PLASMINOGEN ACTIVATOR INHIBITOR 1 AND 2 ARE TUMOR NECROSIS FACTOR/CACHECTIN-RESPONSIVE GENES

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TNF/Cachectin, a monokine released from macrophages after exposure to endotoxin, is an important mediator of the hosts' response to Gram-negative bacteremia (1) and is one of the agents responsible for cachexia observed in chronic infection and metastatic cancer (2). TNF also mediates characteristic features of the inflammatory response (3), and as such, is functionally related to other cytokines, including the IFNs, ILs, and CSFs. The inflammatory response is often associated with the local production of proteolytic enzymes and cytotoxic factors, leading to cell death, extracellular matrix degradation, fibrin deposition, and subsequently the replacement of functional tissue by scar tissue. TNF, as well as IL-1, have been shown to evoke the release of collagenase and prostaglandin E_2 from human synovial cells and dermal fibroblasts (4).

Coagulation disorders frequently observed after exposure to endotoxin or TNF suggest that TNF may also affect the hemostatic balance. In this regard, recent studies have demonstrated that TNF induces tissue factor and inhibits thrombomodulin expression on the surface of endothelial cells (5, 6). An effect on the fibrinolytic system has received relatively little attention. The fibrinolytic system is not only required for the removal of blood clots within the vascular system (natural thrombolysis), but also for the degradation of extravascular fibrin deposits (7). The principal fibrinolytic enzyme is plasmin, which is generated from its zymogen plasminogen by one of two genetically distinct plasminogen activators (PA)[1]: urokinase-type PA (u-PA) and tissue-type PA (t-PA) (8). The rate of plasmin formation is modulated by two specific and fast-acting plasminogen activator inhibitors (PAIs): PAI-1 (9) and PAI-2 (10). PAI-1 is found in plasma, platelets, and in the conditioned media of endothelial and other cultured cells (11). PAI-2 is found in monocytes and granulocytes and is predominantly an intracellular nonglycosylated protein (12). A glycosylated form of PAI-2 is found in low concentrations in the plasma of pregnant women (13) and in the culture supernatants of monocytes/macrophages and the U-937 monocyte-like cell line (14). The physiological role of PAI-2 is still unclear, however, the tissue

This work was supported by the Swiss National Foundation for Scientific Research (grant 3.334–0.86).
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[1] *Abbreviations used in this paper:* PA, plasminogen activator; PAI, plasminogen activator inhibitor; t-PA, tissue-type PA; u-PA, urokinase-type PA.

distribution of this inhibitor suggests that it may function locally during inflammation and wound healing.

To explore a possible effect of TNF on the fibrinolytic enzyme system, we have used the human fibrosarcoma cell line HT-1080, which constitutively expresses both PAs and both PAIs (15). Here, we provide evidence that TNF suppresses constitutively secreted fibrinolytic activity of HT-1080 cells, and that this effect is mediated by the simultaneous transcriptional induction of both plasminogen activator inhibitors in concert with suppression of t-PA gene expression.

Materials and Methods

Cell Culture and Harvesting of Media. HT-1080 cells (originally derived from a fibrosarcoma arising adjacent to the acetabulum of a 35-yr-old caucasian male) were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured to confluency in 150-cm^2 culture flasks (Corning Glass Works, Corning, NY) in 40 ml DME containing 2 mM glutamine and 10% heat-inactivated FCS (Gibco Laboratories, Paisley, UK). Before the start of an experiment, cells were maintained overnight at 37°C in serum-free DME. To start an experiment, medium was replaced with fresh serum-free DME with or without human rTNF (Biogen, Geneva, Switzerland). Cells were subsequently maintained for various times up to 48 h.

Activity Assay. Plasminogen-dependent fibrinolytic activity in conditioned medium was determined by the Fibrin plate assay (16).

Preparation of Cellular Extracts. To quantify cell-associated u-PA, t-PA, PAI-1, and PAI-2, cells were harvested after various incubation times by trypsinization, washed in 20 ml of PBS, centrifuged, and then disrupted by adding 5 ml of 0.5% ice-cold NP-40 lysis buffer as previously described (15). Nuclei were sedimented, and the supernatant containing the cellular extract was removed and stored at −30°C until further use.

Antigen Determinations. Secreted and cell-associated u-PA, PAI-1, and PAI-2 antigen levels were determined by RIA as previously described (13). t-PA antigen was determined using an ELISA kit (Biopool imulyse 5 t-PA; Biopool, Umeå, Sweden), according to manufacturers instructions.

DNA Probes. The probes used were the 1,000-bp *Pst* 1 insert of human PAI-1 cDNA (17) (kindly provided by Dr. David Loskutoff, Scripps Clinic and Research Foundation, La Jolla, CA); the *Eco* R1 insert of pPAI J7, harboring 1,900 bp of the human PAI-2 cDNA (18); the *Bgl* II fragment of pPA 11 4B cDNA harboring 1,948 bp of the human t-PA cDNA (19); the *Eco* R1 fragment of pUK 0321 harboring 1,023 bp of the human u-PA cDNA (15). Mouse β-actin cDNA (20) was a generous gift from Dr. Stefania Petrucco, Swiss Institute for Cancer Research, Epalinges, Switzerland; a human genomic PAI-2 clone (chPAI-2 8) was isolated from a human Charon 4A gene library using the labeled *Eco* R1 insert of pPAI J7 (above). The authenticity of the clone was established by sequencing the first two exons. Metallothionein IIa: Plasmid pH MT-113 (21), was a kind gift from Dr. Rob Richards, Howard Florey Institute, Melbourne, Australia. Labeling of all probes with a ^{32}P dATP was performed by the random priming technique (22). For the "Run-on" transcription assay, probes were linearized and fixed to nitrocellulose as previously described (23). 2 μg of DNA was applied per slot.

Northern Blot Analysis. The isolation of mRNA from cultured cells and agarose gel (0.9%) electrophoresis of mRNA in the presence of 20% formaldehyde followed by Northern blot transfer (25) was performed as previously described (24), but using Gene Screen Plus membrane (New England Nuclear, Boston, MA) in place of nitrocellulose filter paper. Hybridization conditions and processing of filters were performed as described (24).

Run-on Transcription Assay. The methods used were essentially as originally described by Greenberg and Ziff (23) and modified by Medcalf et al. (15). Mean nuclei count for each sample was $\sim 7 \times 10^6$. Filter strips containing cDNA slots were hybridized simultaneously for 36 h at 65°C, then washed, RNase treated, air dried, and exposed to X-ray film for up to 10 d.

Densitometry. Densitometric analysis of transcription and mRNA signals on autoradiograms was performed using a densitometer (Ultrascan XL model 2222; LKB-Pharmacia, Dübendorf, Switzerland). All calculations were expressed relative to an arbitrary value of 1 assigned to the intensity of the signal obtained for each sample at time point 0.

Results

Fibrinolytic activity constitutively present in HT-1080 cell-conditioned medium was suppressed by TNF in a dose-dependent fashion. Over 99% suppression occurred with 2.5 ng/ml TNF, while no fibrinolytic activity could be detected with 25 or 250 ng/ml TNF (Table I). To determine the mechanism by which TNF was exerting this effect, we used specific antigen assays to determine the relative changes in secreted and cell-associated u-PA, t-PA, PAI-1, and PAI-2 in the same conditioned medium (Table I). From these data, it was evident that the loss of fibrinolytic activity was primarily due to an increase of secreted PAI-1, and to a lesser extent, to secreted PAI-2. As with monocytes/macrophages, >90% of PAI-2 is cell associated (12, 14). In this system, intracellular PAI-2 increased 11-fold to 1,900 ng/10^6 cells. Levels of u-PA, the predominant PA in this cell line, were unaffected, whereas levels of t-PA antigen (a relatively minor component in this system) were decreased.

Using cDNA probes, we investigated whether the antigen changes were a reflection of the steady state mRNA levels. Northern blot analysis indicated that, in a dose-dependent manner, TNF increased PAI-2 mRNA, as well as the 3.4- and 2.4-bp species (17) of PAI-1 mRNA, while at the same time suppressing t-PA mRNA (Fig. 1). To determine the time course of this effect, mRNA was extracted from cells after exposure to TNF (50 ng/ml) for various times up to 48 h. Densitometric analysis of the Northern blot autoradiograms demonstrated that TNF evoked a sustained 16-fold and 100-fold increase in PAI-1 and PAI-2 mRNA, respectively, commencing between 4 and 8 h. The suppression of t-PA mRNA followed a similar time course (Fig. 2). Constitutive expression of PAI-1 and PAI-2 mRNA was restored by removing TNF from the culture medium showing the reversibility of the effect (Fig. 3).

Steady-state mRNA levels may change as a result of variations of mRNA stability, gene transcription rates, or a combination of both effects. To determine whether the time-dependent changes in mRNA quantity were the result of a change in gene template activity, we assayed relative changes in PAI-1, PAI-2, and t-PA gene transcription rates in isolated nuclei using the "Run-on" transcription assay (Fig. 4). The results of these experiments demonstrate that the increase in the observed mRNA levels was due to a 4- and 20-fold increase in PAI-1 and PAI-2 gene transcription, respectively, as determined by densitometric analysis of the autoradiograms. This induction was noticeable after 2 h for PAI-2 and after 8 h for PAI-1. For both, maximal levels were attained after 48 h of exposure to TNF. Modulation of t-PA gene transcription could not be assessed with accuracy due to the relatively low basal level of constitutive t-PA gene template activity.

Discussion

The data presented in this report indicate that TNF-Cachectin induces gene transcription of PAI-1 and PAI-2, and simultaneously suppresses gene expression of t-PA. The consequent suppression of fibrinolytic activity would therefore appear to be primarily due to an increase of secreted PAI-1, and to a lesser extent, to secreted

TABLE I

Modulation of Secreted and Cell-associated u-PA, t-PA, PAI-1, and PAI-2 Antigen Levels in HT-1080 Cells after Treatment with Different Concentrations of TNF

		Antigen level							
		u-PA		t-PA		PAI-1		PAI-2	
TNF added	Fibrinolytic activity	Secreted	Cell associated	Secreted	Cell associated	Secreted	Cell associated	Secreted	Cell associated
ng/ml	*U/ml*								
0	30.0	332.0	32.0	5.0	ND*	400	60	18	175
2.5	0.2	320.0	34.0	2.5	ND	800	100	33.5	900
25	ND	276.0	34.0	ND	ND	1,000	140	103.5	1,500
50	ND	396.0	44.0	ND	ND	1,000	90	100	1,300
250	ND	300.0	27.0	ND	ND	1,040	100	124	1,900

Antigen levels expressed as ng/10^6 cells.

* Detection limit: 0.1 U/ml.

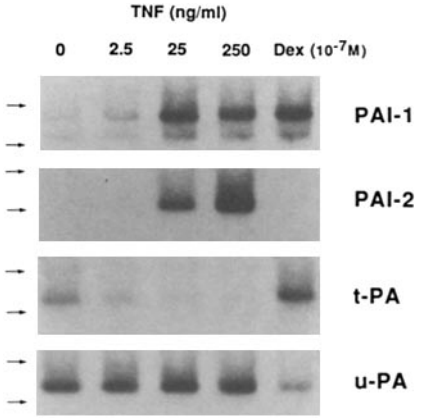

FIGURE 1. Dose-dependent modulation of PAI-1, PAI-2, and t-PA mRNA by TNF. mRNA prepared from HT-1080 cells treated for 24 h with 0, 2.5, 25, or 250 ng/ml human TNF was assessed by Northern blot analysis. Dexamethasone (Sigma Chemical Co., St. Louis, MO, [Dex, 10^{-7} M]), was added to cells as a control for induction of PAI-1 and t-PA mRNA (15) and suppression of u-PA mRNA (24). Relative changes of PAI-1, PAI-2, t-PA, and u-PA were determined by hybridization to random primer labeled PAI-1, PAI-2, t-PA, and u-PA cDNA probes of similar specific activity. Successive hybridizations of each probe to the same membrane are illustrated. Arrows to the left represent the position of the 28 S and 18 S ribosomal RNA markers.

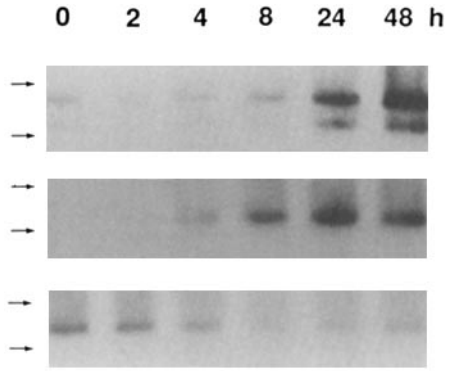

FIGURE 2. Time course of PAI-1 and PAI-2 mRNA accumulation and suppression of t-PA mRNA by TNF in HT-1080 cells. mRNA was prepared from nontreated (0 h) cells or from cells treated with 50 ng/ml TNF for 2, 4, 8, 24, and 48 h. Northern blot analysis of PAI-1, PAI-2, and t-PA mRNA was performed as described in the legend to Fig. 1. Successive hybridizations of each probe to the same membrane are illustrated.

PAI-2. As with monocytes/macrophages, >90% of PAI-2 is cell associated (12, 14). In this system, cell-associated PAI-2 increased 11-fold to 1,900 ng/10^6 cells. Liberation of these large amounts of PAI-2, either by cell death, or by an as yet unknown mechanism, would further contribute to the inhibition of fibrinolytic activity.

Whether the effects of TNF seen here in fibrosarcoma cells are tissue specific or

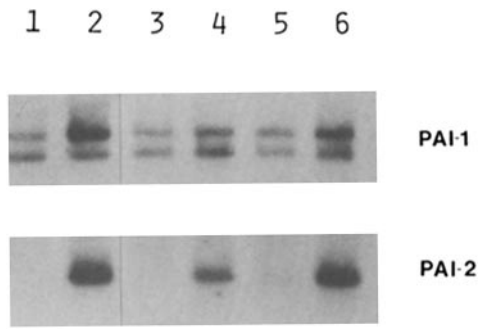

FIGURE 3. Reversibility of the effect of TNF. HT-1080 cells, cultured as described in the legend to Fig. 1, were treated for 24 h in either DME alone or DME containing 50 ng/ml human TNF. Medium was removed and the cell monolayer washed with DME before a further 30-h incubation in either DME or 50 ng/ml TNF (see below). mRNA was then extracted and assessed for PAI-1 and PAI-2 mRNA by Northern blot analysis as described in the legend to Fig. 1. Successive hybridizations of each probe to the same filter are illustrated. (Lane *1*) 24-h DME; (lane *2*) 24-h TNF; (lane *3*) 24-h DME followed by 30 h DME; (lane *4*) 24-h DME followed by 30 h TNF; (lane *5*) 24-h TNF followed by 30 h DME; (lane *6*) 24-h TNF followed by a further 30 h TNF.

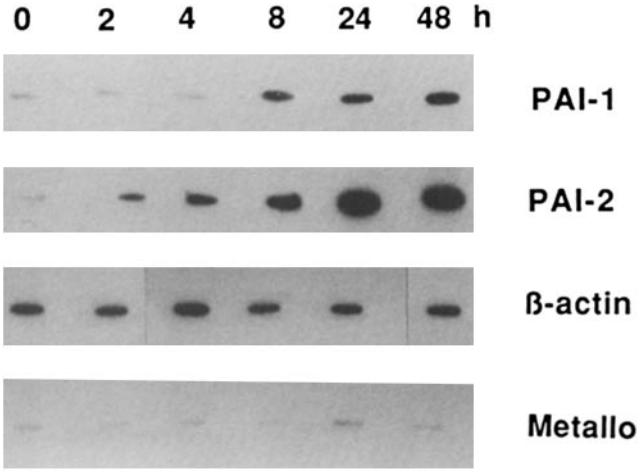

FIGURE 4. Time course of TNF-induced modulation of PAI-1 and PAI-2 gene transcription. HT-1080 cell nuclei were prepared from nontreated (0 h) cells and from cells treated for 2, 4, 8, 24, or 48 h with 50 ng/ml TNF. Elongation and labeling of initiated RNA transcripts in isolated nuclei and hybridization to immobilized cDNA or genomic slot blots (see below) was performed following a modification of the originally described "Run-on" transcription assay (see Materials and Methods). Exposure times were chosen to visually optimize the signals present.

general remains to be determined. A recent report, however, has demonstrated that TNF induces PAI-1 mRNA and suppresses secreted t-PA antigen in bovine aortic endothelial cells (26). Endothelial cells are key components of local hemostasis and fibroblasts play a pivotal role in wound healing and tissue repair. Thus, it seems that TNF released from macrophages under normal circumstances may lead to a localized suppression of the fibrinolytic enzyme system and, thereby, stabilize the fibrin deposited at the wound site. However, the systemic increase of TNF, which occurs in Gram-negative septicemia, has pathogenic consequences. The integrity of the vascular system is not only compromised by the hypofibrinolytic state induced by TNF, but also by the induction of procoagulant activity (tissue factor) on the surface of the endothelial cell (5, 6). The concomitant induction of both PAIs and the suppression of t-PA in endothelial cells would stabilize microthrombi in the microvascular system leading to anoxia and subsequent tissue necrosis. A recent study has provided morphological evidence that thrombus formation is indeed associated with the anti-tumor activity of TNF (27). It is conceivable that agents designed to activate the fibrinolytic system (such as PAs), or to inactivate the PAIs, could be used to counteract the hemostatic complications induced by TNF.

TNF has previously been shown to increase mRNA levels of IL-1 (28) and the histocompatibility antigens, HLA-A,B (29), and suppress mRNA levels of a number of lipogenic genes in adipocytes (30). PAI-1 and PAI-2 represent the first genes where an effect of TNF on gene template activity was directly demonstrated.

The HT-1080 cell line provides a model system for an investigation of TNF-mediated modulation of gene transcription. Isolation and characterization of the PAI gene promoters will allow the identification of TNF modulated *cis*-acting elements and the respective *trans*-acting factors required for the induction of PAI-1 and PAI-2 biosynthesis. Whereas PAI-1 has been shown to be transcriptionally regulated by dexamethasone in HT-1080 cells (15) and PAI-2 by a tumor-promoting phorbol ester in U-937 histiocytic lymphoma cells (18), TNF is the only known compound that induces both inhibitors simultaneously in one cell type. The reversibility of the TNF

effect should encourage the search for TNF-receptor antagonists with possible anti-inflammatory properties.

Summary

Human rTNF/Cachectin was shown to stimulate gene transcription of plasminogen activator inhibitor (PAI)-1 and PAI-2, and simultaneously suppress constitutive gene expression of tissue-type plasminogen activator (t-PA) in human fibrosarcoma cells. We propose that a TNF-mediated reprogramming of gene transcription induces, in appropriate target cells, an anti-fibrinolytic state, which may cooperate with the induction of procoagulant activity (tissue factor) to stabilize the fibrin deposits commonly found in inflamed tissue. PAI genes also provide a model system for a study of the molecular pathways underlying TNF-mediated signal transduction.

We are indebted to Marlies Rüegg and Elizabeth Cousin for their expert technical assistance; Dr. Max Häusermann (LKB-Pharmacia) for densitometric analysis of the autoradiograms; Dr. Alan Shaw (Biogen) for providing human rTNF; and Professor F. Bachmann for advice during the course of this work and for critical reading of the manuscript.

Received for publication 29 March 1988 and in revised form 9 May 1988.

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