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Regulation of Tissue Factor Gene Expression in the Monocyte Procoagulant Response to Endotoxin†

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Tissue factor is the cellular receptor and cofactor for plasma factor VIIa which initiates the coagulation protease cascade on cell surfaces. Although normally absent from all intravascular cell types, tissue factor can be induced to appear on circulating monocytes and vascular endothelial cells by specific inflammatory or immunological mediators. In this study, we have examined the regulation of endotoxin-induced tissue factor gene expression in peripheral blood monocytes.

Formation of a complex between plasma factor VIIa and the glycoprotein tissue factor (TF) on cell surfaces initiates proteolytic activation of factors IX and X, leading ultimately to the generation of thrombin and fibrin (8). Although TF is necessarily absent from the surfaces of cells in contact with the plasma under normal circumstances, T-cell-derived lymphokines (12, 23) and a variety of inflammatory stimuli, including bacterial lipopolysaccharide (LPS) (20), can induce monocyte expression of TF. In addition to its probable role in cellular immune responses (12, 15, 22, 23), pathologic expression of TF in circulating monocytes may play a pivotal role in the generation of microvascular thrombi observed in endotoxin-induced shock, disseminated intravascular coagulation, and local Shwartzman-like reactions (23).

The potential of circulating monocytes to initiate and amplify thrombogenic events in blood vessels demands precise regulation of TF gene expression. Isolation and characterization of cDNA (6, 18, 24, 27) and genomic clones (15a) encoding human TF have recently been accomplished and now provide specific molecular probes for TF studies. TF expression has previously been studied only by measurements of procoagulant activity or factor VII binding. In these studies, we have examined the regulation of TF gene expression in human peripheral blood monocytes in response to bacterial LPS, a potent inflammatory agent and mediator of septic shock.

Nuclear runoff analysis of TF gene transcription. Nuclei were isolated from 8×10^7 gelatin fibronectin-adherent monocytes (7) before and at various times after stimulation with *Escherichia coli*-derived LPS (1 μg/ml, final). Isolated nuclei were used to prepare ^{32}P-labeled runoff transcripts (10). Linearized plasmids (5 μg each) containing TF, interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), and β-actin specific cDNAs were denatured and immobilized on nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.). After hybridization of labeled RNA to each blot for 72 h at 42°C, filters were washed according to the directions of the manufacturer, treated with RNase A at 28°C for 15 min, washed for 10 min each at 28°C in 1× SSPE (0.18 M sodium chloride, 10 mM sodium phosphate [pH 7.7], 1 mM EDTA)–0.1% sodium dodecyl sulfate and 0.1× SSPE, air dried, and exposed to X-ray film (X-Omat AR; Eastman Kodak Co., Rochester, N.Y.) for 7 days at −80°C with intensifying screens. The human TF probe was a 641-base-pair (bp) *Eco*RI fragment from clone λcTF 2 (18) subcloned into the *Pst*I site of pUC8. The human IL-1β probe, a generous gift from Philip Auron, Tufts University School of Medicine, Boston, Mass., was a 920-bp cDNA inserted at the *Pst*I-*Pvu*II site in the Okayama-Berg expression vector II (2). The human TNF-α probe, a generous gift from H. Michael Shephard, Genentech, Inc., San Francisco, Calif., was an 800-bp fragment from clone λ42-4 subcloned into the *Eco*RI site of pSP64 (21). The human β-actin probe, a generous gift from Elaine Fuchs, University of Chicago, Chicago, Ill., was an 819-bp cDNA inserted at the *Pst*I site of pBR322 (13).

Nuclei isolated from resting monocytes demonstrated no detectable transcription of the TF, IL-1β, or TNF-α genes (Fig. 1). Transcription of all three genes was coordinately initiated within 15 min after treatment with LPS and reached a maximum level after 1 h. Although transcription of the TNF-α and IL-1β genes diminished rapidly thereafter, TF gene transcription was observed at a near maximal level for up to 6 h postinduction. The expression of the TF, IL-1β, and TNF-α genes induced by LPS was greatly diminished by 18 h after induction by LPS. Transcription of the β-actin gene was evident in freshly isolated monocytes, diminishing only slightly during the first hour of culture with LPS and then returning to basal levels or slightly higher levels for the rest of the culture period.

Time course of TF activity and mRNA accumulation in monocytes stimulated with LPS. Total cellular RNA (16) was isolated from 2×10^7 to 4×10^7 gelatin fibronectin-adherent monocytes before and at various times after stimulation with LPS. RNA (20 μg per lane) was fractionated in an agarose gel containing formaldehyde, transferred to a GeneScreen (New England Nuclear Research Products, Boston, Mass.) membrane and hybridized to the 641-bp TF cDNA probe at 42°C for 24 h as previously described (18). The TF cDNA probe was radiolabeled, using the random-priming method (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Monocytes from parallel cultures were harvested at the time of RNA extraction (7) and then analyzed for cell surface (viable, intact cells) or total (frozen-thawed and detergent-solubilized cells) TF activity in a single-stage clotting assay (12). Clotting times were converted to milliunits of TF activity by reference to a rabbit brain thromboplastin standard (Difco Laboratories, Detroit, Mich.). For reference, a

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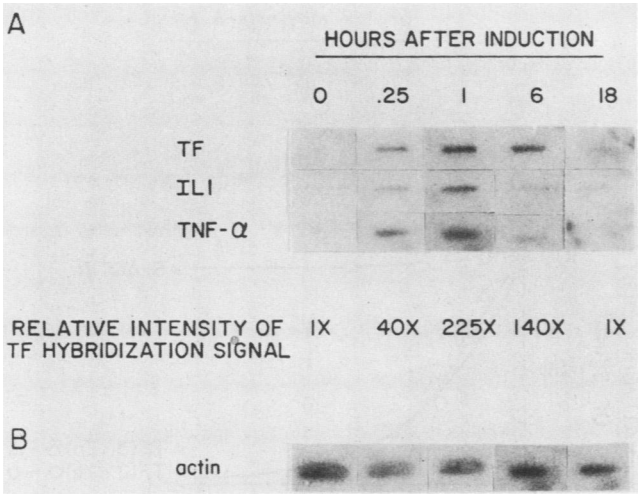

FIG. 1. Nuclear runoff analysis of gene transcription in monocytes stimulated with LPS. (A) Time course of transcription of the TF, IL-1β, and TNF-α genes in nuclei isolated from 8×10^7 monocytes at various times after the addition of 1 μg of LPS per ml. Hybridization to vector DNA (pUC8, pBR322, or pSP64) was not detected nor was hybridization to control IL-2 or gamma interferon cDNAs (data not shown). Hybridization intensities of labeled RNA to TF cDNA were normalized to signals obtained from unstimulated RNA-^{32}P-labeled TF cDNA hybrids (indicated as 1X, which were equivalent to background). (B) Simultaneous runoff analysis of β-actin gene transcription in LPS-stimulated monocytes.

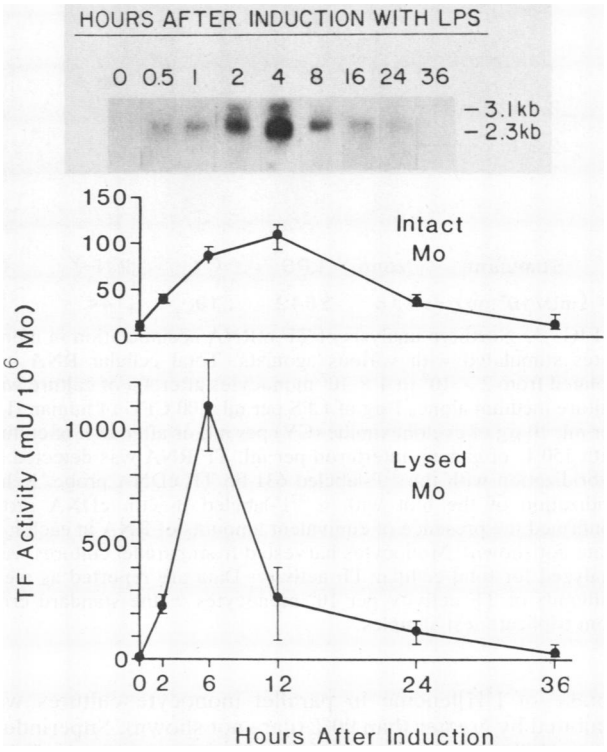

FIG. 2. Time course of TF activity and mRNA levels in monocytes stimulated with LPS. (Top) Northern analysis of total cellular RNA isolated at various times during a 36-h culture of monocytes with 1 μg of LPS per ml and hybridized with the ^{32}P-labeled 641-bp TF cDNA probe. Rehybridization of blots to a ^{32}P-labeled β-actin cDNA probe confirmed the presence of equivalent amounts of RNA in each lane (data not shown). (Bottom) TF activity expressed on the surface (Intact Mo) and total monocyte (Lysed Mo) TF activity expressed in parallel monocyte cultures. Data are reported as the mean milliunits of TF activity per 10^6 monocytes ± the standard error from triplicate test samples.

clotting time of 50 s corresponded to 1,000 mU of TF activity.

Northern (RNA) analysis of total monocyte RNA demonstrated transient accumulation of 2.3- and 3.1-kilobase (kb) TF RNA species in monocytes (Fig. 2). We have detected only the 2.3-kb TF mRNAs in the human fibroblast cell line GM1381 (18), but the human monocytic leukemia cell line THP-1 (11) also produces a major 2.3-kb and one or more other larger (2.7- to 3.2-kb) TF RNAs. The significance of these larger TF RNA species is not presently understood. Several TF cDNA clones recovered from placental libraries have been found to contain unprocessed introns (24), however, suggesting that incomplete processing may result in larger TF RNA species. In LPS-stimulated monocytes, both TF transcripts were detectable by 0.5 h postinduction and accumulated to maximum levels within 4 h. The levels of both TF mRNAs diminished rapidly after reaching their peak accumulation, and by 8 h postinduction only 10 to 20% of the maximum levels was detectable on the basis of densitometry (data not shown). Total cellular TF activity (measured in lysed cells) diminished rapidly over approximately the same period, but TF activity of intact cells persisted at a lower level for at least several hours. Recent studies in our laboratory (T. A. Drake, W. Ruf, J. H. Morrissey, and T. S. Edgington, J. Cell Biol., in press) have demonstrated that prior blocking of surface TF on viable, LPS-stimulated monocytes with anti-TF monoclonal antibodies abolished more than 90% of TF activity of intact as well as lysed cells, demonstrating that nearly all of the biologically active TF is found on the cell surface. In addition, immunoelectron microscopy detected TF antigen only in the surface membranes of monocytes. TF activity is consistently increased when monocytes are disrupted by freeze-thawing, sonication, or partial detergent solubilization (15, 22, 29), and this phenomenon has been documented in other cell types as well (5). Exposure of intact cells to proteolytic enzymes (17) or to sublethal peroxide injury (25) has also been shown to increase apparent TF activity. In synthetic vesicles (19), TF-VIIa catalytic activity has been shown to depend on the qualitative composition of surrounding phospholipids, e.g., the presence of phosphatidyl serine is associated with greater TF-VIIa catalytic activity. Therefore, it is possible that modulation of TF catalytic activity by changes in the surface membrane could represent an additional level of control of cell surface procoagulant activity.

Induction of TF mRNA by various agonists. TF gene expression was not a general consequence of monocyte stimulation. Culture of monocytes with recombinant gamma interferon (150 U/ml) for 18 h failed to induce detectable TF activity or mRNA (Fig. 3), although in parallel experiments we established that gamma interferon was able to prime the monocytes in 48-h cultures for LPS-triggered TNF-α production (data not shown) (14). Recombinant IL-3 (300 CFU/ml) was also unable to induce TF mRNA or activity in 4-h culture with monocytes. Addition of the protein synthesis inhibitor cycloheximide (10 μg/ml) to monocytes resulted in the accumulation of TF mRNA at levels 5 to 10 times greater than that induced by LPS. This induction of TF mRNA by cycloheximide occurred in the absence of any other exogenous agonists. Cycloheximide treatment did not, however, result in the expression of TF procoagulant activity, and the

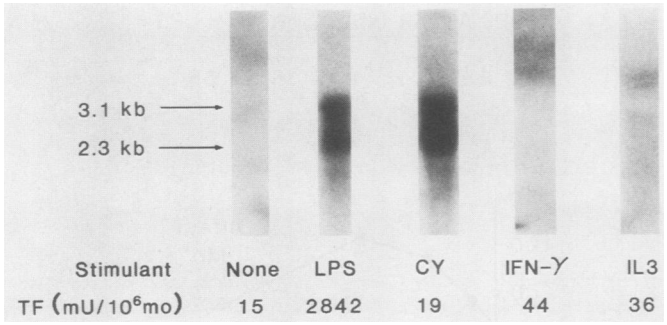

FIG. 3. Northern analysis of TF mRNA accumulation in monocytes stimulated with various agonists. Total cellular RNA was isolated from 2×10^7 to 4×10^7 monocytes after 4 h of culture with culture medium alone, 1 μg of LPS per ml, 300 CFU of human IL-3 per ml, 10 μg of cycloheximide (CY) per ml, or after 18 h of culture with 150 U of gamma interferon per ml. TF RNA was detected by hybridization with the ^{32}P-labeled 631-bp TF cDNA probe. Rehybridization of the blot with a ^{32}P-labeled β-actin cDNA probe confirmed the presence of equivalent amounts of RNA in each lane (data not shown). Monocytes harvested from parallel cultures were analyzed for total cellular TF activity. Data are reported as mean milliunits of TF activity per 10^6 monocytes ± the standard error from triplicate test samples.

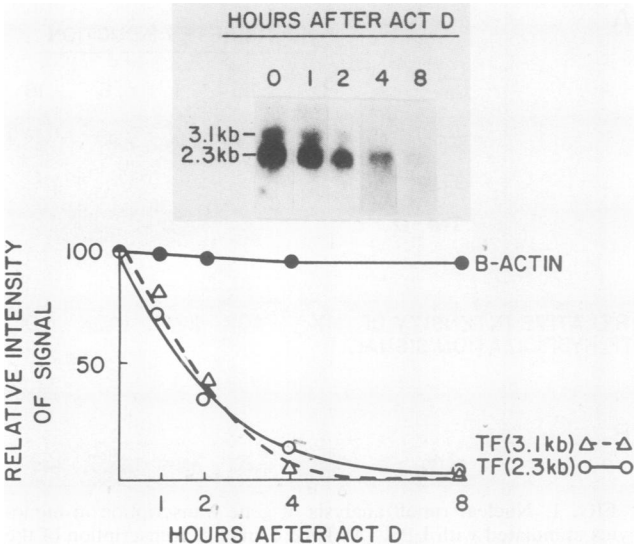

FIG. 4. Northern analysis of TF RNA stability in monocytes stimulated with LPS. Monocytes were cultured for 3 h with 1 μg of LPS per ml; dactinomycin (ACT D) was then added to each culture to a final concentration of 10 μg/ml. Total cellular RNA was isolated from 2×10^7 to 4×10^7 monocytes at 0, 1, 2, 4, and 8 h after dactinomycin treatment. TF RNA was determined by hybridization to the ^{32}P-labeled 641-bp cDNA probe. Rehybridization with a ^{32}P-labeled β-actin cDNA probe confirmed the presence of equivalent amounts of RNA in each lane (data not shown). Relative intensities of the hybridization signals were measured by densitometry.

uptake of [^{3}H]leucine in parallel monocyte cultures was inhibited by greater than 90% (data not shown). Superinduction of TF RNAs by cycloheximide alone suggested that regulation of the TF gene may involve a short-lived repressor protein. We are currently examining the effects of cycloheximide on both the rate of TF gene transcription and the rate of TF RNA degradation.

Stability of TF mRNAs in monocytes stimulated with LPS. Monocytes were stimulated with LPS (1 μg/ml) for 3 h and were subsequently treated with dactinomycin (10 μg/ml) to arrest transcription (Fig. 4). Addition of dactinomycin inhibited incorporation of [^{3}H]uridine into RNA by greater than 90% within 30 min (data not shown). Both the 2.3- and 3.1-kb TF mRNA species decayed rapidly after dactinomycin treatment, and each transcript was found to have an apparent half-life of about 1.5 h. The rapid decay of TF mRNAs did not reflect a general decrease in message stability in the cells, however, as evidenced by the slow decay of β-actin mRNA. The short half-life of TF mRNA is consistent with the rapid decrease in TF mRNA levels that we observed after the cessation of TF gene transcription. The 3′ untranslated region of the TF gene contains an adenosine (A) and thymidine (T)-rich sequence (18), including four copies of the ATTTA motif that has been identified in the genes encoding several other inflammatory mediators and cytokines (4). This sequence motif may target the mRNA transcript for rapid degradation (26).

In previous studies, we have shown that resting human monocytes contain no detectable TF protein and that LPS-stimulated monocytes express approximately 17,000 TF molecules on their surfaces (1). The results presented here demonstrate that TF gene expression in monocytes is regulated primarily at the level of gene transcription. Resting monocytes transcribe little or no TF mRNA, whereas selective exogenous stimuli such as LPS induce a rapid and transient transcription of the TF gene concomitant with transcription of the IL-1β and TNF-α genes. A major 2.3- and a minor 3.1-kb TF mRNA species accumulate in LPS-stimulated monocytes, and both transcripts are rapidly depleted once transcription is halted, due at least in part to the short half-life of TF mRNA. TF functions as the initiator of an inflammatory protease cascade on cell surfaces. Expression of TF may allow cells to modulate or remodel their extracellular environment by assembling an active protease complex on the cell surfaces. Whether the coagulation cascade proceeds completely to the proteolytic cleavage of fibrinogen, induction of TF on the monocyte surface can lead to the production of intermediate, biologically active proteases, such as factor Xa and thrombin. LPS (5, 25), IL-1 (3, 28), TNF-α (9), and thrombin (28) have, in turn, been shown to induce TF expression in cultured endothelial cells. A clear understanding of the role of monocyte/macrophage- and endothelial cell-produced TF in inflammation and immunopathology awaits a comprehensive analysis of TF gene expression in situ as well as purification of the T-cell-derived lymphokine(s) (12, 23) which induce TF expression during cell-mediated immune responses.

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