechallenged with CD40 ligand, but not when the same cells were rechallenged with TNF or IL-1. Similarly, cells desensitized to TNF or IL-1 for 48 h were fully responsive to E-selectin reinduction in response to CD40 ligand. Thus, CD40 ligand also shows homologous desensitization.

Finally, we analyzed reactivation of JNK in response to TNF, IL-1, and CD40 ligand. The results, shown in Fig. 5, suggest that JNK activity also undergoes homologous desensitization. Specifically, cells pretreated with TNF, IL-1, or CD40 ligand showed blunted reactivation of JNK when retreated with the same agents (i.e., compare lanes *2* with *6*, *3* with *11*, and *4* with *16*), and were preferentially reactivated by either of the other agents. Of note, IL-1–pretreated cells also showed a small degree of heterologous desensitization to TNF and CD40 ligand. Again, we point out that such assays may not quantitatively reflect enzyme activity. However, the experimental results do convincingly demonstrate homologous desensitization of this enzyme in HUVECs.

Discussion

We have reported here qualitatively similar patterns of activation of HUVECs in response to the inflammatory cytokines TNF and IL-1 with the response to the ligand for CD40. We find upregulation and induction of the adhesion molecules E-selectin, VCAM-1, and ICAM-1, as well as class I MHC molecules on the EC surface. Although the time course of expression was similar, the magnitude of expression observed with the three activators was very different. The responses showed a consistent hierarchy in which TNF is slightly better than IL-1, and both are much more effective than CD40 ligand. The actions of all three mediators on E-selectin expression appear to be transcriptionally regulated, as shown by transient transfection assays using an E-selectin promoter reporter gene. The analysis of transcription factors by electrophoretic mobility shift assays shows activation of NF-κB, ATF-2/c-Jun, and IRF-1 by TNF and CD40 ligand. TNF, IL-1, and CD40 ligand rapidly and transiently activate JNK in HUVECs. All three agents display homologous desensitization for reinduction of E-selectin expression and for the activation of JNK.

In several assays, we have noted that EC are less effectively activated by CD40 ligand than by TNF or IL-1. In the experiments reported here, we have used an optimal or

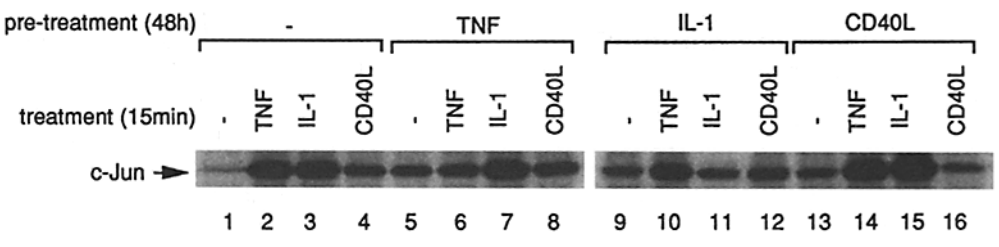

Figure 5. Homologous desensitization of JNK activity in HUVECs by TNF, IL-1, and CD40 ligand. Cells were pretreated for 48 h and treated for 15 min, as described in Materials and Methods, with TNF (100 U/ml), IL-1 (250 U/ml), and CD40 ligand (10 μg/ml), and JNK activity was measured as described in Materials and Methods. The results shown are representative of two similar experiments, both of which were performed in replicate cultures of cells assayed for E-selectin surface expression, as presented in Table 1.

Table 1. *Homologous Desentization of Induction of E-Selectin Expression by CD40 Ligand, TNF-α, and IL-1α*

Pretreatment*	Percent of inhibition after treatment		
	TNF	IL-1	CD40 ligand
TNF (100 U/ml)	91.0‡	None	None
IL-1 (250 U/ml)	None	93.5	None
CD40 ligand (10 μg/ml)	None	None	78.0

*EC were mock-pretreated or pretreated for 44 h with the indicated agents, as described in Materials and Methods. At this time, the ECs were treated for 4 h either with no cytokine, with the same agent, or with one of the other agents as indicated.
‡The values show the percent of inhibition of the increase in mean fluorescence intensities of pretreated cells compared to the cells that were not pretreated, as described in Materials and Methods.
The results shown here are representative of five experiments with similar results

near optimal concentration of soluble CD40 ligand trimer, but we have never approached the level of response seen with TNF and IL-1. We are reluctant to overinterpret this difference because it may reflect the fact that we used a soluble form of CD40 ligand, whereas this molecule normally acts as a cell surface ligand expressed on activated $CD4^+$ T cells (39, 53, 54). Cell–cell interactions may be a much more efficient means of delivering a signal by this molecule. In support of this idea, it has been recently reported that TNF-RII can be activated much more effectively by membrane-bound TNF than by soluble TNF (55).

Signal transduction pathways activated by TNF, IL-1, and CD40 are under intense investigation in many laboratories. Recent studies using molecular cloning with a yeast two-hybrid system have identified "adapter proteins" that associate with TNF receptors and are directly or indirectly involved in coupling receptors to responses (56–59). Specifically, TNF-RI interacts with a protein, called the TNF-RI–associated death domain protein (TRADD), whereas TNF-RII interacts with at least two separate adapter molecules, called TNF receptor–associated factor 1 (TRAF1) and TRAF2. In HUVECs, NF-κB activation and gene expression is mediated largely or entirely through TNF-RI and not through TNF-RII (15, 60). Paradoxically, TRAF2 has been implicated as the adapter protein linked to NF-κB activation. The explanation for this seeming discrepancy is that TRAF2 appears to be involved in TNF-RI–mediated signaling by indirect association via TRADD. A member of the same family of molecules as the TRAFs, called CD40-associated factor 1 (CRAF1, also called TRAF3), is associated with the intracellular domain of CD40 in B cells (61, 62). The function of TRAF3 is not known, but CD40 can also associate with TRAF2 (59), thereby coupling this receptor to NF-κB activation. HUVECs express IL-1-RI, the signaling receptor for this cytokine (63–65). Recently, a kinase has been identified which is associated with the IL-1 RI, called IRAK (66), and functional analysis has suggested that IRAK activation by IL-1 leads to NF-κB activation. It should be noted that neither TRAF2 nor IRAK have been reported in HUVECs as of yet , and the signal transduction pathway by which either adapter molecule is coupled to NF-κB is still unknown.

TNF and IL-1 can both activate JNK in a variety of cells (31, 67). To the best of our knowledge, this pathway has not been previously reported in HUVECs. More significantly, we have presented here several findings that strongly suggest direct involvement of JNK in E-selectin gene regulation. We have shown that JNK in HUVECs is activated by CD40 ligand, TNF, and IL-1; that JNK activation by these mediators, like E-selectin expression, is rapid and transient; and that activation of JNK shows homologous desensitization, a phenomenon that has been also observed for E selectin surface expression. We presume that JNK acts on the ATF-2/c-Jun heterodimeric factor that binds to the E-selectin CRE-like element.

The mechanism of homologous desensitization of E-selectin reinduction has not been identified, but two possibilities can be ruled out. First, the mechanism does not involve receptor inactivation or downregulation, since sustained active cytokine is required for persistent expression of other molecules (e.g., ICAM-1) at times when HUVECs are homologously desensitized. Second, NF-κB inactivation does not seem to be responsible for homologous desensitization, since this factor remains activated even after 24 h of TNF treatment (25). The new studies reported here strongly implicate homologous desensitization of JNK as the basis for homologous desensitization of E-selectin reinduction. The fact that desensitization is homologous may be biologically significant because it would allow events that occur at different times during inflammation, such as $CD4^+$ T cell activation and cytokine production, to reinduce or sustain E-selectin expression, leading to prolonged inflammation.

The molecular basis of JNK desensitization is not known. Since all three receptors activate JNK and show desensitization to their ligand, one possibility is that a receptor-bound adapter protein, such as a TRAF or a G protein that is directly associated with the receptor, becomes inactivated after transducing a signal that leads to activation of JNK. Since the adapter molecule inactivating this pathway is receptor associated, desensitization would be expected to occur in a ligand-specific manner. Separate pools of the same adapter protein could be involved for all three receptors, thereby providing a means of restimulation in response to heterologous signals.

Finally, our observations have potential clinical relevance. New antiinflammatory therapies have been proposed to target the function of endothelial leukocyte adhesion molecules, such as E-selectin. An alternative target is the prevention of adhesion molecule expression. A potential problem with this approach is that proinflammatory signals are redundant and an agent that blocks TNF may not be effective because IL-1 or contact-dependent signals (e.g., such as those mediated by CD40 ligand) can bypass

the blockade. Our data suggest there are contact-dependent pathways that converge with TNF and IL-1 signals to activate ECs, providing common downstream targets. NF-κB has been potentially identified as such a target, but this has a major drawback, namely that TNF-induced cell death, which is independent of NF-κB (56, 57), may in fact be limited by NF-κB–induced genes (68–70). Our new data support the idea that JNK may be an alternative, more selective target, which may have the advantage of targeting tissue factor as well as E-selectin, another endothelial gene that shows rapid and transient induction followed by homologous desensitization (71). We do not as yet know which JNK isoform is most relevant for E-selectin induction, but answers to these and other related questions should be forthcoming in the near future.

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Address correspondence and reprint requests to Jordan S. Pober, MD, PhD, Boyer Center for Molecular Medicine, Box 9812, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06536-0812.

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