Nuclear Factor

Induction of Tissue Factor Expression in Human Endothelial Cells by CD40 Ligand Is Mediated via Activator Protein 1, Nuclear Factor κB, and Egr-1*

Udo Bavendiek‡, Peter Libby‡, Meagan Kilbride‡, Rebecca Reynolds‡, Nigel Mackman§, and Uwe Schönbeck‡‡

Received for publication, April 24, 2002
Published, JBC Papers in Press, April 26, 2002, DOI 10.1074/jbc.M204003200

From ‡The Leducq Center for Cardiovascular Research, Cardiovascular Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115 and the §Department of Immunology and Vascular Biology, The Scripps Research Institute, La Jolla, California 92037

Induction of tissue factor expression in endothelial cells via ligation of CD40 probably figures prominently in the pathogenesis of prevalent inflammatory diseases, including atherosclerosis. However, the molecular mechanisms of tissue factor gene expression triggered by CD40 ligand (CD40L) in this cell type remain unknown. We demonstrate here that the tissue factor promoter region −278 bp to +121 bp contains the CD40L-responsive elements, consisting of activator protein 1 (AP-1)±, nuclear factor (NF) κB-, and Egr-1-binding sites. Mutations of either the AP-1- or NF-κB-binding sites markedly reduced the CD40L-dependent promoter activation. The AP-1 and NF-κB sites displayed constitutive and CD40L-enhanceable DNA binding activity, respectively. Noteworthy, the Egr-1-binding sites were not associated with CD40 signaling, impaired activation of the tissue factor promoter. Accordingly, CD40L strongly induced Egr-1 protein expression and DNA binding activity to all three binding sites. In contrast to CD40L, other established inducers of tissue factor in endothelial cells, interleukin-1β or tumor necrosis factor α, did not increase the expression of Egr-1. In conclusion, induction of tissue factor gene expression in human endothelial cells by CD40L involves AP-1 and NF-κB as well as Egr-1, a pathway previously not implicated in CD40 signaling and distinct from that employed by certain other proinflammatory cytokines.

Thrombosis, resulting in complete or partial vessel occlusion, plays a crucial role in the pathogenesis of cardiovascular diseases such as atherosclerosis and thromboembolism (1, 2). Intravascular coagulation also complicates conditions such as septic shock. For well over a century pathologists have recognized that altered endothelial coagulant potential contributes to thrombotic diathesis. Understanding the molecular details of endothelial coagulability has emerged recently. Expression of procoagulants on the surface of endothelial cells (EC),1 the cell type at the blood interface, has attracted particular attention in this regard. Tissue factor (TF), a 47-kDa membrane-bound glycoprotein, serves as a key initiator of blood coagulation. Of note, EC express little or no TF constitutively in vitro and in vivo. However, levels of this procoagulant increase markedly in the endothelium of the diseased vasculature (3).

Despite its potential pathophysiologic role, knowledge regarding the regulation of TF gene activity in EC remains incomplete. Endotoxin (lipopolysaccharide (LPS)), tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), vascular endothelial growth factor, and phorbol 12-myristate 13-acetate (PMA) induce the expression of this procoagulant in EC (4, 5). Interestingly, the transcriptional regulation of TF expression in different cell types appears to involve distinct signaling pathways (6). Functional studies of the human TF promoter in various cell types, including EC, identified two promoter regions implicated in TF gene expression. The first region, termed the LPS-response element (LRE), contains two activator protein-1 (AP-1)- and one nuclear factor κB (NF-κB)-like binding sites. The LRE mediates inducibility of the TF promoter in response to proinflammatory stimuli (e.g. IL-1β and TNFα), LPS, and PMA (4, 7, 8). The second region, the serum-response region (SRR), contains three Egr-1-binding sites. This region mediates the induction of TF gene expression via Egr-1 in response to serum, PMA, shear stress, low density lipoprotein, hypoxia, vascular endothelial growth factor, and LPS (5, 9–14). Thus, the molecular pathways employed to activate the TF gene appear to depend on the stimulus applied, e.g. proinflammatory cytokines appear not to signal via the Egr-1-binding sites located in the SRR.

Recently, we and others have identified the immunomodulatory dyad CD40/CD40L as a novel regulator of TF protein and activity in various cell types, including EC, in vitro and in vivo (15–19). Further supporting the potential role of CD40L in the modulation of the thrombotic balance of EC, CD40 ligation diminishes the expression of thrombomodulin by this cell type, the “anticoagulant” receptor for thrombin (16, 17). Moreover, in human and experimental atheroma, the expression of CD40L correlates and co-localizes with that of TF in vivo (19, 20).

1 The abbreviations used are: EC, endothelial cells; TF, tissue factor; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; IL-1β, interleukin-1β; PMA, phorbol 12-myristate 13-acetate; LRE, LPS-response element; AP-1, activator protein 1; NF-κB, nuclear factor κB; SRR, serum-response region; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; TBS, Tris-buffered saline.
**CD40L Induces TF Expression via AP-1, NF-κB, and Egr-1 in EC**

Fig. 1. Ligation of CD40 induces expression of TF transcript and protein in human endothelial cells. Endothelial cells, maintained in serum-free medium (16 h), were incubated (4 h for RNA, 6 h for FACS, 24 h for protein extracts) with medium alone (None), PMA (50 ng/ml), or recombinant human CD40L (10 μg/ml). Protein extracts were analyzed by Western blotting (WB) for TF protein or by radioimmunoprecipitation (RIP) for de novo synthesis of TF. Control immunoprecipitation experiments used non-immune Ig. FACS analysis of surface TF protein was performed on EC cultured in the absence (open histogram) or presence of CD40L (solid histogram). The dotted line shows staining performed with control IgG. Total RNA preparations were amplified by reverse transcriptase (RT)-PCR for TF transcript. Three experiments performed with EC from different donors yielded similar results.

Although many cell types can express TF, EC occupy a central position in the homeostasis of blood coagulation as they provide the contact surface of the tissue with coagulation factors containing blood. The CD40 signaling mechanisms mediating TF expression in this cell type, however, remain unknown. Therefore, the present study characterized the cis-acting regulatory elements, which mediate the CD40L-induced TF promoter activity in EC.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Human recombinant IL-1β and TNFα were obtained from Endogen (Cambridge, MA); PMA and polymyxin B were purchased from Sigma. Human recombinant CD40L was obtained from Leinco Technologies (St. Louis, MO).

**Isolation of Culture**—Human vascular EC were isolated from saphenous veins and cultured in dishes coated with gelatin as described elsewhere (21). Cells were maintained in growth medium containing medium 199 (M199; BioWhittaker, Walkersville, MD), supplemented with 1% penicillin/streptomycin (BioWhittaker), 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 μg/ml heparin (Sigma), and 50 μg/ml endothelial cell growth factor (Pel-Freez Biologicals, Rogers, AR), and used throughout passages 2–4. Culture media and fetal bovine serum contained less than 40 pg of endotoxin/ml as determined by chromogenic limulus amebocyte assay (QLC-1000; BioWhittaker). Purity of EC cultures was ≥99% as characterized by immuno-staining with anti-vasa Willebrand factor monoclonal antibody (Dako, Carpinteria, CA) or by flow cytometry with anti-CD31 monoclonal antibody (BD PharMingen). EC were cultured 18 h before the experiments in M199 supplemented with 0.1% human serum albumin (Immuno-US, Rochester, MI). All experiments employing recombinant CD40L were performed in the presence of 1 μg/ml polymyxin B.

**Isolation of RNA and Reverse Transcriptase-PCR**—Total RNA was isolated from EC cultures employing RNazol (Tel-Test, Friendswood, TX) and was reverse transcribed (2 μg of total RNA, 50 min, 42 °C) in 20 μl of total reaction mixture (200 units of Superscript II reverse transcriptase, 25 μg/ml oligo(dT)12–18 primers, 0.5 mM DTT, 10 mM dNTPs, 4 μl of first strand buffer; all from Invitrogen, Rockville, MD). Aliquots (2 μl) of the reverse transcriptase reaction were applied to 24 µl of reaction mixture (1.5 mM MgCl2, 0.2 mM dNTPs, 2.5 units of platinum Taq DNA polymerase, and 5 μl of 10× PCR buffer; all from Invitrogen). TF primers (0.2 μM) employed in PCR analysis were: forward, 5′-CTACTGTTTCCAGTTCACACGTGA-3′ and reverse, 5′-CAGTGAATATAGCATTAGCAGTACG-3′ (22). PCR was performed for 35 cycles at 94 °C (60 s), 55 °C (60 s), and 72 °C (90 s) after hot start. Aliquots (10 μl) of the PCR products were run on 1.5% agarose gels and visualized by UV transillumination.

Radioimmunoprecipitation—Vascular EC were washed with medium lacking methionine/cysteine. Subsequently, medium containing 10% fetal bovine serum and 50 μCi/ml [35S]methionine/cysteine (PerkinElmer Life Sciences) was added to the cells for 24 h. Immunoprecipitation buffer (50 mM Tris-HCl, 0.1% SDS, 0.1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 20 mg/ml soybean trypsin inhibitor, 0.1% serum, 0.2 units/ml aprotinin, 0.025% sodium azide) was added to the cultures, and cells were harvested. Subsequently, non-immune mouse serum (Vector, Burlingame, CA) was added (24 h, 4 °C) to preclude the samples. Antigens in cell extracts were immunoprecipitated (2 h, 4 °C) with the specific mouse anti-human TF antibody (American Diagnostica, Greenwich, CT) and pelleted by subsequent addition of rabbit anti-mouse IgG (18 h, 4 °C) as well as protein A-Sepharose beads (2 h, 4 °C). The beads were washed four times in 50 mM Tris-HCl, and finally 50 μl of SDS-PAGE loading buffer (200 mMol/liter Tris, 5% glycerol, 0.1% SDS, 3% β-mercaptoethanol, 0.1 mg/ml bromphenol blue) was added. After heating the samples for 5 min at 95 °C, supernatants were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Blots were dried and exposed to x-ray film (Eastman Kodak Co.) for detection of the immunoprecipitated antigen.

Flow Cytometry—Human vascular EC were harvested by trypsinization, fixed (PBS, 4% paraformaldehyde, 15 min), washed once with ice-cold PBS, 0.2% bovine serum albumin (Ca2+/Mg2+-free), and subsequently incubated (30 min, 4 °C) with fluorescein isothiocyanate-conjugated IgG (Axxell, Bayport, MN) or TF antibody (American Diagnostica, Greenwich, CT) and pelleted by subsequent addition of rabbit anti-mouse IgG (18 h, 4 °C) as well as protein A-Sepharose beads (2 h, 4 °C). The beads were washed four times in 50 mM Tris-HCl, and finally 50 μl of SDS-PAGE loading buffer (200 mMol/liter Tris, 5% glycerol, 0.1% SDS, 3% β-mercaptoethanol, 0.1 mg/ml bromphenol blue) was added. After heating the samples for 5 min at 95 °C, supernatants were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Blots were dried and exposed to x-ray film (Eastman Kodak Co.) for detection of the immunoprecipitated antigen.

**Plasmids**—Plasmid used in this study was as previously described: pBluescript vector (pTF− /−), pTF− (194), pTF− (153), pTF− (111), pTF− (67), and pTF− (21) were 5′-deletion constructs of the human TF promoter (7); pTF− (227) contained the wild-type human TF promoter; pTF− (227)AP−1m and pTF− (227)NF−κBm constructs contained mutations of both AP-1 sites and the NF-κB site, respectively (8). The wild-type human TF promoter (−278 bp to +14 bp) and a construct containing mutations in each of the Egr-1 sites (Egr−1m) were described elsewhere (14). pSV-β-Gal vector and pGL2basic were obtained from Promega (Madison, WI). Plasmids used for transfections were prepared with the EndoFreeTM plasmid kit (Qiagen Inc., Valencia, CA).

**Transfections**—Human vascular EC were subcultured the day before transfection (1:2) into 150-cm2 flasks. For transfection, cells were harvested by trypsinization, washed once in electroporation medium (Opti-MEM I, Invitrogen; supplemented with 1% fetal bovine serum), and 3 × 106 cells/well (6-well plate) were incubated (30 min, 4 °C) with lucifer-
ase plasmid and pSV-β-Gal (5 μg each) in electroporation medium. Electroporation was performed in electroporation cuvettes with a gap of 0.4 cm in a Gene-Pulser (Bio-Rad) at a time constant of 32 ms. After electroporation, cells were incubated for 30 min at room temperature, resuspended in growth medium, and equally distributed to 6-well plates. Following a 20-h recovery, EC were cultured (16 h) in serum-free medium as described above. Following stimulation with 10 μg/ml recombinant CD40L (6 h), cell lysates were assayed for luciferase (BD PharMingen) and β-galactosidase activity (Tropix, Galecto-Light Plus, Bedford, MA) in a luminometer (Berthold, Bad Wildbad, Germany). Luciferase activity of each sample was normalized to β-galactosidase activity. Five experiments performed with EC from different donors yielded similar results.

**FIG. 2.** CD40L induces TF promoter activity in human endothelial cells. A, location of transcription factor-binding sites in the wild-type TF promoter, pTF (-278), and 5′-deletion constructs of the promoter used in this study. B, human EC were transiently transfected with plasmids containing vector (p19Luc), the wild-type (-278 to +121 bp), or 5′-deletion constructs of the human TF promoter. After overnight recovery in growth medium, cells were cultured (16 h) in serum-free medium and subsequently in the absence or presence (6 h) of recombinant human CD40L (10 μg/ml). Luciferase activity in cell lysates was normalized to β-galactosidase activity. Five experiments performed with EC from different donors yielded similar results.
FIG. 3. CD40L-induced TF promoter activity in human endothelial cells involves AP-1- and NF-κB-binding sites. A, human EC cultures were transiently transfected with plasmids containing the wild-type TF promoter, pTF(-227), or those with either mutation in the AP-1 sites (AP-1m) or NF-κB site (NF-κBm). Induction of TF promoter activity by CD40L (10 μg/ml, 6 h) is expressed as percent of the wild-type promoter. Six experiments performed with EC from different donors yielded similar results.

B–D, confluent human EC were cultured (16 h) in serum-free medium and then in the absence or presence (1 h) of recombinant CD40L (10 μg/ml). Protein-DNA binding was performed with nuclear extracts (AP-1, 2 μg; NF-κB, 10 μg) and oligonucleotides containing binding sites for the proximal (AP-1 P) and distal (AP-1 D) AP-1 as well as NF-κB sites of the TF promoter. To confirm the specificity of the protein-DNA complexes, a 25-fold molar excess of non-labeled competitor oligonucleotides containing AP-1, NF-κB, or Sp1 consensus binding sites or specific antibodies (4 μg) was applied (arrowhead indicates supershift, n.s. indicates nonspecific bands). Protein-DNA complexes were separated using 6% nondenaturing acrylamide gels. Three experiments performed with EC from different donors yielded similar results.
with CD40L further induced the expression of TF mRNA, suggesting transcriptional modulation via this cytokine (Fig. 1). To identify putative CD40L response elements in the TF promoter in human EC, we performed promoter reporter gene analysis utilizing 5′-deletion constructs of the wild-type TF promoter (−278 to +121 bp) (Fig. 2A). Ligation of CD40 on human EC transiently transfected with wild-type TF promoter (pTF(−278)) markedly increased the promoter activity compared with non-treated cells (fold induction: 2.70 ± 0.78; n = 5, Fig. 2B). However, 5′-deletion of the wild-type promoter between −278 and −194 bp diminished the constitutive and CD40L-inducible promoter activity significantly compared with the wild-type promoter (53 ± 11 and 16 ± 23%, respectively; n = 5, p < 0.05). Deletion of the promoter region to −111 bp further decreased the basal promoter activity (21 ± 7%; n = 5) but continued to show diminished inducibility of the promoter by CD40L compared with wild-type (28 ± 24%; n = 5). Deletion of the promoter to −67 or −21 bp eventually resulted in the loss of detectable promoter activity with and without exposure to CD40L (Fig. 2B). These data indicated that a region of the human TF promoter downstream of −278 bp contains cis-acting elements required for induction of the promoter by CD40L in human EC.

The Induction of Human Tissue Factor Promoter Activity by Recombinant CD40L in Human Endothelial Cells Requires Functional AP-1- and NF-κB-binding Sites—The significantly impaired inducibility of the TF promoter activity by CD40L associated with 5′-deletions indicated the involvement of the LRE region. This region contains two AP-1 and one NF-κB binding sites. To determine whether these sites indeed participate in the CD40L-mediated induction of TF, we transiently transfected EC with plasmids containing mutations in the proximal and distal AP-1 (pTF(−227)AP-1m) or NF-κB (pTF(−227)NF-κBm) binding sites. Mutations in either binding site significantly and independently diminished the CD40L-mediated induction of TF promoter activity compared with wild-type (AP-1, 33 ± 18%; NF-κB, 18 ± 17%, n = 6, p < 0.05; Fig. 3A). These findings demonstrated an obligatory role for both AP-1- and NF-κB-binding sites in the induction of TF gene expression following CD40 ligation.

More detailed analysis of AP-1 and NF-κB binding activity in EC following CD40 ligation used EMSA employing oligonucleotides containing the TF promoter-specific proximal (AP-1p) and distal (AP-1p) AP-1 as well as NF-κB DNA-binding sites. Nuclear extracts from unstimulated as well as CD40L-stimulated EC formed a prominent protein-DNA complex with the distal and proximal AP-1 sites (Fig. 3, B and C). Specificity of this complex for AP-1 was demonstrated by competition with a 25-fold molar excess of unlabeled AP-1 but not of Sp1 consensus oligonucleotides (Fig. 3, B and C). To determine the composition of the AP-1 complexes formed at the proximal and distal AP-1 sites, specific antibodies to members of the AP-1 transcription factor family were added to the DNA-protein binding assay. Supershifted protein-DNA complexes occurred after addition of antisera against c-Jun and JunD, but not JunB, c-Fos, and FosB to the DNA binding reaction performed with either the proximal or distal AP-1 sites (Fig. 3, B and C). The observation that nuclear extracts from unstimulated and CD40L-stimulated EC yielded similar results suggested constitutive binding of c-Jun and JunD to the AP-1 sites of the TF promoter in human EC.
complex formation, which ceased in the presence of an excess of an unlabeled oligonucleotide containing a NF-κB but not an AP-1 consensus binding site. Furthermore, addition of antibodies against c-Rel and p65, but not of p50, RelB, and p52, diminished formation of the CD40L-enhanced complex (Fig. 3D), indicating that CD40 ligation enhanced binding of a c-Rel/p65 heterodimer to the TF-specific NF-κB site in EC.

Egr-1 Modulates Induction of the Human Tissue Factor Promoter by Recombinant CD40L in Human Endothelial Cells—
The analysis of TF promoter activity employing the 5′-deletion constructs described above did not permit evaluation of the involvement of binding sites located in the SRR because of the loss of inducibility in these markedly shortened constructs. Although the three Egr-1-binding sites may participate in the induction of TF promoter activity in EC by shear stress and vascular endothelial growth factor (5, 11), previous work has not associated these sites with TF induction by proinflammatory cytokines (25).

Exploration of Egr-1 involvement employed TF promoter constructs mutated in all three Egr-1-binding sites. Surprisingly, mutations in the Egr-1 sites of the SRR significantly reduced the induction of the promoter activity by CD40L compared with wild-type (46 ± 31%, n = 5; p < 0.05, Fig. 4A). These results indicated that Egr-1 indeed contributes to the CD40L-dependent induction of TF gene expression in human EC.

To test CD40L-inducible protein-DNA binding activity to the Egr-1 sites, we performed EMSA employing oligonucleotides containing overlapping regions (RI, RII, and RIII) of the SRR, each containing a single Egr-1-binding site. Incubation of nuclear extracts from CD40L-stimulated EC with labeled oligonucleotides containing RI, RII, or AP-1 consensus binding sites (A) or specific antibodies (B) (4 μg) were applied (arrowhead indicates supershift). Protein-DNA complexes were separated using 6% nondenaturing acrylamide gels. Three experiments performed with EC from different donors yielded similar results.

Fig. 5. CD40L induces binding of Egr-1 to three Egr-1-binding sites of the TF promoter in human endothelial cells. Confluent human EC were cultured (1 h) in the absence or presence of recombinant CD40L (10 μg/ml) or PMA (50 ng/ml). Protein-DNA binding was performed with nuclear extracts (2 μg) and oligonucleotides containing Egr-1-binding sites located at regions I, II, or III (RI, RII, and RIII) in the human TF promoter. To confirm the specificity of protein-DNA complexes, a 25-fold molar excess of nonlabeled competitor oligonucleotides containing Egr-1, Sp1, or AP-1 consensus binding sites (A) or specific antibodies (B) (4 μg) were applied (arrowhead indicates supershift). Protein-DNA complexes were separated using 6% nondenaturing acrylamide gels. Three experiments performed with EC from different donors yielded similar results.
CD40L Induces TF Expression via AP-1, NF-κB, and Egr-1 in EC

studies demonstrated that the ligation of CD40 induced the formation of protein-DNA complexes specific for Egr-1.

CD40L Induces the Expression of Egr-1 Protein in Human Vascular Endothelial Cells—To verify our surprising observation that ligation of CD40 on EC promotes TF expression via Egr-1, we further analyzed whether CD40L induced the expression of Egr-1 protein in human EC. Incubation of EC with CD40L indeed triggered accumulation of the Egr-1 protein in nuclear extracts, a previously unsuspected function. Notably, this increase in Egr-1 protein resembled that induced by PMA (Fig. 6A). Interestingly, IL-1β and TNFα did not elevate either expression of Egr-1 protein (Fig. 6A) or Egr-1-specific protein-DNA complexes (Fig. 6B), as demonstrated by Western blot and EMSA, respectively.

DISCUSSION

Various lines of evidence support a role for CD40 signaling in regulating the thrombotic balance in blood vessels (15–20). However, the molecular pathways of CD40L-induced TF expression have remained unknown. We demonstrate here that optimal induction of TF expression in human EC requires not only binding activity of AP-1 and NF-κB family members but also of Egr-1, a transcription factor previously not associated with CD40 signaling and not employed by other established mediators of TF expression, namely IL-1β and TNFα (Fig. 7).

Promoter-reporter gene studies employing 5′-deletion constructs localized CD40L-sensitive elements to a region downstream of −278 bp in the human TF promoter. This region contains the recognized LRE as well as the SRR containing the main regulatory cis-elements for TF gene expression (AP-1 and NF-κB as well as Egr-1-binding sites, respectively) (6). Notably, induction of TF promoter activity by proinflammatory cytokines, such as IL-1β and TNFα, utilizes binding sites located in the LRE (4), whereas those in the SRR are employed by mediators, such as serum, shear stress, hypoxia, and vascular endothelial growth factor (5, 9, 12, 13). Of further interest, here-tofore only PMA and LPS have been reported to utilize both elements, LRE and SRR, to induce TF gene expression (4, 9, 14, 24).

Promoter reporter gene studies demonstrated the involvement of AP-1- and NF-κB-binding sites in CD40L-mediated TF expression, in common with other proinflammatory cytokines, such as IL-1β and TNFα (6). Notably, the constitutive binding of c-Jun and JunD to the AP-1 sites of the TF promoter was not affected by CD40L, whereas CD40 ligation markedly enhanced the binding of c-Rel/p65 proteins to the NF-κB site (Fig. 3). The constitutive binding of c-Jun and JunD proteins to the AP-1 sites agrees with the basal TF promoter activity. Nevertheless, CD40L-induced phosphorylation of bound c-Jun by the stress-activated protein kinase c-Jun NH2-terminal kinase (26) might contribute to increased transcriptional activity of c-Jun (27).

The CD40L-dependent activation of NF-κB binding to the TF promoter most likely depends on the inactivation of IkBα via IKK as demonstrated for other cytokines in various cell types, including EC (28, 29). The requirement for members of both the AP-1 and NF-κB/Rel transcription factor family to maximally induce the TF promoter by CD40L might indicate the necessity of cooperative binding of c-Jun/JunD to the AP-1 sites and c-Rel/p65 to the NF-κB site, both located closely in the LRE of the TF promoter. This hypothesis gains support from recent studies that demonstrated interaction of c-Jun as well as JunD with p65 exhibiting enhanced functional DNA binding activity (30, 31). In addition, modifying the spacing between the AP-1 sites and the NF-κB site in the LRE by insertion of additional base pairs ablated induction of the TF promoter by LPS in monocytes (32).

We further tested whether CD40L, besides PMA and LPS, might utilize binding sites in both elements, LRE and SRR, for optimal induction of TF gene expression, indicating that CD40 signaling differs from that of previously studied proinflammatory mediators, e.g. IL-1β and TNFα. In contrast to the proinflammatory cytokines IL-1β or TNFα, CD40L surprisingly induced Egr-1 protein expression and DNA binding activity in EC, notably to a degree similar to that achieved by PMA. Although a recent report suggested the activation of Egr-1 by

FIG. 6. Egr-1 protein expression in human endothelial cells is induced by CD40L but not by IL-1β or TNFα. Confluent human EC were cultured (1 h) in the absence and presence of IL-1β (10 ng/ml), TNFα (50 ng/ml), recombinant CD40L (10 μg/ml), or PMA (50 ng/ml). A, nuclear extracts (20 μg) were separated by SDS-PAGE, and expression of Egr-1 protein was determined by Western blotting using anti-Egr-1 rabbit polyclonal antibody (1:500). B, protein-DNA binding was performed with nuclear extracts (2 μg) employing oligonucleotides containing either an Egr-1 or AP-1 consensus binding consensus DNA-binding sites for Egr-1. To confirm specificity of formed protein-DNA complexes specific for Egr-1. To verify our surprising observation that ligation of CD40 on EC promotes TF expression via Egr-1, we further analyzed whether CD40L induced the expression of Egr-1 protein in human EC. Incubation of EC with CD40L indeed triggered accumulation of the Egr-1 protein in nuclear extracts, a previously unsuspected function. Notably, this increase in Egr-1 protein resembled that induced by PMA (Fig. 6A). Interestingly, IL-1β and TNFα did not elevate either expression of Egr-1 protein (Fig. 6A) or Egr-1-specific protein-DNA complexes (Fig. 6B), as demonstrated by Western blot and EMSA, respectively.

DISCUSSION

Various lines of evidence support a role for CD40 signaling in regulating the thrombotic balance in blood vessels (15–20). However, the molecular pathways of CD40L-induced TF expression have remained unknown. We demonstrate here that optimal induction of TF expression in human EC requires not only binding activity of AP-1 and NF-κB family members but also of Egr-1, a transcription factor previously not associated with CD40 signaling and not employed by other established mediators of TF expression, namely IL-1β and TNFα (Fig. 7).

Promoter-reporter gene studies employing 5′-deletion constructs localized CD40L-sensitive elements to a region downstream of −278 bp in the human TF promoter. This region contains the recognized LRE as well as the SRR containing the main regulatory cis-elements for TF gene expression (AP-1 and NF-κB as well as Egr-1-binding sites, respectively) (6). Notably, induction of TF promoter activity by proinflammatory cytokines, such as IL-1β and TNFα, utilizes binding sites located in the LRE (4), whereas those in the SRR are employed by mediators, such as serum, shear stress, hypoxia, and vascular endothelial growth factor (5, 9, 12, 13). Of further interest, here-tofore only PMA and LPS have been reported to utilize both elements, LRE and SRR, to induce TF gene expression (4, 9, 14, 24).

Promoter reporter gene studies demonstrated the involvement of AP-1- and NF-κB-binding sites in CD40L-mediated TF expression, in common with other proinflammatory cytokines, such as IL-1β and TNFα (6). Notably, the constitutive binding of c-Jun and JunD to the AP-1 sites of the TF promoter was not affected by CD40L, whereas CD40 ligation markedly enhanced the binding of c-Rel/p65 proteins to the NF-κB site (Fig. 3). The constitutive binding of c-Jun and JunD proteins to the AP-1 sites agrees with the basal TF promoter activity. Nevertheless, CD40L-induced phosphorylation of bound c-Jun by the stress-activated protein kinase c-Jun NH2-terminal kinase (26) might contribute to increased transcriptional activity of c-Jun (27).

The CD40L-dependent activation of NF-κB binding to the TF promoter most likely depends on the inactivation of IkBα via IKK as demonstrated for other cytokines in various cell types, including EC (28, 29). The requirement for members of both the AP-1 and NF-κB/Rel transcription factor family to maximally induce the TF promoter by CD40L might indicate the necessity of cooperative binding of c-Jun/JunD to the AP-1 sites and c-Rel/p65 to the NF-κB site, both located closely in the LRE of the TF promoter. This hypothesis gains support from recent studies that demonstrated interaction of c-Jun as well as JunD with p65 exhibiting enhanced functional DNA binding activity (30, 31). In addition, modifying the spacing between the AP-1 sites and the NF-κB site in the LRE by insertion of additional base pairs ablated induction of the TF promoter by LPS in monocytes (32).

We further tested whether CD40L, besides PMA and LPS, might utilize binding sites in both elements, LRE and SRR, for optimal induction of TF gene expression, indicating that CD40 signaling differs from that of previously studied proinflammatory mediators, e.g. IL-1β and TNFα. In contrast to the proinflammatory cytokines IL-1β or TNFα, CD40L surprisingly induced Egr-1 protein expression and DNA binding activity in EC, notably to a degree similar to that achieved by PMA. Although a recent report suggested the activation of Egr-1 by

FIG. 7. Induction of tissue factor by IL-1β, TNFα, and CD40L is mediated via differential pathways.

FIG. 7. Induction of tissue factor by IL-1β, TNFα, and CD40L is mediated via differential pathways.
TNFα in EC (33), lack of Egr-1 inducibility via TNFα in this cell type reported here is in accordance with the previous report of Pendurthi et al. (25). Notably, all three Egr-1-binding sites located downstream of the LRE in the human TF promoter appear to function in CD40L-mediated TF expression.

Therefore, optimal induction of the TF promoter by CD40L in EC probably requires not only interaction with the transcription factors of the AP-1 and c-Rel/NF-κB families but also Egr-1. The finding that members of all three transcription factor families participate in the induction of TF expression agrees with recent observations in LPS-stimulated monocytic cells (14).

The present findings indicate that CD40 ligation on EC results in broader activation of intracellular signaling pathways targeting the TF gene than the “classical” cytokines IL-1β and TNFα. This finding may further help to understand why CD40L activates certain genes (e.g. caspase-1 (ICE) and stromelysin-3) (21, 34) not affected by IL-1β or TNFα.

The present results provide new insight into the transcriptional regulation of endothelial TF expression following CD40 ligation and illustrate a role for Egr-1 activation in the regulation of the thrombogenic potential of endothelial cells by CD40L. Thus, pharmacologic inhibition of CD40L signaling pathways might provide new therapeutic options to prevent thrombotic complications in inflammatory processes such as atherosclerosis.

Acknowledgments—We thank Maria Muszynski, Anna Papautsky, Samantha LaClair, and Elissa Simon-Morrissey (Brigham and Women’s Hospital) for skillful technical assistance, and Karen Williams for editorial assistance.

REFERENCES

1. Fuster, V., Badimon, I., Badimon, J. J., and Chesebro, J. H. (1992) N. Engl. J. Med. 326, 242–250
2. Fuster, V., Badimon, L., Badimon, J. J., and Chesebro, J. H. (1992) N. Engl. J. Med. 326, 310–318
3. Edgington, T. S., Mackman, N., Brand, K., and Ruf, W. (1991) Thromb. Haemostasis 66, 67–79
4. Parry, G. C., and Mackman, N. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 612–621
5. Mechtcheriakova, D., Wlauch, A., Holzmuller, H., Binder, B. R., and Hofer, E. (1999) Blood 93, 3811–3823
6. Mackman, N. (1997) Thromb. Haemostasis 77, 747–754
7. Mackman, N., Brand, K., and Edgington, T. S. (1991) J. Exp. Med. 174, 1517–1526
8. Oeth, P. A., Parry, G. C., Kunsch, C., Nantenmet, P., Rosen, C. A., and Mackman, N. (1994) Mol. Cell. Biol. 14, 7572–7582
9. Cui, M. Z., Parry, G. C., Edgington, T. S., and Mackman, N. (1994) Arterioscler. Thromb. 14, 807–814
10. Cui, M. Z., Penn, M. S., and Chislem, G. M. (1999) J. Biol. Chem. 274, 32705–32702
11. Houston, P., Dickson, M. C., Ludbrook, V., White, B., Schwachtmann, J. L., McVey, J. H., Mackman, N., Reese, J. M., Gorman, D. G., Campbell, C., and Bradock, D. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 281–288
12. Lin, M. C., Almus-Jacobs, F., Chen, H. H., Parry, G. C., Mackman, N., Shyy, J. Y., and Chien, S. (1997) J. Clin. Invest. 99, 737–744
13. Yan, S. F., Zou, Y. S., Gao, Y., Zhai, C., Mackman, N., Lee, S. L., Milbrandt, J., Pinsky, D., Kistel, W., and Stern, D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8298–8303
14. Guha, M., O’Connell, M. A., Pawlinski, R., Hollis, A., McGovern, P., Yan, S. F., Stern, D., and Mackman, N. (2001) Blood 98, 1429–1439
15. Mach, F., Schonbeck, U., Benzeley, J. V., Pober, J. S., and Libby, P. (1997) Circulation 96, 396–399
16. Miller, D. L., Yaron, R., and Yellin, M. J. (1998) J. Leukocyte Biol. 63, 373–379
17. Slupsky, J. R., Kalhas, M., Wissweil, A., Henn, V., Kroczek, R. A., and Muller-Berghaus, G. (1998) Thromb. Haemostasis 80, 1008–1014
18. Zhou, L., Stordeur, P., de Lavareille, A., Thielemans, K., Capel, P., Goldman, M., and Pradier, O. (1998) Thromb. Haemostasis 79, 1025–1028
19. Schonbeck, U., Mach, F., Sukhwa, G. K., Herman, M., Gruber, P., Kehry, M. R., and Libby, P. (2000) Am. J. Pathol. 156, 7–14
20. Aikawa, M., Voglic, S. J., Sugiyama, S., Rabkin, E., Taubman, M. B., Fallon, J. T., and Libby, P. (1999) Circulation 100, 1215–1222
21. Schonbeck, U., Mach, F., Sukhwa, G. K., Atkinson, E., Akimian, E., Levesque, E., Herman, M., Gruber, P., Basset, P., and Libby, P. (1999) J. Exp. Med. 189, 843–853
22. Potgens, A. J., Luhsen, N. H., van Altena, G., Schoenmakers, J. G., Ruitter, D. J., and de Waal, R. M. (1994) Thromb. Haemostasis 71, 208–213
23. Read, M. A., Cordle, S. R., Veach, R. A., Carlisle, C. D., and Hawiger, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8987–8991
24. Cui, M. Z., Parry, G. C., Oeth, P., Larsson, H., Smith, M., Huang, R. P., Adamson, E. D., and Mackman, N. (1994) J. Biol. Chem. 271, 2731–2738
25. Pendurthi, U. R., Williams, J. T., and Rao, L. V. (1997) Arterioscler. Thromb. Vasc. Biol. 17, 3406–3413
26. Karmann, K., Hughes, C. M., Schechner, J., Fanalow, W. C., and Pober, J. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4342–4346
27. Karin, M. (1995) J. Biol. Chem. 270, 16483–16486
28. Bennett, B. L., Lacson, R. G., Chen, C. C., Cruz, R., Wheeler, J. S., Kletzien, R. R., Tomasselli, A. G., Heinrikson, R. L., and Manning, A. M. (1996) J. Biol. Chem. 271, 19680–19688
29. Karin, M. (1999) J. Biol. Chem. 274, 27339–27342
30. Stein, B., Baldwin, A. S., Jr., Ballard, D. W., Greene, W. C., Angel, P., and Herrlich, P. (1993) EMBO J. 12, 3873–3881
31. Rahmani, M., Peron, P., Weitzman, J., Bakiri, L., Lardeux, B., and Bernaua, D. (2001) Oncogene 20, 5132–5142
32. Oeth, P., Parry, G. C., and Mackman, N. (1997) Arterioscler. Thromb. Vasc. Biol. 17, 365–374
33. Mechtcheriakova, D., Schabbauer, G., Lucerna, M., Claus, M., De Martin, R., Binder, B. R., and Hofer, E. (2001) FEBS J. 18, 230–242
34. Schonbeck, U., Mach, F., Benzeley, J. V., Lappnow, H., Flad, H. D., and Libby, P. (1997) J. Biol. Chem. 272, 19569–19574
Induction of Tissue Factor Expression in Human Endothelial Cells by CD40 Ligand Is Mediated via Activator Protein 1, Nuclear Factor κB, and Egr-1
Udo Bavendiek, Peter Libby, Meagan Kilbride, Rebecca Reynolds, Nigel Mackman and Uwe Schönbeck

J. Biol. Chem. 2002, 277:25032-25039.
doi: 10.1074/jbc.M204003200 originally published online April 26, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204003200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 21 of which can be accessed free at http://www.jbc.org/content/277/28/25032.full.html#ref-list-1