

Thrombin Enhances Monocyte Secretion of Tumor Necrosis Factor and Interleukin-1 Beta By Two Distinct Mechanisms

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ABSTRACT. Thrombosis and disseminated intravascular coagulation (DIC) are common complications of infections. Abnormal activation of coagulation is due in part to expression of tissue factor on intravascular cells in response to cytokines, including interleukin-1 beta (IL1 β) and tumor necrosis factor (TNF). Both TNF and IL1 β are thought to play significant roles in producing the pathologic manifestations of sepsis. Therefore, we examined the effects of thrombin on TNF and IL1 β secretion by monocytes, and the ability of monocyte products to promote tissue factor expression by endothelial cells. Human monocytes were treated with thrombin or a thrombin receptor agonist peptide (SFLLRN), and/or bacterial lipopolysaccharide (LPS). The agonists were removed, and monocytes cultured 18 hours. The monocyte-conditioned supernatants were assayed for TNF and IL1 β antigen, and for their ability to induce tissue factor expression on human umbilical vein endothelial cells and the Ea.hy endothelial cell line.

Thrombin alone did not promote monocyte TNF or IL-1 β secretion. However, thrombin enhanced LPS-induced TNF and IL1 secretion. Supernatants from monocytes exposed to LPS plus thrombin promoted greater tissue factor expression on endothelial cells than supernatants from those treated with LPS only. SFLLRN did not increase TNF secretion in response to LPS, but did enhance LPS-induced IL1 β secretion and tissue factor-inducing activity. Neither SFLLRN nor active thrombin augmented the level of mRNA for TNF above that induced by LPS alone. However, both increased the LPS-induced level of IL1 β message. Thus, thrombin enhanced LPS-induced TNF and IL1 β secretion by monocytes. Unexpectedly, the effects on these two cytokines were mediated by different mechanisms. Enhancement of LPS-induced IL1 β secretion was largely mediated via the tethered ligand type thrombin receptor and correlated with an increase in the steady state level of mRNA. By contrast, enhanced TNF required proteolytically active thrombin, but was not mediated by the tethered ligand receptor.

These data demonstrate that physiologically relevant amounts of thrombin can synergize with endotoxin to stimulate monokine release. Thrombin could thereby play a role in the complex network of mediators involved in the pathophysiology of sepsis. We speculate that limiting thrombin activity during DIC could be a beneficial adjunct in the management of sepsis.

Keywords: tumor necrosis factor, interleukin 1, blood coagulation, endotoxin, disseminated intravascular coagulation, thrombin receptor

INTRODUCTION

Sepsis is the clinical syndrome that results from the host's response to serious infection, usually with gram negative bacterial organisms. Manifestations of sepsis include coagulation abnormalities, metabolic abnormalities, dys-

function of multiple organ systems, hypotension and shock. Many of these manifestations are not the direct result of bacterial infection, but are toxic effects of monocyte/ macrophage-derived cytokines released in response to bacterial endotoxins. Tumor necrosis factor (TNF) levels rise rapidly after exposure to

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endotoxin *in vivo* (1), and outcome in septic patients has been correlated with circulating TNF levels (2). A neutralizing antibody against TNF prevents mortality in baboon models of lethal gram negative sepsis (3, 4). Anti-TNF antibody also prevents or attenuates most of the cardiovascular, metabolic and coagulation changes resulting from sublethal endotoxemia (5). IL1 β levels also increase following endotoxin administration, but more slowly than TNF levels. Administration of an IL1 receptor antagonist can reduce the hematologic manifestations of a non-lethal dose of endotoxin (6), and prevent mortality when given before a lethal dose of Gram negative organisms in rabbits (7). In spite of the beneficial effects of antagonizing TNF and IL1 β in animal models of sepsis, clinical trials of anti-IL1 and anti-TNF therapies in humans have been disappointing [reviewed in (8)]. Thus, effective management of clinical sepsis may require simultaneous attack on multiple mediator systems.

We hypothesized that thrombin generated during DIC might play a role in the complex network of cytokine responses to severe Gram negative bacterial infection. In addition to its ability to clot fibrinogen, thrombin has cytokine-like effects on monocytes and other cells. Thrombin is chemotactic for endothelial cells, alters endothelial permeability and promotes expression of endothelial adhesion molecules (9-11). Thrombin also has receptor-mediated chemotactic and mitogenic activities for monocytes and macrophages (12, 13). Some, but not all, of the biological activities of thrombin are mediated by a unique tethered ligand-type receptor, first cloned from platelets (14). The receptor is proteolytically cleaved by thrombin near its amino terminal end. The newly revealed amino terminus then binds to a cell surface receptor and transmits a signal to the cell. Thus, thrombin must be proteolytically active to trigger cellular activities via the tethered ligand type receptor. Peptides based on the amino-terminal sequence of the cleaved thrombin receptor (such as SFLLRN)

are agonists for the receptor, and can mimic many of thrombin's activities (15, 16). These peptides have no proteolytic activity, and can be used as specific tools to investigate those biological activities of thrombin which are mediated by the tethered ligand type receptor. Monocytes possess a tethered ligand type thrombin receptor, and respond to thrombin receptor agonist peptides with an increase in intracellular calcium (17).

Monocytes synthesize IL-1 β (18, 19) and TNF- α , (20) as larger precursors. Jones & Geczy (21) found that thrombin and coagulation factor Xa enhanced the LPS-induced release of IL-1-like activity by monocytes as measured by a lymphocyte mitogenic assay. They proposed that thrombin might enhance LPS-induced IL-1 activity by cleaving an IL-1 precursor to release active IL-1. IL-1 β is the principle form of IL-1 produced by monocytes/macrophages. While TNF is expressed as a transmembrane protein which is cleaved in its extracellular portion to release soluble cytokine (22), it is now known that the IL-1 β precursor is primarily processed intracellularly by the interleukin converting enzyme (reviewed in (23)). Therefore, we thought that effects of thrombin on IL-1 β release were unlikely to be the result of precursor proteolysis, but might be receptor-mediated. The purpose of the current work was to compare the effects of thrombin on monocyte release of TNF and IL-1 β , and determine whether such effects are receptor-mediated or a direct result of proteolysis.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle Medium (DMEM) and Eagle's Minimum Essential Medium (EMEM) were purchased from the Tissue Culture Facility of the Lineberger Cancer Research Center at The University of North Carolina-Chapel Hill. Mono-Poly Resolving Medium was purchased from Flow Laboratories, Inc. Lipopolysaccharide (LPS) from *E. coli* was

purchased from Sigma Chemical Co. (St. Louis, MO). An inhibitory monoclonal antibody against human tissue factor was purchased from American Diagnostica, IC (Greenwich, CT). Immunoaffinity purified factor X was purchased from Enzyme Research Laboratories (South Bend, IN). Spectrozyme FXa chromogenic substrate was purchased from American Diagnostica (Greenwich, CT). Thrombin was the kind gift of Dr. Frank Church (University of North Carolina), and was purified from outdated fresh frozen human plasma as previously described (24). PPACK-thrombin was prepared by treating thrombin with a 100-fold molar excess of PPACK overnight, followed by exhaustive dialysis to remove any unreacted inhibitor. Residual thrombin activity was <0.01% of the starting material. Recombinant human factor VIIa was the kind gift of Novo Nordisk (Gentofte, Denmark).

TNF antigen levels in monocyte supernatants were assayed using BIOTRAK™ (Amersham Corp., Arlington Heights, IL) or Cytoscreen™ Innotech (Innogenetics N.V., Zwijndrecht, Belgium) ELISA kits according to the manufacturers instructions. IL1β was assayed using an ELISA kit from AMAC, Inc. (Westbrook, ME).

DNA probes for TNF (clone pAW783) and IL-1 (clone IL-1 X-14) were obtained from the American Type Tissue Collection (Rockville, MD), and the probe for actin from Clontech (San Francisco, CA). The probes were labeled with ³²P using the RadPrime DNA Labeling System (GIBCO BRL, Gaitersburg, MD). RNase was from Boehringer Mannheim (Indianapolis, IN), ribonucleoside vanadyl complexes, Sarkosyl and Antifoam A from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of high commercial grade.

Monocyte Isolation

Blood was collected from medication-free healthy volunteers into citrate anticoagulant, and separated on density gradients (Mono-Poly Resolving Medium, MPRM) as described (25).

The resulting mononuclear cell preparation was analyzed by flow cytometry on a FACScan flow cytometer (Becton-Dickinson, Mountainview, CA) after staining with CD45-FITC/CD14-PE Dual Color Reagent (Olympus Immunochemicals, Lake Success, NY). Sufficient mononuclear cells were plated to give 1×10^6 monocytes per well for mRNA analyses, or 1×10^5 monocytes per well for all other experiments. Monocytes were separated from lymphocytes by plating for 1 hour at 37°C in DMEM with 5% neonatal bovine serum (NBS, Biocell, Inc.), then removing nonadherent cells by washing with PBS. All subsequent incubations were carried out in DMEM without NBS. All buffers, sera and media contained <0.06 ng/mL endotoxin.

Endothelial Cell Culture

The Ea.hy 926 cell line is an immortalized human-derived cell line, produced by hybridizing endothelial cells with a lung carcinoma cell line. The Ea.hy cell line displays many features characteristic of endothelial cells (26-29). Ea.hy cells were cultured in EMEM with 10% fetal calf serum (FCS). Second passage human vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA), and maintained as directed by the supplier.

For the tissue factor expression experiments, Ea.hy or HUVEC cells were plated in 96-well plates and grown to confluence. Monocyte supernatants were diluted 1:4 with fresh EMEM/10% FCS and added to triplicate wells of endothelial cells. All of the experiments were conducted with a single lot of FCS. The cell monolayers were cultured with the supernatants for 18 hrs. They were washed thoroughly with HEPES buffered saline (pH=7.4) containing 1% bovine serum albumin before being assayed for tissue factor activity.

To verify that changes in endothelial tissue factor activity were not due to tissue factor introduced in the monocyte supernatants, supernatants were added to empty wells, which

had been preincubated with EMEM/FCS. The supernatants were incubated in the wells for 18 hours, washed, and both the supernatants and wells were assayed for tissue factor activity. The monocyte supernatants contained small amounts of tissue factor activity; ranging from 2 to 4% of the activity of the monocyte monolayers. A small amount of tissue factor activity was also present in the wells, which was never more than 5% of the activity of the endothelial cells.

Activity Assays

Tissue factor activity was assayed as factor VIIa-dependent activation of factor X, as previously described (30). Briefly, monocyte or endothelial cell monolayers were incubated for up to 1 hour in 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS) containing 3.5 mM CaCl_2 , 170 nM purified Factor X (approximately plasma concentration) and either 0 or 200 pM Factor VIIa. Aliquots were removed and Xa activity was measured as cleavage of the chromogenic substrate, Spectrozyme FXa. Any baseline factor Xa activity (cleavage of the chromogenic substrate in the absence of added factor VIIa) was subtracted from the values measured for all of the other wells. To verify that the factor X-activating activity was due to tissue factor, cultured cells were also assayed in the presence of an inhibitory anti-tissue factor antibody. Tissue factor activity was expressed relative to the activity of Innovin™ recombinant human thromboplastin reagent (Dade, Miami, FL). A standard curve of serial dilutions of the Innovin was assayed with each set of experimental samples. The activity of undiluted Innovin in this assay was designated as 1000.

Dot Blot Hybridization for Cytokine RNA

Initially, northern blots were performed on RNA isolated from preparations of mononuclear cells in suspension (10×10^6 /treatment) cultured for four hours with 500 ng/ml LPS. Total cellular RNA was isolated by lysis in

guanidinium isothiocyanate (4M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, 0.1 M β -mercaptoethanol, 1 drop/12 ml Antifoam A), followed by sedimentation through cesium chloride (5.7 M CsCl, 0.1 M EDTA, 25 mM sodium acetate) (31). The RNA was electrophoresed on formaldehyde-1% agarose gels, transferred to nylon membranes (Hybond N+, Amersham), and blocked with hybridization buffer (0.15 M NaCl, 50 mM NaH_2PO_4 , 5mM EDTA, 0.2% Ficoll, 0.2% PVP, 0.2% BSA, 0.1 mg/ml salmon testis DNA, 2% SDS, 50% formamide, pH 7.4). Messenger RNA for IL-1 and TNF was detected using ^{32}P -labeled cDNA probes. A single band of the appropriate size was detected using each probe.

Therefore, dot blot analysis was subsequently used for detection of mRNA levels for TNF and IL-1, as previously applied to human monocytes by Ziegler-Heitbrock et al (32). For dot blot analysis, about 1×10^6 monocytes per well were cultured with LPS, thrombin and/or SFLLRN. The cells were then lysed for 10 minutes in a one volume of a buffer containing 0.5% SDS, 2x SSC, 1.5 mM MgCl_2 , 10 mM Tris, 10 mM vanadyl ribonucleoside complexes, pH 8.6. One volume of 10x SSC/18.5% formaldehyde was added. After a 15-20 minute incubation at 65° C, one volume of 3M potassium acetate (pH 4.8) was added. Samples were incubated 30 minutes on ice, then spun twice for 10 minutes each in a microfuge to sediment precipitated proteins. Serial dilutions of the samples were applied to nylon membranes using a dot blot apparatus (Minifold II, Schleicher and Schull, Dassel, Germany). The membranes were air-dried, then heated at 80° C for 2 hours. Pre-hybridization and hybridization were performed as for the northern blot analysis using ^{32}P -labeled cDNA probes. The membranes were washed, then exposed to X-Omat x-ray film (Sigma Chemical Co.). The intensity of the hybridization signals was quantitated by digitizing the images with a Microtek Scanmaker scanner (Microtek Lab, Torrance, CA), then integrating the region of each dot using ScanAnalysis™ software

(BIOSOFT, Ferguson, MO). Each membrane was sequentially hybridized with the probes for TNF, IL-1 β and actin. Each probe was stripped from the nylon membranes by boiling in 40 mM NaCl, 1% SDS for 15 minutes. The membrane was then washed five times with 250 mM NaCl, 2% SDS before being reprobred. The staining intensity of the samples showed a linear relationship to the dilution over the range tested for each probe. Thus, the dot blots were appropriate for quantitating mRNA levels. Control samples were treated with DNase-free RNase at the time of cell lysis. No signal for TNF, IL-1 or actin was detected in these samples.

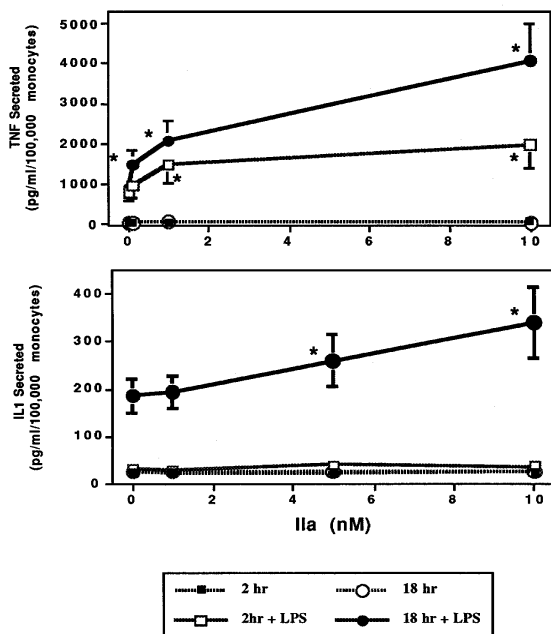


Figure 1. The effect of different doses of thrombin on TNF (upper panel) and IL1 β (lower panel) secretion by monocytes exposed to 100 ng/ml LPS. Monocytes were isolated, plated and cultured as described in "Methods" for 2 hours with the indicated agonists, then for another 18 hours after the removal of the agonists. The supernatants were assayed for TNF and IL1 β antigen levels by ELISA as described in "Methods". Data are the means \pm sd of five determinations (* $p < 0.05$ compared to monocytes cultured with LPS alone; Dunnett's procedure for multiple comparisons with a control).

Data Analysis

Data analysis was carried out using Data Desk⁴ (Data Description, Inc. Ithaca, NY). The statistical significance of differences between

treatment means was evaluated using Dunnett's procedure for multiple comparisons with a control (33), or the Kruskal-Wallis test for the non-parametric comparison of several independent samples (34).

RESULTS

Thrombin Augments Monocyte TNF Secretion in Response to LPS.

As shown in the upper panel of figure 1, treatment of monocytes with thrombin alone, up to 10 nM, did not increase TNF secretion. Monocytes treated with 100 ng/ml LPS showed increased TNF secretion at 2 hours and 18 hours. The addition of increasing concentrations of thrombin along with the LPS resulted in a dose-dependent enhancement of TNF secretion. The addition of 1 nM thrombin doubled LPS-induced TNF secretion at 18 hours, and 10 nM thrombin had increased TNF secretion about 4-fold.

The lower panel of figure 1 shows monocyte release of IL1 β antigen in response to LPS and/or thrombin. This data is consistent with the observation of Jones and Geczy (21) that thrombin augmented LPS-induced secretion of IL1-like (lymphocyte mitogenic) activity, and confirms that this activity was at least partly due to increased release of IL1 β . We found that the time course of IL1 β release and dose of thrombin required to augment IL1 β secretion differed from those required for enhancement of TNF secretion. Unlike TNF, very little IL1 β was detected in the monocyte supernatants after 2 hours of incubation. Significant IL1 β was detected 18 hours after exposure to 100 ng/ml LPS, and the amount secreted was significantly increased by exposure to 5 or 10 nM thrombin. However, the augmentation was not as great as that seen with TNF. The addition of 10 nM thrombin increased LPS-induced IL1 β secretion less than 2-fold.

The effects of different concentrations of LPS on TNF secretion in the presence and absence of 1 nM thrombin are shown in figure

2. Thrombin enhanced secretion of TNF at LPS concentrations between 10 and 1000 ng/mL. While augmentation of LPS-induced TNF secretion by thrombin was evident after 2 hours, augmentation was even more pronounced 18 hours after removal of the thrombin and LPS. Boiled thrombin, at the same concentrations used for the experiments shown in figures 1 and 2, did not significantly augment TNF or IL1 β secretion. Thus, the effect of thrombin on monokine secretion was not due to contaminating endotoxin. Treatment of thrombin with PPACK to inhibit its proteolytic activity also dramatically reduced its ability to augment both TNF and IL1 β secretion. PPACK-thrombin (10 nM) increased LPS-induced TNF secretion by only 24 \pm 10% (compared to 344 \pm 100% for active thrombin) and increased IL1 β secretion by 26 \pm 8% (compared to 72 \pm 40% for active thrombin). This suggests that thrombin must be proteolytically active to effectively augment LPS-induced monokine secretion.

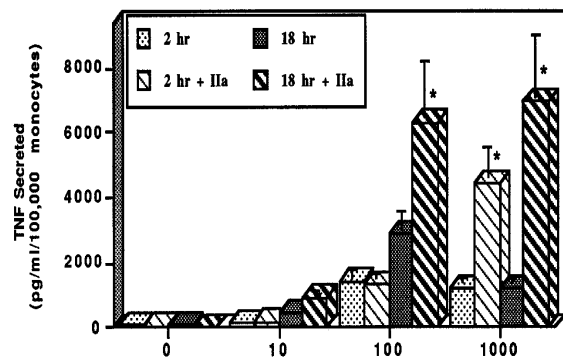


Figure 2. The effect of different concentrations of LPS on TNF secretion by monocytes exposed to 1 nM thrombin. Monocytes were isolated, plated and cultured as described in "Methods" for 2 hours with the indicated agonists, then for another 18 hours after the removal of the agonists. The supernatants were assayed for TNF by ELISA as described in "Methods". Data are the means \pm sd of five experiments (* $p < 0.05$ compared to monocytes cultured with the same concentration of LPS alone; Dunnett's procedure for multiple comparisons with a control).

There are two direct mechanisms by which proteolytically active thrombin could promote cytokine release. Thrombin could cleave

cytokine precursors, or it could interact with the tethered ligand thrombin receptor, which must be cleaved to be activated. To distinguish between these two possibilities, we incubated monocytes with a thrombin receptor agonist peptide, SFLLRN, in the presence and absence of LPS. SFLLRN directly and specifically stimulates the tethered ligand thrombin receptor, without the necessity for proteolysis. In contrast to the results with thrombin, SFLLRN did not augment LPS-induced TNF secretion (figure 3). However, SFLLRN did enhance IL1 β secretion in response to LPS, and this effect reached statistical significance at an SFLLRN concentration of 10 μ g/ml. The concentrations of SFLLRN tested were sufficient to induce a maximal increase in the level of intracellular calcium in fluo-3AM-loaded monocytes, as previously described (17). Thus, the tethered ligand thrombin receptor mediates the potentiation of LPS-induced IL1 β secretion, but not TNF secretion.

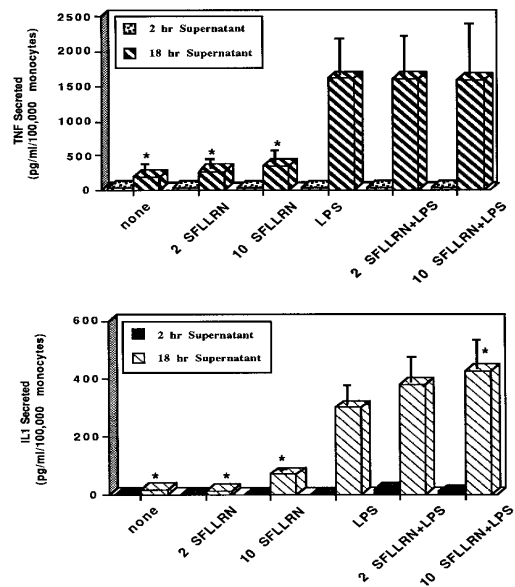


Figure 3. The effect of the thrombin receptor agonist peptide, SFLLRN (2 or 10 μ g/ml), on TNF and IL1 β secretion by monocytes with or without 100 ng/ml LPS. Monocytes were isolated, plated and cultured as described in "Methods" for 2 hours with the indicated agonists, then for another 18 hours after the removal of the agonists. The supernatants were assayed for TNF and IL1 β antigen levels by ELISA as described in "Methods". Data are the means \pm sd of six determinations. (* $p < 0.05$ compared to monocytes cultured with LPS alone; two-sided Dunnett's t -statistic).

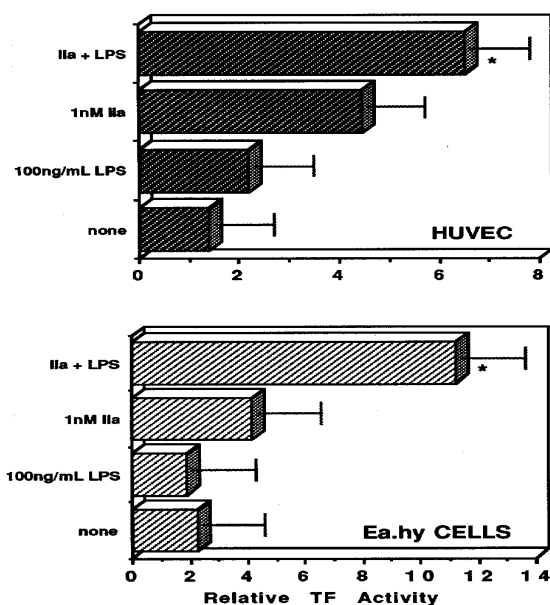


Figure 4. The effect of supernatants from monocytes cultured with thrombin and/or LPS on tissue factor activity of endothelial cells. Ea.hy and HUVEC were cultured for 18 hours with 1:4 dilutions of 18-hour monocyte supernatants. The intact endothelial cultures were washed and assayed for tissue factor activity as described in "Methods". The upper panel shows the results for HUVEC, and the lower panel for Ea.hy cells. Data are the means \pm sd of five determinations. (* $p < 0.05$ compared to cells cultured with conditioned medium from monocytes cultured with LPS alone; two-sided Dunnett's t -statistic). Tissue factor activity for both cell types is expressed relative to the activity of Innovin recombinant human tissue factor (designated as 1000).

Effect of Monocyte Supernatants on Endothelial Cell Tissue Factor Activity.

Tissue factor is the major physiologic initiator of coagulation, and is also of prime importance in initiating disseminated intravascular coagulation (DIC), which often accompanies sepsis (35, 36). It is expressed constitutively in extravascular tissues, but is not normally present on cells in contact with the blood (37). TNF has the ability to induce expression of tissue factor on monocytes (38, 39) and vascular endothelial cells (40, 41). As an assessment of the functional effects of thrombin-augmented monokine release, we measured the effect of monocyte supernatants on endothelial tissue factor expression.

Since endothelial cells themselves possess thrombin receptors (42), the direct effects of

thrombin on endothelial tissue factor expression were first assessed. Exposure to LPS at 100 ng/ml for 18 hours in culture had no significant effect on endothelial cell tissue factor. However, 500 ng/ml LPS induced a 2 to 3-fold increase in activity. Thrombin, at concentrations up to 10 nM, alone or in combination with LPS, had no effect on endothelial tissue factor activity (data not shown).

The effect of monocyte supernatants on endothelial tissue factor activity was then tested. As in previous experiments, monocytes were treated for 2 hours with agonists, washed, then cultured an additional 18 hours. The 18-hour monocyte supernatants contained no detectable thrombin activity. Endothelial monolayers were cultured with 1:4 dilutions of the 18-hour monocyte supernatants, then assayed for tissue factor activity. As shown in figure 4, supernatants from monocytes treated with a suboptimal concentration of LPS (100 ng/ml) did not significantly increase tissue factor expression by endothelial cells. Supernatants from thrombin (1nM)-treated monocytes appeared to increase tissue factor expression, although this effect did not reach statistical significance. Supernatants from monocytes treated with both thrombin and LPS did increase endothelial tissue factor expression significantly. Endothelial cells cultured with TNF are reported to express most of their tissue factor on the basal side of the cells (43). Therefore, the endothelial were lysed by two freeze-thaw cycles to expose additional tissue factor that was not expressed on their apical surface. Lysing the cells increased the amount of tissue factor activity in all wells by a similar proportion (about 10-fold), but did not change the relative effects of the different treatments (data not shown).

As in the experiments with thrombin, monocytes were treated with LPS (100 ng/mL) alone or in combination with SFLLRN (2 or 10 mg/mL). As shown in figure 5, supernatants from monocytes treated with SFLLRN with 100 ng/ml LPS induced a significant increase in

endothelial tissue factor activity, while supernatants from monocytes treated with SFLLRN or LPS alone did not. This effect of SFLLRN is similar to that seen with proteolytically active native thrombin when HUVEC were used as the indicator cell. However, the tissue factor activity of Ea.hy cells did not increase as much in response to LPS/SFLLRN-treated monocyte supernatants as to those from LPS/thrombin-treated monocytes.

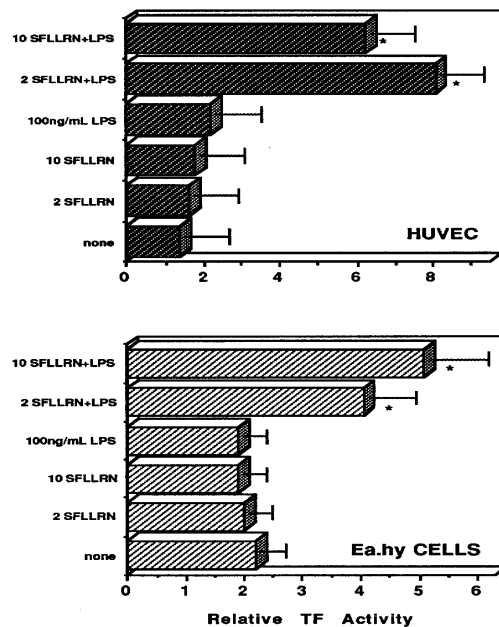


Figure 5. The effect of supernatants from monocytes exposed to SFLLRN (2 or 10 $\mu\text{g/ml}$) and/or LPS (100 ng/ml) on endothelial tissue factor activity. Ea.hy and HUVEC were cultured 18 hours with 1:4 dilutions of monocyte supernatants. The intact monolayers were washed and tissue factor activity assayed as described in "Methods". The upper panel shows the results for HUVEC, and the lower panel for Ea.hy cells. Data are the means \pm sd of five experiments. (* $p < 0.05$ compared to cells cultured with medium from monocytes treated with LPS alone; two-sided Dunnett's t -statistic). Tissue factor is expressed relative to the activity of Innovin recombinant human tissue factor (designated as 1000).

Effect of Thrombin and SFLLRN on Monocyte mRNA for TNF and IL-1 β .

To explore the mechanisms by which thrombin augments LPS-induced cytokine secretion, we measured the levels of mRNA for TNF and IL1 β in monocytes. Monocytes incubated with medium alone had no detectable

mRNA for TNF or IL1 β . Initial 6-hour time-course experiments showed that the mRNA for both IL-1 β and TNF increased after 1 hour incubation with 100 or 500 ng/ml LPS. The level of IL-1 β mRNA peaked between 2 and 4 hours, then began to gradually decline. The message for TNF peaked at 2 to 3 hours, and remained slightly below peak levels for the next 3 hours. We used an incubation time of 2 hours in experiments to examine the level of mRNA in response to different combinations of LPS, thrombin and/or SFLLRN. As shown in figure 6, increasing concentrations of SFLLRN or thrombin increased the amount of mRNA for IL1 β in LPS-treated monocytes. This enhancement was statistically significant at 10 $\mu\text{g/ml}$ SFLLRN and at 1 nM and 10 nM thrombin. By contrast, neither SFLLRN nor thrombin had any significant effect on the level of TNF mRNA in LPS-treated monocytes. Thrombin or SFLLRN in the absence of added LPS did not induce a change in the amount of mRNA for either TNF or IL1 β . Thus, activating the tethered ligand thrombin receptor with SFLLRN enhanced the LPS-induced increase in IL1 β mRNA. However, mechanisms in addition to changes in the mRNA level must be involved in determining IL1 β release, since SFLLRN induced a greater increase in the level of mRNA (about 3-fold) than thrombin (about 1 1/2 times LPS alone), at concentrations that resulted in equal enhancement in protein release.

DISCUSSION

The current study revealed that thrombin alone did not stimulate monocyte TNF and IL1 β secretion, but enhanced secretion in response to LPS. Augmentation of secretion persisted even after the thrombin and LPS had been washed off the monocytes. Most, or perhaps all, of thrombin's ability to augment monocyte secretion of TNF and IL1 β required proteolytic activity. The medium from monocytes exposed to LPS induced tissue factor procoagulant activity on cultured endothelial cells, and

thrombin augmented LPS-induced secretion of procoagulant monokines. The tethered ligand receptor agonist, SFLLRN, did not augment LPS-induced TNF mRNA or protein release. Therefore, the effect of thrombin on TNF release was not mediated by the tethered ligand type receptor. By contrast, we found that SFLLRN did enhance LPS-induced IL1 β mRNA and protein secretion, as well as augmenting the ability of monocyte supernatants to promote endothelial tissue factor expression. IL-1 β has potent tissue factor-inducing activity for endothelial cells (44, 45) and was reported to be the major cytokine responsible for tissue factor induction in LPS-treated cocultures of monocytes and endothelial cells (46). Thus, IL-1 β appeared to be responsible for induction of the major proportion of endothelial tissue factor expression in the current experiments, as well, and augmentation of IL-1 β secretion was mediated via the tethered ligand thrombin receptor.

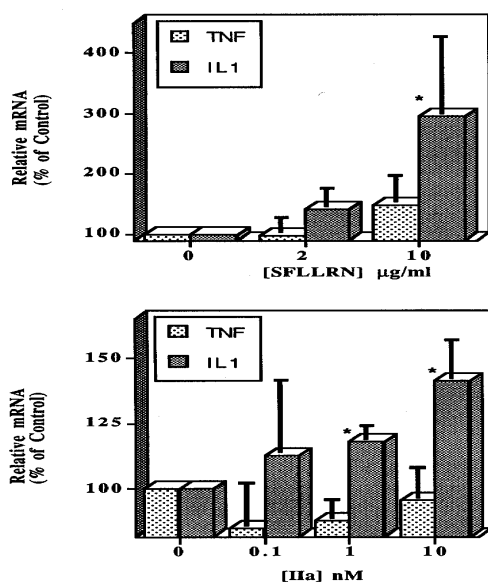


Figure 6. Effect of thrombin, SFLLRN and LPS on monocyte mRNA for TNF and IL1 β . Monocytes were cultured 2 hrs with 100 ng/ml LPS and thrombin or SFLLRN. Lysates were applied to nylon membranes and hybridized sequentially with 32 P-labeled cDNA probes for TNF, IL-1 β and actin as described in "Methods". Control samples treated with RNase had no signal above background. Signal intensity was quantitated by scanning the films and analyzing with ScanAnalysisTM. The data are the means \pm sd of four experiments, and the values are corrected for loading as indicated by the intensity of the actin signal. (* $p < 0.05$ compared to cells cultured with medium from monocytes treated with LPS alone; two-sided Dunnett's t -statistic).

Thrombin also had to be proteolytically active to promote LPS-induced TNF secretion but, surprisingly, SFLLRN did not reproduce this activity. This suggests that thrombin might promote TNF secretion by cleaving the membrane-bound form of the cytokine to release the mature form into the supernatant. Thrombin usually cleaves at an arginine residue. There is an arginine two amino acids nearer the carboxy terminal than the authentic cleavage site of TNF (47). This location should be accessible in the membrane-bound form of TNF, and might be cleaved by thrombin to release a form of TNF very similar to the normal one. This possibility remains to be investigated.

In conclusion, these results show that thrombin has the ability to enhance LPS-induced secretion of TNF and IL1 β . The effects of thrombin on IL1 β and TNF secretion have different time courses and are mediated by different mechanisms. TNF has many potent cardiovascular, metabolic, procoagulant and inflammatory effects that are significant in producing the pathology of septicemia. IL1 β also plays an important role in mediating the effects of endotoxemia (6). The current data suggest that locally generated thrombin could enhance the deleterious effects of sepsis by synergizing with bacterial endotoxins to augment monocyte release of TNF, IL1 β and possibly other cytokines as well. While proteolytic activity of thrombin is probably inhibited rapidly by plasma protease inhibitors under normal conditions, thrombin could remain active for a longer period of time when DIC has depleted the levels of natural inhibitors. In addition, receptor-mediated effects of thrombin could persist long after the thrombin itself had been inactivated. Thus, thrombin could have long-lasting biological effects in the setting of sepsis. The potential exists to develop a positive feedback loop in which cytokines induce tissue factor expression, which leads to thrombin formation and promotes additional cytokine release. Common wisdom holds that it is unnecessary or futile to treat DIC in

patients with sepsis, and that therapy should be aimed at eliminating the source of infection. In support of this concept is a study showing that inhibition of thrombin formation, by itself, did not reduce the mortality of *E. coli* sepsis in baboons (48). However, other studies showed that the addition of a specific thrombin inhibitor (hirudin) (49), or antithrombin III (50) to antibiotic therapy reduced DIC and organ damage, and increased survival in a rat model of *Klebsiella* sepsis. We speculate that inhibiting thrombin generation or activity, as an adjunct to other therapies, might be beneficial in the management of septicemia by preventing thrombin-mediated enhancement of monokine release.

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