

INTERLEUKIN 1 INDUCES ENDOTHELIAL CELL
SYNTHESIS OF PLASMINOGEN ACTIVATOR INHIBITOR

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Endothelial cells, when activated by endotoxin or IL-1, support a procoagulant generating system oriented at the surface membrane (1-5). Endothelial cells also play an important role in fibrinolysis (6) by secreting plasminogen activators and a rapidly acting inhibitor of plasminogen activator (7-9). The modulation of the synthesis of these several activities in different pathophysiologic circumstances may significantly influence the anticoagulant or procoagulant role of the vascular endothelium. Fibrinolytic inhibitors directed against plasminogen activators have been reported in several cell types, including bovine aortic endothelial cells (8), umbilical venous endothelium (10), human platelets (11), mouse macrophages (12), and rat hepatoma cells (13). In this study we report the induction of plasminogen activator inhibitor (PAI) synthesis by IL-1 stimulation of human cultured endothelial cells.

Materials and Methods

Materials. rIL-1 (pI 7.0 or "beta" form) with expression from amino acid 112 to 269 was obtained as described previously (14). This rIL-1 was homogenous on SDS-PAGE and contained 60 pg of endotoxin per mg protein. Recombinant tissue plasminogen activator (TPA) was kindly provided by Genentech Inc. (San Francisco, CA). Urokinase (UK) was a gift from The Sterling Winthrop Research Institute (Rensselaer, NY).

Cell Culture. Early passage (P₂-P₅) human umbilical vein endothelial cells (HUVEC) were grown to confluency on gelatin-coated 24-w311 tissue culture plates as previously described (15-16). The cells were plated at a density of $\sim 5 \times 10^4$ cells/well, reached confluency by 48-72 h, and were used within 3-5 days of plating. The culture medium consisted of Medium 199 containing 50 U/ml penicillin-50 μ g/ml streptomycin, 2.5 μ g/ml fungizone, 2.0 mM L-glutamine, 20 μ g/ml endothelial cell growth factor, 90 μ g/ml porcine intestinal mucosa heparin (170 USP U/mg), and 20% (vol/vol) pooled human serum.

For cell passage, monolayers on 25 or 75 cm² flasks were washed with Hepes-buffered saline (HBS; 11 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, 137 mM NaCl, 4 mM KCL, 11 mM glucose, pH 7.4) and then incubated for 5 min in HBS containing 0.05% Type I collagenase, 0.01% EDTA, and 0.25% BSA at 37°C. After addition of an equal volume of human serum containing medium, cells were washed once, resuspended, and replated.

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In each experiment, endothelial cells were used at confluency, at which time they exhibited typical polygonal, cobblestone appearance. Each 1.7-cm well contained $\sim 2 \times 10^5$ cells. The monolayers were washed twice with sterile Medium 199 containing 5 mg/ml BSA (essentially free of fatty acid and globulin) (M199/BSA) at 37°C. For IL-1 stimulation, we added various concentrations of rIL-1 (in M199/BSA) and incubated the cells at 37°C in a humidified atmosphere containing 6% CO₂ for various periods of time. After incubation, the postculture medium was collected, made 0.01% with Triton X-100, and for some experiments, stored at -70°C until assayed.

To examine the effect of cycloheximide on the production of PAI, cultures were incubated for 30 min with 0.1 mM cycloheximide (Sigma Chemical Co., St. Louis, MO) in M199/BSA or M199/BSA alone. The cells were then washed three times with warm M199/BSA and incubated for an additional 18 h with M199/BSA with or without rIL-1 (5.32 ng/ml). Postculture medium was then harvested as described above.

Quantitative PAI Assay. ¹²⁵I-fibrin plates were prepared by the method of Unkeless et al. (17), as previously described (18). We added 200 μ l ¹²⁵I-conjugated bovine fibrinogen to 24-well tissue culture plates (100 μ g/ml, 3-5 $\times 10^5$ CPM/well). The wells were then air-dried at 37°C, clotted with thrombin (0.04 μ g/ml), and extensively washed. Immediately before use they were washed three times, incubated with lys-plasminogen (10 μ g/ml) at 20°C for 30 min, then washed again. Endothelial cell postculture medium (diluted in preculture medium) was incubated with purified TPA (0.5 U/ml) at 37°C for 1 h in the presence of 0.01% Triton X-100 and then added in duplicate to wells in a volume of 0.2 ml. At 10 min time intervals 10- μ l aliquots were removed and counted to quantify serial release of fibrin degradation products. The time to half maximum lysis (S₅₀), which in this system where plasminogen and fibrin concentrations are constant is a reproducible measure of TPA activity, was then determined from the sigmoidal lysis curves. Residual TPA activity was then determined from standard curves relating S₅₀ to TPA concentration. Standard curves were obtained for each set of plates. A unit of PAI was defined as the amount of inhibitor required to inhibit one unit of TPA in this assay, i.e., for each well [(total TPA - residual TPA) \times dilution = PAI units/well].

TPA Antigen Levels. TPA antigen levels in endothelial cell conditioned medium were determined by a double antibody sandwich ELISA using the Immunbind TPA kit. (American Diagnostica Inc., Greenwich, CT).

Reverse Fibrin Autography. Reverse fibrin autography was performed essentially as described by Levin and Loskutoff (19). SDS-PAGE was performed by the procedure of Laemmli (20) using 7.5 or 9.0% resolving gels and 3.9% stacking gels. *M_r* standards included myosin (200,000), β -galactosidase (116,250), phosphorylase B (92,500), BSA (66,200), OVA (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000), and lysozyme (14,400). After soaking for 90 min in 2.5% Triton X-100, gels to be analyzed were applied to the surface of a freshly formed fibrin agar gel containing (final concentrations): agarose 1.25%, fibrinogen 1.74 mg/ml, plasminogen 25 μ g/ml, thrombin 0.25 U/ml, and TPA 10 U/ml. Each fibrin agar gel measured 5.5 \times 10.5 cm and occupied a volume of 9.6 ml. Incubated gels were stained with amido black 0.05% in 40% ethanol/10% acetic acid, destained with 90% ethanol/5% acetic acid, photographed, and then analyzed.

Demonstration of PAI-UK Complex Formation by SDS-PAGE Autoradiography. 10 μ l of ¹²⁵I-UK (0.3 U; 5,000 cpm/U) was incubated for 30 min at 37°C with either 30 μ l M199/BSA, postculture medium from nonstimulated endothelial cells, or postculture medium from IL-1- (5.3 ng/ml) stimulated cells. Samples were then incubated with Cibacron Blue-Sepharose for 30 min at 37°C. The supernatants were then electrophoresed under nonreducing conditions, as described above, using 7.5% acrylamide with prestained molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD). The gels were then dried and autoradiographs obtained.

Results

PAI activity was detectable at low levels in the conditioned medium obtained from resting endothelial cell monolayers. After IL-1 stimulation, there was a 10-fold increase in the PAI released into the conditioned medium (Fig. 1). This stimulatory effect of IL-1 was dose-dependent, with a minimal effect detected at

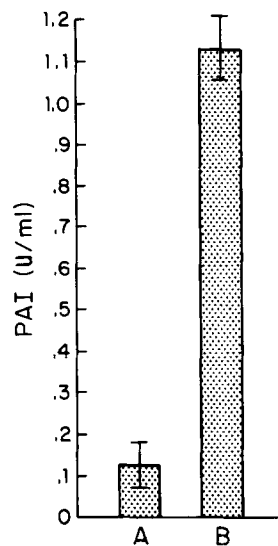


FIGURE 1. Effect of IL-1 on the PAI content of conditioned medium from cultured endothelial cells. Confluent human endothelial cell monolayers were incubated at 37° in M199/BSA for 18 h with or without IL-1 (5.32 ng/ml). At the completion of the incubation period, the postculture medium was collected, made 0.01% with Triton X 100, centrifuged at 1,200 *g* to remove cell debris, and assayed for PAI activity by preincubation with TPA (0.5 U for 1 h at 37°) and measuring the residual TPA activity on ¹²⁵I-fibrin plates containing plasminogen. (A) Post culture medium from nonstimulated cells. Mean = 0.125 U/ml ± 0.042 (SEM). *n* = 4. (B) Postculture medium from IL-1-stimulated cells. Mean = 1.13 U/ml ± 0.075 (SEM). *n* = 6.

3–4 h and a maximum effect detected after 18 h of IL-1 incubation. IL-1-stimulated PAI release into the conditioned medium was dependent on endothelial cell protein synthesis. Preincubation of endothelial cell monolayers with 0.1 mM cycloheximide for 30 min followed by 18 h of stimulation with IL-1 abolished the release of PAI into the conditioned medium.

Plasminogen activator activity as assayed in the ¹²⁵I-fibrin plate system was not detected either in resting or in IL-1-stimulated monolayers. The TPA antigen level in IL-1-stimulated, conditioned medium was unchanged compared with medium obtained from resting cells (Fig. 2). This reflects the fact, as previously observed (10), that TPA released from human endothelial cells is complexed with PAI, which appears to be present in excess in the conditioned medium from these cells.

The PAI synthesized by the IL-1-stimulated endothelial cells was equally active against both TPA and urokinase, and was not inhibited by DFP (10 mM) nor inactivated by acid exposure (pH 3.0 for 1 h), a treatment that destroys the protease inhibitors, α-2-antiplasmin (21), and protease nexin (22). By reverse fibrin autography, the PAI in conditioned medium was stable to SDS treatment and had an approximate molecular weight of 63,000 (Fig. 3). Incubation of ¹²⁵I-UK with postculture medium from stimulated endothelial cells showed formation of a radioactive complex of approximate molecular weight 110,000 (Fig. 4). Thus, this PAI is likely the same as that described by Loskutoff et al. (8).

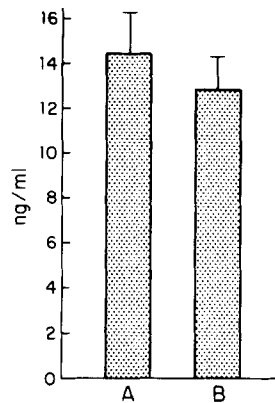


FIGURE 2. Effect of IL-1 on TPA antigen levels of conditioned medium from cultured endothelial cells. Confluent human endothelial cell monolayers were incubated at 37°C in M199/BSA for 18 h with or without IL-1 (5.32 ng/ml). The postculture medium was made 0.01% with Triton X-100, centrifuged at 1,200 *g* to remove cell debris, and assayed for TPA antigen levels using a sandwich double antibody ELISA. (A) Postculture medium from nonstimulated cells. (B) Postculture medium from stimulated cells. Mean \pm SEM. *n* = 6.

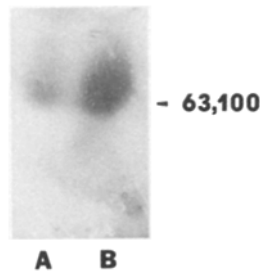


FIGURE 3. Identification of PAI by reverse fibrin autorography. Detergent-treated postculture medium from (A) nonstimulated cells and (B) IL-1-stimulated cells (5.32 ng/ml, 18 h) was concentrated fivefold using Centricon 10 filters (60 min; 1,000 *g*; starting volume, 1 ml). Samples were run on a 7.5% Laemmli slab gel, soaked in Triton X-100 (2.5%, 90 min), and applied to a fibrin agar gel as described in Materials and Methods. The gel and the overlay were incubated in a moist chamber at 37°C for 4 h, stained, destained, then compared with M_r markers run in the same gel and stained with Coomassie blue.

Discussion

Recent studies have suggested that IL-1 stimulates endothelial cells *in vitro*, leading to the biosynthesis and surface expression of a tissue factor like procoagulant, as well as surface adhesion molecules that mediate the attachment of polymorphonuclear leukocytes and monocytes (4, 23). In addition, IL-1 infusion in rabbits has been shown to down regulate the protein C-thrombomodulin anticoagulant pathway on endothelial cell surfaces (24). It therefore appears that IL-1, a potent mediator of inflammatory responses, has a markedly prothrombotic effect on endothelial cell function. The auto-regulatory effects of procoagulant events occurring at the endothelial cell surface have been pointed out by Stern et al. (1). Thus endothelial cells produce IL-1 after exposure to thrombin (1), and thrombin has been shown to lead to the induction of PAI in cultured human endothelial cells (10). Our studies showing IL-1 induction of PAI synthesis and release by endothelial cells further emphasizes positive feedback modulation of procoagulant events at the endothelial cell surface. Thrombin induction of

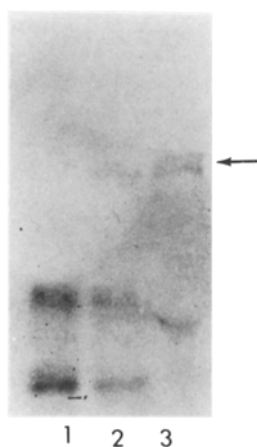


FIGURE 4. Demonstration of PAI-UK Complex Formation. SDS-PAGE autoradiogram of ^{125}I -UK (0.3 U; 5000 CPM/U) incubated for 30 min at 37°C with either M199/BSA (lane 1), postculture medium from nonstimulated cells (lane 2), or postculture medium from IL-1-stimulated cells (lane 3). The arrow indicates the 110,000 mol. weight complex.

endothelial cell IL-1 can lead to a series of prothrombotic signals, including synthesis of PAI, which further potentiates thrombosis in the immediate microenvironment. It may be useful to regard these IL-1-induced changes as an early manifestation of endothelial cell injury.

Summary

Human endothelial cells activated with IL-1 express a surface membrane-oriented procoagulant generating system characterized by increased tissue factor synthesis and decreased thrombomodulin activity. We now report that IL-1 also stimulates endothelial cell synthesis of plasminogen activator inhibitor. This array of IL-1-induced activities shifts the balance at the endothelial cell surface to a prothrombotic influence and may reflect an early response of the blood vessel wall to injury.

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References

1. Stern, D. M., I. Bank, P. P. Nawroth, J. Cassimeris, W. Kisel, J. W. Fenton, C. Dinarello, L. Chess, and E. A. Jaffe. 1985. Self-regulation of procoagulant events on the endothelial cell surface. *J. Exp. Med.* 162:1223.
2. Stern, D., P. Nawroth, D. Handley, and W. Kisel. 1985. An endothelial cell dependent pathway of coagulation. *Proc. Natl. Acad. Sci. USA.* 82:2523.
3. Crutchley, D. J., and L. B. Conanan. 1986. Endotoxin induction of an inhibitor of plasminogen activator in bovine pulmonary artery endothelial cells. *J. Biol. Chem.* 261:154.
4. Bevilacqua, M. P., J. S. Pober, G. R. Majeau, R. S. Cotran, and M. H. Gimbrone. 1984. Interleukin induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. *J. Exp. Med.* 160:618.
5. Bevilacqua, M. P., J. S. Pober, M. E. Wheeler, R. S. Cotran, and M. H. Gimbrone. 1985. Interleukin-1 activation of vascular endothelium. *Am. J. Pathol.* 121:394.

6. Erickson, L. A., R. R. Schleef, T. Ny, and D. J. Loskutoff. 1985. The fibrinolytic system of the vascular wall. *Clin. Haematol.* 14:513.
7. Philips, M., A. G. Juul, and S. Thorsen. 1984. Human endothelial cells produce a plasminogen activator-inhibitor complex. *Biochim. Biophys. Acta.* 802:99.
8. Loskutoff, D. J., J. H. van Mourik, L. A. Erickson, and D. Lawrence. 1983. Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. *Proc. Natl. Acad. Sci. USA.* 80:2956.
9. Levin, E. G. 1983. Latent tissue plasminogen activator produced by human endothelial cells in culture. Evidence for an enzyme inhibitor complex. *Proc. Natl. Acad. Sci. USA.* 80:6804.
10. Gelehrter, T. D., and R. Sznycer-Laszuk. 1986. Thrombin induction of plasminogen activator-inhibitor in cultured human endothelial cells. *J. Clin. Invest.* 77:165.
11. Erickson, L. A., M. H. Ginsberg, and D. J. Loskutoff. 1984. Detection and partial characterization of an inhibitor of plasminogen activator in human platelets. *J. Clin. Invest.* 74:1465.
12. Chapman, H. A., and O. L. Stone. 1985. Characterization of a macrophage derived plasminogen-activator inhibitor. *Biochem. J.* 230:109.
13. Coleman, P. L., P. A. Barowski, and T. D. Gelehrter. 1982. The dexamethasone-induced inhibitor of fibrinolytic activity in hepatoma cells. A cellular product which specifically inhibits plasminogen activation. *J. Biol. Chem.* 257:4260.
14. Auron, P. E., A. C. Webb, L. J. Rosenwaser, S. F. Mucei, A. Rick, S. M. Wolff, and C. A. Dinarello. 1984. Nucleotide sequence of human monocyte interleukin-1 precursor cDNA. *Proc. Natl. Acad. Sci. USA.* 84:7907.
15. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. *J. Clin. Invest.* 52:2745.
16. Maciag, T., G. A. Hoover, M. B. Sterman, and R. Weinstein. 1981. Serial propagation of human endothelial cells in vitro. *J. Cell Biol.* 91:420.
17. Unkeless, J. C., A. Tobia, L. Ossowski, J. P. Quigley, D. Rifkin, and E. Reich. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. *J. Exp. Med.* 137:88.
18. Silverstein, R. L., L. L. K. Leung, P. C. Harpel, and R. L. Nachman. 1984. Complex formation of platelet thrombospondin with plasminogen. *J. Clin. Invest.* 74:1625.
19. Levin, E. G., and D. J. Loskutoff. 1982. Cultured bovine endothelial cells produce urokinase and tissue-type plasminogen activator. *J. Cell Biol.* 94:631.
20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680.
21. Travis, J., and G. S. Salvesen. 1983. Human plasma proteinase inhibitors. *Ann. Rev. Biochem.* 52:655.
22. Eaton, D. L., R. W. Scott, and J. B. Baker. 1984. Purification of human fibroblast urokinase proenzyme and analysis of its regulation by proteases and protease nexin. *J. Biol. Chem.* 259:6241.
23. Bevilacqua, M. P., J. S. Pober, M. E. Wheeler, R. S. Cotran, and M. A. Gimbrone. 1985. Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. *J. Clin. Invest.* 76:2003.
24. Nawroth, P. P., D. H. Handley, C. T. Esmon, and D. M. Stern. 1986. Interleukin-1 induces endothelial cell procoagulant while suppressing cell surface anticoagulation activity. *Proc. Natl. Acad. Sci. USA.* In Press.