Thrombin Stimulation of the Vascular Cell Adhesion Molecule-1 Promoter in Endothelial Cells Is Mediated by Tandem Nuclear Factor-κB and GATA Motifs*

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The goal of this study was to delineate the transcriptional mechanisms underlying thrombin-mediated induction of vascular adhesion molecule-1 (VCAM-1). Treatment of human umbilical vein endothelial cells with thrombin resulted in a 3.3-fold increase in VCAM-1 promoter activity. The upstream promoter region of VCAM-1 contains a thrombin response element, two nuclear factor κB (NF-κB) motifs, and a tandem GATA motif. In transient transfection assays, mutation of the thrombin response element had no effect on thrombin induction. In contrast, mutation of either NF-κB site resulted in a complete loss of induction, whereas a mutation of the two GATA motifs resulted in a significant reduction in thrombin stimulation. In electrophoretic mobility shift assays, nuclear extracts from thrombin-treated endothelial cells displayed markedly increased binding to the tandem NF-κB and GATA motifs. The NF-κB complex was supershifted with anti-p65 antibodies, but not with antibodies to RelB, c-Rel, p50, or p52. The GATA complex was supershifted with antibodies to GATA-2, but not GATA-3 or GATA-6. A construct containing tandem copies of the VCAM-1 GATA motifs linked to a minimal thymidine kinase promoter was induced 2.4-fold by thrombin. Taken together, these results suggest that thrombin stimulation of VCAM-1 in endothelial cells is mediated by the coordinate action of NF-κB and GATA transcription factors.

Vascular adhesion molecule-1 (VCAM-1) is a 110-kDa cell surface glycoprotein that was first identified as an adhesion molecule expressed by cytokine-activated endothelial cells (1). In response to inflammatory mediators, VCAM-1 interacts with its integrin counter-receptor, very late antigen-4, very late antigen-4 to mediate the recruitment of mononuclear leukocytes to the endothelium. In addition to its physiological role in cell trafficking, VCAM-1 has been implicated in a number of chronic inflammatory disease states, including rheumatoid arthritis and atherosclerosis (2–5).

The VCAM-1 promoter has been cloned and well characterized (6). Previous studies have shown that two tandem NF-κB elements located at position −77 and −63, relative to the transcriptional start site are necessary for mediating response to inflammatory mediators (6–8). Other studies have invoked an important role of co-stimulators in mediating cytokine response, including Sp1 (9), activating protein-1 (10), interferon regulatory factor-1 (11), and GATA-binding proteins (12, 13).

Thrombin, a multifunctional serine protease derived from the zymogen prothrombin, is a key component of the coagulation cascade, serving to catalyze the conversion of fibrinogen to fibrin. In addition, thrombin plays a role in inflammation by activating a variety of cell types, including endothelial cells, smooth muscle cells, and leukocytes. Most of these cellular effects are mediated by PAR1, a G-protein-coupled receptor. Thrombin cleaves the receptor, unmasking a tethered ligand, which is then free to activate the receptor (14, 15).

Thrombin-stimulated endothelial cells bind more avidly to polymorphonuclear cells (16–19), and monocytes (20). The effect of thrombin on endothelial-polymorphonuclear cell interactions may be explained, at least in part, by the ability of thrombin to induce expression of E-selectin (21), P-selectin (17, 19), and intercellular adhesion molecule-1 (ICAM-1) (18, 21), whereas the effect on monocyte adhesion has been attributed to increased levels of ICAM-1 and VCAM-1 (20). Given that chronic inflammation is characterized by increased monocyte-endothelial cell interactions, an understanding of the molecular mechanisms by which thrombin induces VCAM-1 expression may provide important insight about the link between coagulation and chronic inflammatory disease states.

In the present study, we have examined the transcriptional mechanisms that underlie thrombin-mediated induction of VCAM-1 in endothelial cells. We show that the effect of thrombin on VCAM-1 expression is mediated by a combination of NF-κB and GATA motifs in the upstream promoter region. These findings are the first to demonstrate a link between thrombin signaling and GATA binding activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human thrombin, leech hirudin, angiotensin II, and thrombin receptor activation peptide (TRAP; SFLIRNPNDKYPF) were obtained from Sigma. Human vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF)-BB were obtained from Peprotec (Rocky Hill, NJ). Mouse tumor necrosis factor (TNF-α) was obtained from Life Technologies, Inc. Anti-TNF-α antibody was obtained from Calbiochem (San Diego, CA).

**Cell Culture**—Human umbilical vein endothelial cells (HUVEC) (Clonetics, La Jolla, CA) were cultured in EGM-2 MV complete medium. Human embryonic kidney (HEK)-293 cells (ATCC CRL-1573) were cultured in Dulbecco’s modified Eagle’s medium supplemented with...
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10% heat-inactivated fetal bovine serum (FBS). HUVEC were used within the first 8 passages.

Plasmids—For construction of the VCAM-1-luc plasmid, human VCAM-1 promoter, a region spanning -1716 to +119 bp (generously provided by Dr. Tsutahiko Kodama, Tokyo University, Tokyo, Japan) was cloned into pGL3-basic vector (Promega, Madison, WI). A series of point mutations were introduced into the VCAM-1 promoter by polymerase chain reaction methodology. To generate NF-κB-mut-luc, two polymerase chain reaction fragments (A and B) were amplified from VCAM-1-luc. Fragment A was generated from forward primer with an NsiI site (5′-AAATACGATCATGTTAATGATGAGT-3′) and reverse primer with a PstI site (5′-GGATCTTTCGAGGTTCCCTG-3′) and reverse primer with an NcoI site (5′-CCCCATGTCACCTGAACT-3′) and reverse primer with a SacII site (5′-CAGATAACCGGATGAACTAGAAAAGTCTG-3′). Fragment A was digested with NsiI and PstI, whereas fragment B was digested with PstI and SacII. Fragments A and B were then inserted into NsiI/SacII-digested VCAM-1-luc in a three-way ligation. The resulting plasmid introduced two NF-κB point mutations: GGGTTTCCCC and GGGATTTCGG and GGGATTTCGG → GCCATTTCGG. A similar strategy was used to introduce the following mutations into 5′ NF-κB-mut: GGGTTTCCCC → GCCATTTCGG; 3′ NF-κB-mut, GGGATTTCGG → GCCATTTCGG; thymidylate response element (TRE) mut, GCCMCTTC → CCCGATG; and GATA mut, TTAATCT → TTAAAT and AGATAG → ATTTAG. All point mutations were confirmed by automated DNA sequencing. To generate the pGEM-vCAM1, a 254-bp human VCAM-1 cDNA fragment, a covered region from bp 1755 to 2009, was amplified from reverse-transcribed HUVEC total RNA and subcloned into pGEM/T easy vector (Promega). pTRI-hGAPDH was purchased from Ambion (Austin, TX). For construction of the herpes simplex virus thymidine kinase (TK)-luc, a BglII/HindIII fragment of pRL-TK (Promega) was cloned into the pGEM-T easy vector. To generate pGEM-hVCAM1, a 254-bp probe, 1 kb of poly(dI-dC), and 3 templates B mut-luc, two simplex virus thymidine kinase (TK)-luc, a double-stranded oligonucleotide containing two copies of the tandem GATA motifs from the VCAM-1 promoter (5′-ATTGTCTTTATATTCGAGGTTCCCTG-3′) was cloned into the pGL3-basic vector. To generate GATA-1-TK-luc, a double-stranded oligonucleotide containing two copies of the tandem GATA motifs from the VCAM-1 promoter (5′-ATTGTCTTTATATTCGAGGTTCCCTG-3′) was cloned into the Nhel-digested TK-luc plasmid vector. Insert direction was confirmed by automated DNA sequencing.

RNA Isolation and RNase Protection Assays—HUVEC were cultured in 100-mm culture dishes, and then the culture medium was replaced with serum-starved medium (EBM-2 plus 0.5% FBS). 18 h later, HUVEC were incubated with 1.5 units/ml human thrombin (Sigma), with 20 ng/ml TNF-α (Life Technologies, Inc.), or with serum-starved medium alone. Cells were harvested for total RNA 4, 7, and 24 h following thrombin treatment and 4 h following TNF-α treatment, using the Trizol reagent (Life Technologies). For in vitro transcription, VCAM-1- and GAPDH-specific 32P-labeled riboprobes were synthesized from pGEM-hVCAM1 and pTRI-hGAPDH, respectively. Both riboprobes were synthesized using SP6 RNA polymerase (Ambion) and purified with a G-50 spin column (Amersham Pharmacia Biotech). RNase protection assays were performed with a RPA III kit (Ambion), according to the manufacturer’s instructions.

Transfections and Analysis of Luciferase Activity—HUVEC and HEK-293 cells were transfected using FuGENE 6 reagent (Roche Molecular Biochemicals) as instructed by the manufacturer. Either 1 × 105 cells/well of HUVEC or 2 × 105 cells/well of HEK-293 cells were seeded in 12-well plates 18–24 h before transfection. For HUVEC transfections, 0.5 μg of the reporter gene construct and 50 ng of a co-transfected plasmid containing the Renilla luciferase reporter gene under the control of a cytomegalovirus enhancer/promoter (pRL-CMV) (Promega) were incubated with 2 μg of FuGENE 6. 24 h later, the cells were washed with phosphate-buffered saline two times and cultured for 18 h in serum-starved medium (EBM-2 plus 0.5% FBS). The cells were then incubated in the presence or absence of cytokines for 6 h, at which time cells were lysed and assayed for luciferase activity using the dual luciferase reporter assay system (Promega) and Lumat LB 9507 lumino-meter (Berthold, Gautersburg, MD). For HEK-293 cell transfections, 0.05 pmol of the test construct, 0.075 pmol of the GATA expression vector, and 30 ng of pRL-CMV were incubated with 1.5 μl of FuGENE 6. 2 days later, the cells were lysed and assayed for luciferase activity as described above.

Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared as described previously (22). Double-stranded oligonucleotides were labeled with [α-32P]dCTP and Klenow fragment and purified by spin column (Amersham Pharmacia Biotech). 5 μg of HUVEC nuclear extracts were incubated with 10 fmol of 32P-labeled probe, 1 μg of poly[dI-dC], and 3 μl of 10× binding buffer (100 mM Tris-HCl [pH 7.5], 50% glycerol, 10 mM dithiothreitol, 10 mM EDTA) for 20 min at the room temperature, followed by 30 min at 4 °C. To test the effect of antibodies on DNA-protein binding, nuclear extracts were pre-incubated with antibodies of p65, RelB, c-Rel, p50, and p52 (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature, or with antibodies of GATA-2 (a generous gift from Dr. Stuart Orkin, Harvard Medical School, Boston, MA) and GATA-3 (Santa Cruz Biotechnology) for 1 h at 4 °C. In competition studies, nuclear extracts were pre-incubated with 50-fold molar excess of unlabelled wild-type or mutant oligonucleotides for 20 min at room temperature, then added to the reaction mixture. DNA-protein complexes were resolved on a 5% non-denaturing polyacrylamide gel containing 5% glycerol in 0.5× TBE (50 mM Tris, 50 mM boric acid, and 1 mM EDTA). The loaded gel was fixed with 10% methanol and 10% acetic acid, then autoradiographed.

RESULTS

Thrombin-mediated Up-regulation of VCAM-1 mRNA—In a previous study, thrombin was shown to induce VCAM-1 expression in endothelial cells (20). To confirm these results, we employed RNase protection assays with a probe that is specific for the human VCAM-1 gene and total RNA derived from control and thrombin- and TNF-α-treated HUVEC. As shown in Fig. 1 (A and B), the incubation of HUVEC in the presence of 1.5 units/ml thrombin or 20 ng/ml TNF-α for 4 h resulted in 39.8- or 48.0-fold stimulation of VCAM-1 mRNA, respectively (Fig. 1A, lanes 3 and 7). Following thrombin treatment,

FIG. 1. Endogenous VCAM-1 expression in endothelial cells is induced by thrombin. A, HUVEC were serum-starved and then incubated in the absence or presence of 1.5 units/ml thrombin or 20 ng/ml TNF-α for the indicated times. In RNase protection assays, a [α-32P]UTP-labeled 331-bp human VCAM-1 antisense riboprobe was incubated with 10 μg of yeast RNA (lane 1) or 10 μg of total RNA from untreated HUVEC (lanes 2 and 6), thrombin-treated HUVEC (lanes 3–5), respectively, or TNF-α-treated HUVEC (lane 7). The protected fragment (254 bp) represents the human VCAM-1 transcript (hVCAM-1). A [α-32P]UTP-labeled human GAPDH antisense riboprobe (hGAPDH) was hybridized with total RNA as an internal control. B, quantitation of the RNase protection assays. Densitometry was used to calculate the ratio of VCAM-1 and GAPDH signals. The results are expressed as -fold induction relative to untreated cells (—). The data represent the average from two independent experiments.
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VCAM-1 mRNA levels remained elevated at 7 h and approached base-line levels by 24 h (Fig. 1A, lane 5).

Thrombin-mediated Up-regulation of VCAM-1 Promoter—We next wished to determine whether the effect of thrombin on VCAM-1 mRNA expression was mediated by the VCAM-1 promoter. HUVEC were transiently transfected with the VCAM-1-luc plasmid that contains a 1.8-kilobase pair region of the human VCAM-1 promoter (between −1716 and +119) coupled to the luciferase reporter gene. Transfected HUVEC were grown in the absence or presence of thrombin and assayed for luciferase activity 6 h later. As shown in Fig. 2A, thrombin resulted in a dose-dependent stimulation of reporter gene activity, with maximal induction occurring at a concentration of 1.5 units/ml. Furthermore, the addition of the specific thrombin inhibitor, hirudin, resulted in a dose-dependent inhibition of the thrombin-mediated VCAM-1 promoter activity (Fig. 2B). In subsequent studies, we analyzed the effects of TRAP, a 14-amino acid peptide representing the new amino terminus of PAR-1 generated after thrombin cleavage, on VCAM-1 promoter activity. As shown in Fig. 3, thrombin and TRAP induced VCAM-1 promoter activity by 3.3- and 3.5-fold, respectively. These results suggest that thrombin-induced activation of the VCAM-1 promoter is mediated by cleavage of PAR-1. The VCAM-1 promoter was induced 4.3-fold by TNF-α. The addition of VEGF resulted in a slight increase in promoter activity, whereas PDGF-BB and angiotensin II had no such effect (Fig. 3).

Finally, to rule out the possibility that thrombin stimulation of the VCAM-1 promoter was mediated by TNF-α release, transfected cells were treated with TNF-α or thrombin in the absence or presence of neutralizing anti-TNF-α antibodies. In these experiments, thrombin-mediated induction of promoter activity was unaltered by pre-incubation with antibody at concentrations that otherwise inhibited the effects of TNF-α (data not shown).

Thrombin-mediated Up-regulation of the VCAM-1 Promoter Is Not Mediated by the Thrombin Response Element—The human VCAM-1 promoter contains a consensus TRE at −319 relative to the start site of transcription (Fig. 4A). To determine whether this regulatory element was involved in transducing the thrombin signal, a plasmid containing a point mutation of the TRE sequence was transfected into HUVEC. As shown in Fig. 4B, the TRE mutant retained thrombin and TRAP responsiveness, suggesting that this sequence is not essential for thrombin-mediated activation of VCAM-1. In electrophoretic mobility shift assays, a radiolabeled VCAM TRE element probe was incubated with nuclear extracts derived from HUVEC treated in the presence or absence of thrombin. DNA binding activity was similar in both treated and untreated cells (Fig. 4C, lanes 2 and 5).

Thrombin-mediated Up-regulation of the VCAM-1 Promoter Is Mediated by the NF-κB Motifs—The VCAM-1 promoter has tandem NF-κB motifs located at the positions −65 and −75 relative to the transcriptional start site (Fig. 5A). To assess the role of the NF-κB elements in mediating thrombin stimulation of the VCAM-1 promoter, the two NF-κB sites were mutated and the resulting plasmid was transfected into HUVEC. The double mutation resulted in complete abrogation of response to thrombin, TRAP, and TNF-α (Fig. 5B). To determine the relative contribution of the two NF-κB motifs in transducing the thrombin response, the 5’ or 3’ NF-κB motifs were individually mutated. As shown in Fig. 5B, each mutation completely blocked the response to thrombin, TRAP, and TNF-α. Taken together, these results suggest that both NF-κB motifs on the VCAM-1 promoter are necessary for thrombin-mediated activation of the VCAM-1 promoter.
Thrombin Induces DNA-binding Levels on NF-κB Motifs in VCAM-1 Promoter

To determine the effect of thrombin on NF-κB binding activity, electrophoretic mobility shift assays were carried out. To that end, a radiolabeled probe containing the two adjacent NF-κB sites (Fig. 6A) was incubated with nuclear extracts derived from untreated and thrombin-treated HUVEC. Incubation with nuclear extracts from untreated, thrombin-treated, or TNF-α-treated HUVEC resulted in specific DNA-protein complexes (open arrows, Fig. 6B, lanes 2, 5, and 8). These DNA-protein complexes were inhibited by the addition of a 50-fold molar excess of unlabeled self-competitor (Fig. 6B, lanes 3, 6, and 9), but not by the same concentration of unlabeled NF-κB mutant competitor (Fig. 6B, lanes 4, 7, and 10). The faster migrating DNA-protein complex (closed arrow) was inhibited by a 50-fold molar excess of unlabeled self-probe (lanes 3, 6, and 9) or mutant TRE probe (lanes 4, 7, and 10) was added to the reaction mixture. The arrow indicates the specific DNA complex. The results are representative of two independent experiments.

FIG. 5. The tandem NF-κB motif is necessary for mediating thrombin stimulation of the VCAM-1 promoter. A, schematic representation of the 5′-flanking region of the human VCAM-1 gene. VCAM-1-luc represents the wild type promoter, whereas NF-κB mut-luc, 5′ NF-κB mut-luc, and 3′ NF-κB mut-luc contain mutations of one or both NF-κB motifs. B, HUVEC were transiently transfected with 0.5 μg of each plasmid and exposed to 1.5 units/ml thrombin, 20 nmol/ml TRAP, and 20 ng/ml TNF-α for 6 h. The results show the means and standard deviations of luciferase light units (relative to untreated cells) obtained in triplicate from at least three independent experiments.

Thrombin Induces DNA-binding Levels on NF-κB Motifs in VCAM-1 Promoter—To determine the effect of thrombin on NF-κB binding activity, electrophoretic mobility shift assays were carried out. To that end, a radiolabeled probe containing the two adjacent NF-κB sites (Fig. 6A) was incubated with nuclear extracts derived from untreated and thrombin-treated HUVEC. Incubation with nuclear extracts from untreated, thrombin-treated, or TNF-α-treated HUVEC resulted in specific DNA-protein complexes (open arrows, Fig. 6B, lanes 2, 5, and 8). These DNA-protein complexes were inhibited by the addition of a 50-fold molar excess of unlabeled self-competitor (Fig. 6B, lanes 3, 6, and 9), but not by the same concentration of unlabeled NF-κB mutant competitor (Fig. 6B, lanes 4, 7, and 10). The faster migrating DNA-protein complex (closed arrow) was inhibited by a 50-fold molar excess of unlabeled self-probe (lanes 3, 6, and 9) or mutant TRE probe (lanes 4, 7, and 10) was added to the reaction mixture. The arrow indicates the specific DNA complex. The results are representative of two independent experiments.

TNF-α for 6 h. The results show the mean and standard deviation of luciferase light units (relative to untreated cells) obtained in triplicate from three independent experiments. C, electrophoretic mobility shift assays were performed with 32P-labeled TRE probe (5′-TTCTCTC-CCCACCCTCTT-3′) and 5 μg of nuclear extract from HUVEC treated in the absence (−) or presence of 1.5 units/ml thrombin (+Thrombin) or 20 ng/ml TNF-α (+TNF-α). In competition assays, 50-fold molar excess of unlabeled self-probe (lanes 3, 6, and 9) or mutant TRE probe (5′-TTCTCTCCTTATGCTTA-3′) (lanes 4, 7, and 10) was added to the reaction mixture. The arrow indicates the specific DNA complex. The results are representative of two independent experiments.
FIG. 6. Thrombin induces binding of p65 homodimers to the tandem NF-κB motif. A, schematic representation of the probe sequences used in electrophoretic mobility shift assays. The consensus NF-κB motifs are underlined. The mutated bases are represented by asterisks. B, electrophoretic mobility shift assays were performed with 32P-labeled NF-κB probe and 5 μg of nuclear extract from HUVEC treated in the absence (−) or presence of 1.5 units/ml thrombin (+Thrombin) or 20 ng/ml TNF-α (+TNF-α). In competition assays, a 50-fold molar excess of unlabeled wild type (lanes 3, 6, and 9) or mutant (lanes 4, 7, and 10) NF-κB probe was added to the reaction mixture. The open arrows indicate specific DNA-protein complexes. The closed arrow indicates nonspecific DNA-protein complex. C, electrophoretic mobility shift assays were performed as described above, using radiolabeled wild type NF-κB probe (lanes 1, 4, 7, and 10), 5' NF-κB mut (5' mut) probe (lanes 2, 5, 8, and 11), or 3' NF-κB mut (3' mut) probe (lanes 3, 6, 9, and 12). The open arrows indicate specific DNA-protein complexes. The closed arrow indicates nonspecific DNA-protein complex. D, nuclear extracts from untreated (−) or thrombin-treated (+Thrombin) were incubated in the absence (lanes 2 and 3) or presence of antibodies against p65 (lane 4), RelB (lane 5), c-Rel (lane 6), p50 (lane 7), and p52 (lane 8) as described under “Experimental Procedures.” The asterisk indicates the supershifted complex. E, nuclear extracts from untreated (−) or TNF-α-treated (+TNF-α) HUVEC were incubated with antibodies against p65 (lane 4), RelB (lane 5), c-Rel (lane 6), p50 (lane 7), and p52 (lane 8). The asterisk indicates the supershifted complex. The results are representative of two independent experiments.
was nonspecific, as it was inhibited by the addition of both wild type and mutant competitors (Fig. 6B, lanes 2–10). Importantly, the addition of thrombin or TNF-α resulted in a marked increase in DNA binding activity (Fig. 6B, compare lanes 5 and 8 with lane 2). To test the relative importance of the two NF-κB motifs in mediating this effect, mobility shift assays were carried out with radiolabeled probes containing a mutation of either the 5′ or 3′ NF-κB motif. As shown in Fig. 6C, each single mutation resulted in a significant reduction of basal as well as thrombin- and TNF-α-stimulated DNA-protein complex formation. Together with the transfection data, these results suggest that both NF-κB motifs are involved in mediating the thrombin response.

We next performed supershift experiments with specific antibodies to p65 (RelA), RelB, c-Rel, p50, and p52 to determine the identity of proteins in the thrombin-induced NF-κB binding complex. The addition of anti-p65 antibody resulted in a supershift of the specific DNA-protein complexes (asterisk, Fig. 6D, lane 4), whereas the addition of antibodies to RelB, c-Rel, p50, and p52 had no such effect. A similar pattern was observed in the TNF-α-treated cells (asterisk, Fig. 6E, lane 4). To verify that the anti-p50 antibody was capable of supershifting DNA-protein complexes under these conditions, mobility shift assays were carried out with nuclear extracts from untreated, thrombin-treated, or TNF-α-treated cells resulting in specific DNA-protein complexes (closed and open arrows, Fig. 6B, lanes 2, 5, and 8). The DNA-protein complexes were inhibited by the addition of a 100-fold molar excess of unlabeled self-competitor (Fig. 6B, lanes 3, 6, and 9). In contrast, DNA-protein binding was unaltered in the presence of 100-fold molar excess of unlabeled probe containing a mutation of the GATA sites (Fig. 6B, lanes 4, 7, and 10). Nuclear extracts from thrombin- or TNF-α-treated endothelial cells displayed markedly increased GATA binding activity (Fig. 6B, compare lanes 5 and 8 with lane 2).

To determine the identity of proteins in the thrombin-induced GATA motif binding complex, the binding reactions were incubated with anti-GATA-2 or anti-GATA-3 antibodies. The addition of an anti-GATA-2 antibody from Dr. Stuart Orkin resulted in a supershift of the upper DNA-protein complex (Fig. 8B, lane 12), whereas the addition of anti-GATA-3, anti-
suggest that the slower migrating complex (open arrow, Fig. 8A) of the GATA mutant probe (data not shown). Together with the previous results, these findings suggest that the GATA motifs are both necessary and sufficient for mediating the thrombin response.

The VCAM-1 Promoter Is Transactivated by GATA-2—GATA-2 has a tissue-specific expression pattern. To study the correlation of the vascular selective expression of VCAM-1 and GATA-2, we performed transactivation assays in which VCAM-1-luc construct was co-transfected with human GATA-2 expression plasmid (pMT2-hGATA2) in HEK-293 cells. As shown in Fig. 10, co-transfection of GATA-2 induced the VCAM-1 promoter activity by 7.3-fold. In contrast, VCAM-1-luc construct containing a mutation of GATA sites and pGL3-basic backbone plasmid failed to respond to GATA-2 overexpression. These findings indicate that GATA element on the VCAM-1 promoter has important role for the expression of VCAM-1 in the vasculature.

DISCUSSION

Recent studies have underscored the importance of crosstalk between the coagulation and inflammatory pathways. Inflammatory mediators induce the expression of tissue factor on circulating monocytes (24), thereby contributing to the initiation of coagulation. Moreover, cytokines and other inflammatory mediators activate endothelial cells, resulting in local imbalances of endothelial-derived anticoagulants and procoagulants (25, 26). In turn, serine proteases of the coagulation cascade are capable of amplifying the pro-inflammatory response. For example, factors VIIa and Xa and thrombin have each been shown to activate endothelial cells by binding to membrane-associated protease-activated receptors (27).

The interaction of thrombin with endothelial cells has been reported to result in increased permeability (28), the release of soluble mediators, including platelet-activating factor (29), interleukin-8 (21, 30), monocyte chemotactic protein-1 (31–33), growth factors, and matrix metalloproteinases (34). In addition, thrombin induces the expression of adhesion molecules, including E-selectin (21), VCAM-1 (20), and ICAM-1 (18, 20).

Most of thrombin’s actions are mediated through PAR1, a G-protein-coupled receptor. Thrombin cleaves the receptor, unmasking a tethered ligand that then activates the receptor (15). TRAP is a 14-amino acid peptide that corresponds to the newly exposed tethered ligand and reproduces many of the same cellular processes as thrombin.

Thrombin-mediated induction of gene expression has been reported to involve one of three transcriptional networks. Thrombin stimulation of the PDGF B chain and tissue factor has been linked to the activation of a Y box-binding protein DNA-binding protein (dbpB) and subsequent binding to CCACCC consensus sequence in the upstream promoter region (35, 36). Thrombin-mediated induction of ICAM-1 has been shown to involve the binding of p65 homodimers to a single NF-xB site in the upstream promoter region (18). In the latter study, a construct containing tandem NF-xB sites coupled to a heterologous core promoter was sufficient for transducing the thrombin response. Finally, thrombin has been reported to increase the expression of a chemokine, termed 9E3/cCAF, through the induction of phosphorylated Elk-1:DNA protein interactions (37).

The VCAM-1 promoter contains a potential binding site for dbpB (consensus sequence CCACCC). However, thrombin treatment of HUVEC did not result in increased nuclear protein binding to this sequence. Moreover, a mutation of the CCACCC sequence had no effect on thrombin stimulation of
the VCAM-1 promoter. These results argue against an important role of the CCACCC sequence in mediating the thrombin response of VCAM-1.

In contrast, the two adjacent NF-κB sites in the 5′-flanking region of the VCAM-1 promoter were shown to be necessary for mediating the thrombin response. In electrophoretic mobility shift assays, NF-κB binding was significantly induced by thrombin treatment. Moreover, a mutation of either site resulted in a complete loss of thrombin stimulation in transient transfections. NF-κB is a proinflammatory rapidly inducible transcription factor, which up-regulates several genes involved in endothelial cell activation. The NF-κB/Rel family consists of five distinct DNA-binding proteins, including p50, p52, p65 (RelA), c-Rel, and Rel-B. In quiescent cells, NF-κB is seques-
The finding that the NF-κB DNA-protein complexes were supershifted by antibodies to p65, but not p50, suggests that thrombin-mediated induction of VCAM-1 is dependent on p65 homodimers. These observations are consistent with previous studies showing that the p65 homodimer, but not the p50-p65 heterodimer, is a potent transactivator of the ICAM-1 promoter (38). Indeed, the p50 subunit has been proposed to function as a negative transcriptional regulator of the VCAM-1 gene, by virtue of its ability to inhibit the transcriptional potential of p65 (38).

We have shown that a tandem GATA site in the upstream promoter region of VCAM-1 is necessary for optimal levels of thrombin induction. A mutation of the two GATA sites in the upstream promoter region of VCAM-1 resulted in a reduction of thrombin stimulation. Moreover, nuclear extracts from thrombin-treated endothelial cells revealed significantly higher GATA binding activity compared with control, untreated cells. We have also demonstrated that the VCAM-1 GATA motifs are sufficient for transducing the thrombin signal. When linked to a minimal core promoter, the tandem GATA sites retained thrombin responsiveness. Previous studies have implicated a role for GATA proteins in mediating VCAM-1 response to LPS (13) and TNF-α (12). In this latter study, TNF-α treatment of HUVEC resulted in increased GATA-2 and GATA-6 mRNA levels and decreased GATA-3 mRNA levels, raising the interesting possibility that TNF-α-mediated induction of VCAM-1 involves the selective up-regulation and down-regulation of specific GATA isoforms. In the present report, we have shown that thrombin-stimulated GATA DNA-protein complexes contained GATA-2. In contrast, we did not detect a supershift with anti-GATA-3 or anti-GATA-6 antibodies. These results suggest that thrombin stimulation of VCAM-1 is mediated, at least in part, by a GATA-2-dependent mechanism. The extent to which the effect is mediated by increased GATA-2 mRNA levels or post-translational modification(s) remains to be determined.

Although GATA-binding proteins were initially characterized as constitutively active transcription factors involved in mediating cell type-specific gene expression and lineage determination, recent studies have uncovered a potentially important role for GATA proteins in temporal gene regulation. GATA DNA binding activity and/or GATA mRNA expression has been shown to increase in response to a number of mediators, including insulin-like growth factor 1 (39), follicle-stimulating hormone (40), endothelin-1 (41), interleukin-3 (42), and interleukin-4 (43). GATA activity has been reported to decrease in response to other mediators, such as estrogen (44) and TGF-β (45).

As a general rule, GATA proteins serve as positive acting transcription factors. Therefore, temporal alterations in GATA binding activity are normally coupled to parallel changes in downstream target gene expression. However, in some cases, increased GATA binding has been shown to inhibit gene expression. Certain genes contain a GATA motif in place of the consensus TATAAA site, including platelet factor 4 and erythropoietin (46). GATA proteins interact with the core promoter GATA motif and inhibit generation of preinitiation complexes, resulting in transcriptional repression (46). In a recent study, the uremic toxin, N\textsuperscript{6}-monomethyl-L-arginine was shown to repress erythropoietin gene expression at the level of the core promoter by up-regulation of GATA-2 (47). Although full induction of VCAM-1 (and perhaps other GATA-responsive genes) by thrombin is dependent on the positive action of GATA-binding proteins, it is conceivable that a subset of genes is repressed through the competitive inhibition of DNA binding.

The current study, along with previous investigations, underscores important differences in the regulatory mechanisms of the ICAM-1 and VCAM-1 genes. The ICAM-1 gene is expressed in many cell types, whereas VCAM-1 expression is largely limited to endothelial cells. In the case of ICAM-1, TNF-α and thrombin response has been mapped to a single NF-κB site in the upstream promoter region (18). In contrast, TNF-α and thrombin-mediated induction of VCAM-1 induction by is dependent on two adjacent NF-κB sites. Moreover, full induction of VCAM-1 is achieved by combinatorial interactions of NF-κB with other transcriptional activators. In the present study, we have demonstrated that optimal thrombin stimulation requires a combination of NF-κB and GATA binding activity. Given that GATA-2 is expressed predominantly in the
endothelium, it is tempting to speculate that the endothelial cell-specific nature of VCAM-1 induction is mediated, at least in part, by a GATA-dependent co-stimulatory pathway. Future investigations will be aimed at determining whether thrombin-mediated induction of VCAM-1 involves a physical interaction between NF-κB and GATA proteins and/or the combinatorial effect of other transcription factors, such as Sp1, activating protein-1, or interferon regulatory factor-1.

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