Tissue factor is critically important for initiating the activation of coagulation zymogens leading to the generation of thrombin. Quiescent endothelial cells do not express tissue factor on their surface, but many stimuli including cytokines and coagulation proteases can elicit tissue factor synthesis. We challenged human endothelial cells simultaneously with tumor necrosis factor α (TNFα) and thrombin because many pathophysiological conditions, such as sepsis, diabetes, and coronary artery disease, result in the concurrent presence of circulating inflammatory mediators and activated thrombin. We observed a remarkable synergy in the expression of tissue factor by thrombin plus TNFα. This was due to altered regulation of the transcription factors c-Jun and c-Fos. The activation of c-Jun was greater and more sustained than that obtained with either thrombin or TNFα alone. Thrombin-stimulated expression of c-Fos was both enhanced and prolonged by the concurrent presence of TNFα. These changes support the increased availability of c-Jun/c-Fos AP-1 complexes for mediating transcription at the tissue factor promoter. Transcription factors downstream of the extracellular signal-regulated kinases as well as changes in NFκB regulation were not involved in the synergistic increase in tissue factor expression by thrombin and TNFα. Thus, concurrent exposure of vascular endothelial cells to cytokines and procoagulant proteases such as thrombin can result in greatly enhanced tissue factor expression on the endothelium, thereby perpetuating the prothrombotic phenotype of the endothelium.

Many diseases with a pronounced inflammatory component, such as sepsis, type II diabetes, coronary artery disease, heparin-induced thrombocytopenia, and anti-phospholipid syndrome, also exhibit enhanced activation of coagulation enzymes (1–5). These serine proteases, most notably thrombin, stimulate protease-activated receptors (PARs) on the surface of endothelial cells. Signaling through the PARs shifts the endo-

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EXPERIMENTAL PROCEDURES

Antibodies—Antibodies used were goat anti-human tissue factor (American Diagnostica, Inc., Stamford, CT); mouse anti-human NFκB p65, mouse anti-human IκB α, mouse anti-human pc-Jun, rabbit anti-human c-Fos (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit anti-active MAPK (extracellular signal-regulated kinase (ERK), p38, and JNK (Promega Corp., Madison, WI); and rabbit anti-MAPKAPK-2 (Cell Signaling Technology, Beverly, MA). All secondary antibodies...
were obtained from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA).

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were isolated and characterized as described by Jaffe et al. (18). Cells were grown in Medium 199 containing 10% fetal calf serum, 1 mM glutamine, 12 units/ml heparin, 100 μg/ml crude endothelial cell growth supplement, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C on fibronectin-coated tissue culture dishes. Confluent, quiescent HUVEC were used at passages 1–4.

Cell Treatment—Where indicated, cultures were preincubated for 1 h in human endothelial serum-free medium (Invitrogen) with 10 μM U0126, 10 μM SB202190, 5 μM BAPTA/AM (Biomol Research Laboratories, Inc., Plymouth Meeting, PA) or 25 μM SP600125 (Calbiochem). Cultures were incubated without or with 5 or 15 ng/ml TNFα (R&D Systems, Inc., Minneapolis, MN), 0.5 or 2 units/ml thrombin (Enzyme Research Laboratories, South Bend, IN), 100 μM TFLLR (Bachem Biosciences, Inc., Plymouth Meeting, PA), or 100 nM ionicmycin (Biomol Research Laboratories, Inc.), as indicated.

Cell Extraction—HUVEC monolayers were washed with ice-cold Dulbecco’s PBS containing 0.7 mM CaCl2 and 0.5 mM MgCl2 (DPBS) prior to scraping in extraction buffer A: 1% Triton X-100, 60 mM octyl glycoside, 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 30 mM Na4P207, 50 mM NaF, 1 mM Na2VO4, 2 mM CaCl2, 0.2 mM H2O2, plus mammalian protease inhibitor mixture (Sigma). After solubilization on ice for 15 min with intermittent vortexing, the extract was microcentrifuged for 10 min and the supernatant recovered.

Nuclear and Cytosolic Fractionation—Nuclear and cytosolic fractions were prepared by a protocol modified from the procedure of Dignam et al. (19, 20). HUVEC monolayers were washed three times with DPBS, then scraped in DPBS and centrifuged at 1500 × g for 10 min. The cells were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.1% Nonidet P-40) and allowed to swell for 30 min on ice and briefly vortexed. Nuclei were pelleted through a 10% sucrose layer. The pelleted nuclei were lysed in extraction buffer A for 10 min on ice, microcentrifuged for 15 min at 4 °C, and the supernatant recovered.

Immunoblotting—Samples containing equal amounts of protein (5 μg/μl) were solubilized in Laemmli sample buffer (21), separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Immunoblots were blocked with 5% milk, allowed to swell for 30 min on ice and briefly vortexed. Nuclei were solubilized in Laemmli sample buffer A for 10 min on ice, microcentrifuged for 10 min at 4 °C, and the supernatant recovered.

RESULTS

HUVEC monolayers were challenged without or with 15 ng/ml TNFα, 2 units/ml thrombin, or the combination of TNFα plus thrombin for 5 h, an optimal time for tissue factor expression in endothelial cells. In the absence of any stimulus, tissue factor was almost undetectable (Fig. 1). TNFα was a potent stimulus for TF expression, while thrombin produced a more modest response, as has been observed by others (9). We were surprised, though, by the pronounced synergy in tissue factor expression (6.2-fold ± 0.8 S.E.) induced by thrombin plus TNFα. Similar results were obtained when HUVEC were treated with 5 ng/ml TNFα and 0.5 unit/ml thrombin. The thrombin receptor-activating peptide TFLLR also potentiated TNFα-stimulated TF expression (data not shown), consistent with thrombin initiating the signaling through PARs and not through other potential thrombin receptors on the surface of endothelial cells.

Signaling through the different MAP kinase families is known to regulate the expression of tissue factor in a stimulus and cell context dependent manner (11, 22–24). Thrombin and TNFα alone or in combination activate the ERKs as well as the stress-activated kinases p38 and JNK in human endothelial cells. To determine whether signaling through any of the MAP kinase pathways was responsible for the synergistic interactions of thrombin and TNFα on endothelial cell TF expression, we utilized selective pharmacological inhibitors for each family of MAP kinases. When confluent HUVEC were pretreated with 10 μM U0126, a concentration of inhibitor that completely blocks ERK activation in HUVEC, and then exposed to TNFα alone or TNFα plus thrombin, TF expression was not significantly inhibited (Fig. 2). Thrombin-stimulated TF expression was attenuated by U0126 by about 35%, although significant variability was observed between experiments. Thus, although some diminution of thrombin-stimulated tissue factor expression was observed when ERK activation was blocked, signaling through the ERK pathway was not important for the synergistic increase in tissue factor expression elicited by the combination of TNFα plus thrombin.

Inhibition of either MAP kinase p38 with 10 μM SB202190 or JNK with 25 μM SP600125 resulted in significant attenuation of TF expression elicited by TNFα and thrombin, individually or in combination (Fig. 2). Pretreatment of HUVEC with both SB202190 and SP600125 almost completely ablated synthesis of tissue factor stimulated by TNFα and thrombin. Clearly, activation of both p38 and JNK is critical for TNFα- and thrombin-stimulated tissue factor expression in human endothelial cells.

The temporal patterns of activation of the MAP kinases affect the regulation of downstream signaling molecules, both at the level of transcription and post-translational modification (25, 26). Based on the inhibitor studies described above, we next examined the activation of p38 and JNK over a time course of 5 h using immunoblot analysis with phosphospecific antibodies for the active kinases. For both thrombin and TNFα, peak activation of p38 occurred between 5 and 15 min, followed by a drop in activity that was sustained above the basal level through 5 h (Fig. 3A). TNFα was a more potent initiating stimulus than thrombin. The combination of thrombin plus TNFα exhibited a similar pattern of p38 activation. Activation of a downstream target of p38, MAPKAPK-2, closely mimicked the pattern of p38 activation (data not shown). In contrast, the pattern of JNK activation was markedly different in endothelial cells stimulated either with TNFα or thrombin (Fig. 3B). TNFα stimulation of JNK resulted in a sharp peak of JNK activity at 15 min, followed by a precipitous drop almost to

FIG. 1. TNFα plus thrombin synergistically stimulated tissue factor expression in human endothelial cells. A, HUVEC were incubated without or with TNFα (15 ng/ml), thrombin (2 units/ml), or TNFα plus thrombin for 5 h. Cells were extracted and immunoblotted for tissue factor. The immunoblot is representative of 6 experiments. B, analysis of TF expression from 6 experiments using independent isolates of HUVEC. Values ± S.E. are relative to the value for TF expression elicited by TNFα, which was designated as 1. C, control.
basal levels by 30–60 min. After this, JNK activity steadily increased over the next 4 h. Thrombin-stimulated activation of JNK gradually increased to a peak at 1 h and then dropped slightly to a plateau. Thus the time of peak thrombin-stimulated JNK activity occurred at the nadir of TNFα/H9251-stimulated JNK activation. In endothelial cells challenged with both thrombin and TNFα/H9251, JNK activity peaked dramatically at 15 min, then dropped to a plateau well above baseline that was maintained through 5 h. The phosphorylation of c-Jun, an immediate downstream target of activated JNK and a component of AP-1 transcription factor dimers, also was studied (Fig. 3). The patterns of c-Jun activation in thrombin- or TNFα/H9251-stimulated cells mimicked those observed for JNK, with peak activation by thrombin occurring at 1 h when TNFα/H9251 activation of c-Jun was minimal. Interestingly, when the endothelial cells were stimulated with the combination of TNFα plus thrombin, c-Jun phosphorylation increased steadily, reaching a peak at 1 h that diminished very gradually over the next 4 h. Thus, the combined stimulus of thrombin plus TNFα resulted in the continuous, enhanced activation of c-Jun, allowing sustained c-Jun homodimer and heterodimer interaction at AP-1 sites on the TF promoter for periods ≥4 h.

An important component of AP-1 transcription factor heterodimers in endothelial cells is c-Fos (27). In HUVEC, very little c-Fos was expressed by cells treated with TNFα alone (Fig. 5, A and B). Thrombin stimulated an increase in c-Fos expression within 30 min, peak expression occurred at 1 h, and c-Fos was barely detectable 2 h after stimulation (Fig. 5C). The combined presence of TNFα with thrombin both prolonged the time course of c-Fos expression and significantly enhanced the quantity of c-Fos expressed.
Thrombin plus TNFαSynergize for Tissue Factor Expression

FIG. 5. c-Fos expression was induced by thrombin and TNFα plus thrombin but not by TNFα alone. TNFα increased the magnitude and duration of thrombin-stimulated c-Fos expression. A, HUVEC were incubated for 1 h without or with TNFα, thrombin, or TNFα plus thrombin. Cells were extracted and immunoblotted for c-Fos. C, control. B, analysis of c-Fos expression from 3 independent experiments. Values ± S.E. are relative to the value for c-Fos expression elicited by thrombin, which was designated as 1. C, control. C, HUVEC were incubated for 0, 0.5 h, 1 h, 2 h, or 3 h with thrombin or TNFα plus thrombin. Cells were extracted and immunoblotted for c-Fos.

Thrombin is a potent mediator of calcium mobilization in endothelial cells (6, 28), a second messenger that is important for c-Fos expression (29). We hypothesized that thrombin-stimulated calcium mobilization was important for the synergistic stimulation of tissue factor expression by thrombin plus TNFα. To test this hypothesis, we pretreated HUVEC with the cell-permeable calcium chelator BAPTA/AM for 1 h prior to challenge with thrombin plus TNFα. Tissue factor expression was significantly attenuated (~70%) by chelation of intracellular calcium, as was c-Fos expression (Fig. 6). Phosphorylation of c-Jun, in contrast, was unaffected by the presence of the intracellular calcium chelator.

To confirm our hypothesis that the potentiation of TNFα-stimulated tissue factor expression by thrombin in endothelial cells is related to the capacity of thrombin to increase intracellular calcium and, thereby, c-Fos expression, we used the calcium ionophore ionomycin in place of thrombin. Ionomycin treatment alone resulted in detectable TF expression (~25% relative to TNFα alone). When HUVEC were treated with both ionomycin and TNFα, there was a significant increase (3-fold) in tissue factor expression above that obtained with TNFα alone (Fig. 7). This correlated with detectable c-Fos expression, which was observed when HUVEC were treated with TNFα, plus thrombin (9, 15, 16). We examined NFκB mobilization to the nucleus after stimulating endothelial cells for 1 h, a time of maximal degradation of the cytosolic regulator of NFκB, IκB. Thrombin proved to be a very poor stimulus compared with TNFα for promoting the nuclear translocation of NFκB (Fig. 8). The combination of thrombin plus TNFα did not significantly increase the nuclear localization of NFκB when compared with TNFα alone. In contrast, the TNFα-premobilized mediates increases in c-Jun activation and c-Fos expression were clearly apparent in the same experiment. Thus, the synergistic increase in TF expression by thrombin plus TNFα is not dependent upon altered NFκB nuclear localization.

DISCUSSION

We have discovered a novel synergy between thrombin and TNFα that results in greatly enhanced tissue factor expression by endothelial cells. Knowledge of the mechanisms that underlie this synergy are likely to be crucial for understanding the potentially life-threatening thrombotic events that can occur in individuals with inflammatory pathologies.

When we observed the synergistic increase in tissue factor expression in endothelial cells exposed to the combination of thrombin plus TNFα, we hypothesized that the synergy was due to selective differences in signaling by the two mediators. Thrombin activates G protein-coupled receptors, the PARs, enlisting a variety of G protein subtypes that can signal through many different networks. TNFα clusters and activates TNF receptors, which recruit a distinctive set of adapter proteins (TRADD, RIP, TRAF2) to mediate TNFα signaling (30, 31). Thrombin and TNFα, although they initiate signaling by different subsets of proteins, both activate many of the same signaling networks. Therefore, we looked for temporal or functional changes in signaling pathways that were utilized by both thrombin and TNFα (MAP kinase cascades) or signaling pathways that were preferentially activated by either thrombin (calcium mobilization) or TNFα (NFκB nuclear localization).
Tissue factor expression is regulated by the transcription factors Egr-1, AP-1, and NFκB, some of which are immediate early gene products (15, 16, 32). Recent studies (25, 26) have suggested that the regulation of immediate early genes, such as Egr-1 and c-Fos, is contingent upon the magnitude and duration of activation of ERK. This, then, can translate into increased expression, stability, nuclear localization, and/or transcriptional activation of the particular immediate early gene. Others (17, 23, 33, 34) have shown that ERK activation by TNFα or thrombin can increase Egr-1 and/or TF expression in endothelial cells. Although we observed significant activation of ERK by thrombin and TNFα alone or in combination, ablation of the ERK signal had little effect upon the synergistic stimulation of tissue factor expression by TNFα plus thrombin. As a corollary to this, Egr-1, a downstream target of ERK, is unlikely to be a major mediator in thrombin plus TNFα-mediated synergistic up-regulation of TF.

Perhaps not surprisingly, the MAP kinases important for signaling increased expression of the procoagulatory and inflammatory protein tissue factor in endothelial cells are the stress-activated kinases p38 and JNK. When we inhibited either p38 or JNK activities, there was a 50–75% decrease in tissue factor expression. Inhibition of both p38 and JNK essentially ablated tissue factor expression. Although p38 activity is important for tissue factor synthesis in HUVEC, we did not detect any substantial changes in the regulation of p38 or its downstream substrate MAPKAPK2 when the cells were treated with TNFα and thrombin. In contrast, the temporal pattern of activation of JNK and its downstream substrate c-Jun differed significantly depending upon the stimulus. TNFα appears to program the endothelial cell shift toward an inflamed phenotype by eliciting a distinctive pattern of JNK/c-Jun activation, a rapid spike and drop in JNK/c-Jun activity which was completed within 1 h followed by a progressive recovery of JNK/c-Jun activity over a time course of 4 h. Thrombin alone, although a less effective stimulus of JNK/c-Jun activation than TNFα, exhibited peak activities at 1 h, the nadir for TNFα-stimulated JNK/c-Jun phosphorylation. Thus, the combined stimulus of TNFα plus thrombin resulted in elevated, sustained c-Jun phosphorylation relative to TNFα or thrombin alone. This was most notable at 1 h, the time frame when the AP-1 partner of c-Jun, c-Fos, is expressed. Sustained, elevated levels of active c-Jun allow prolonged transcription, both as homodimers or heterodimers, at AP-1 sites on the tissue factor promoter. This pattern of c-Jun activation, then, provides one mechanism for the observed synergistic stimulation by TNFα plus thrombin of tissue factor expression in human endothelial cells.

c-Jun/c-Fos dimers appear to be the preferred AP-1 dimer at both the proximal and distal AP-1 sites on the tissue factor promoter of human endothelial cells (16). We, as others (29), observed that thrombin can induce the transient expression of c-Fos in a calcium-dependent manner in endothelial cells. TNFα, while a poor stimulus for c-Fos synthesis, greatly potentiated the thrombin-elicited signal, augmenting the magnitude and temporal expression of c-Fos. Such increased c-Fos expression provides another mechanism by which the combined signaling by thrombin plus TNFα augments tissue factor expression, again functioning at the AP-1 sites.

Regulation of the classic inflammatory mediator NFκB, although important for TF expression in endothelial cells (9, 15, 16), does not appear to be involved in the synergistic induction of tissue factor expression by thrombin plus TNFα. Our data for the nuclear mobilization of NFκB demonstrated that TNFα was a potent stimulus while thrombin was a comparatively poor stimulus, in agreement with studies by other investigators (35). The combination of thrombin plus TNFα did not significantly increase NFκB nuclear import compared with TNFα alone.

These studies, then, in conjunction with data accumulating from other investigators begin to provide a framework for understanding why patients with pathophysiologicals that result in increased levels of circulating cytokines are primed for life-threatening thrombotic events. Circulating cytokines impact on all cells within the vascular compartment. Up-regulation of tissue factor, whether on circulating blood cells or the endothelium, will support some level of activation of coagulation factors due to the initiating event of circulating Factor VII binding to its receptor, tissue factor. The consequent exposure of the endothelium to thrombin in the presence of cytokines will ensure the dramatic up-regulation of tissue factor on the surface of the endothelium, thereby amplifying the probability of increased thrombin generation and inappropriate development of thrombi. Our investigations would suggest that pharmacological interventions that attenuate or block JNK activation in the endothelium might prove beneficial in decreasing the thrombotic complications in individuals with known risks associated with elevated circulating cytokines.

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