

## SELF-REGULATION OF PROCOAGULANT EVENTS ON THE ENDOTHELIAL CELL SURFACE

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Endothelium has been thought to play a passive role in hemostatic and inflammatory phenomena. Clinical and laboratory observations, however, have suggested that endothelium can play an active role in the response of the inflammatory and coagulation systems to perturbation and that these responses are linked. A component of the delayed hypersensitivity response, for example, includes induction of procoagulant activity (1). The Shwartzmann reaction, a basic phenomenon in the biology of bacterial infections, includes tissue infiltration by leukocytes and prominent thrombotic pathology. Another example is the clinical picture of systemic lupus erythematosus, which includes a vasculitic syndrome and multiple coagulation abnormalities in addition to autoimmune phenomena (2).

As a potent mediator of inflammatory and immunologic events (3–6), interleukin 1 (IL-1)<sup>1</sup> is likely to have a role in the physiology of these phenomena. Recent studies have shown that IL-1 can promote endothelial cell synthesis and expression of tissue factor (7), a cofactor in the initiation of coagulation (8). Once activation of coagulation is initiated on the cell surface, endothelium can promote an entire pathway of coagulation leading to the formation of fibrin (9). Furthermore, three recent reports have suggested that IL-1 can be produced by endothelium (10–12). These findings have prompted us to consider the hypothesis that IL-1 is an integral part of the endothelial cell response to injurious stimuli. If this were true, then endothelial cell IL-1 could exert a regulatory effect on inflammatory phenomena while simultaneously inducing procoagulant activity in the vessel wall.

These considerations have led us to study factors that influence the production

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<sup>1</sup>Abbreviations used in this paper: DIP, diisopropyl fluorophosphate; IL-1, interleukin 1.

of IL-1 by endothelial cells. The results indicate that cultured human umbilical vein endothelial cells treated with lipopolysaccharide synthesize and elaborate enhanced amounts of IL-1. In addition, the coagulation enzyme thrombin, a physiological vessel wall perturbant generated by the activated coagulation system, induced comparable amounts of IL-1 activity. The generation of IL-1 by perturbed endothelium indicates a new mechanism by which the systems involved in coagulation and inflammation can influence each other via the endothelium. This emphasizes the regulatory role that endothelium can play in coagulation and inflammation.

### Materials and Methods

**Cell Culture.** Endothelial cells derived from human umbilical cord veins were prepared according to the method of Jaffe et al. (13, 14) as modified by Thornton et al. (15). In brief, after harvesting endothelial cells from umbilical veins, the cells were added to tissue culture flasks pretreated with gelatin (0.2%) (gelatin solutions contained polymyxin B at 1  $\mu\text{g}/\text{ml}$ ) and grown in medium 199 (Gibco Laboratories, Grand Island, NY), containing heparin (90  $\mu\text{g}/\text{ml}$ ; Sigma Chemical Co., St. Louis, MO), endothelial cell growth factor (20  $\mu\text{g}/\text{ml}$ ; Meloy Laboratories, Springfield, VA), human serum (10%; prepared from normal human volunteers), fetal calf serum (10%; Sterile Systems, Logan, UT), and penicillin-streptomycin (50 U/ml–50  $\mu\text{g}/\text{ml}$ ; Gibco Laboratories). Where indicated, fibronectin was used as a substrate for cell growth in place of gelatin. The cells were grown at 37°C in a humidified atmosphere with CO<sub>2</sub>/air (5%/95%). At confluence ( $1.0\text{--}1.3 \times 10^5$  cells/cm<sup>2</sup>), cells were prepared for subculture with trypsin-EDTA (0.25–0.05%; Gibco Laboratories). Cultures were characterized as endothelial cells based on morphologic criteria (13) and by positive immunofluorescent staining (16) with a specific anti-factor VIII/von Willebrand factor antibody (generously provided by Drs. Chopek and Davie, University of Washington, Seattle, WA). Endothelial cell cultures contained no monocyte/macrophage-contaminating cells, as judged by morphologic criteria and by cytofluorometry using OKM2, a monoclonal antibody reactive with the majority of human monocytes/macrophages (17). Absence of OKT3-positive cells (18) by cytofluorometry of endothelial cell cultures indicated that T lymphocytes were not present.

Bovine aortic endothelial cells were grown from aortae of newborn calves as described (19) and were used from passages 2–6. Bovine capillary adrenal cells, prepared by the method of Folkman (20), were provided by Drs. Furie and Silverstein (Columbia University, New York) at passage 4 (21).

**Treatment of Endothelial Cells with Endotoxin and Thrombin.** Experiments were carried out in 35-mm tissue culture dishes 24 h after endothelial cells reached confluence, using primary cultures and serially passaged cells (passages 1–18). Monolayers were washed three times with Hanks' balanced salt solution (Gibco Laboratories), and then fresh medium 199 (1 ml/dish) with fetal calf serum (2%) and endotoxin (a phenolic extract of *Escherichia coli* serotype 026:B6; Sigma Chemical Co.) were added. In experiments using cycloheximide (1  $\mu\text{g}/\text{ml}$ ; Sigma Chemical Co.), this inhibitor was added simultaneously with the endotoxin. Culture supernatants were harvested at the indicated times, centrifuged to remove cellular debris, filtered (0.22  $\mu\text{m}$ ), and assayed for IL-1 activity as described below. Throughout these experiments, endothelial cell viability was >95%, as assessed by trypan blue exclusion. When thrombin or modified thrombins were used as the perturbing agents in place of lipopolysaccharide, the incubation medium contained only medium 199 with bovine serum albumin (fatty acid free) (0.5%; Sigma Chemical Co.) and polymyxin B (1  $\mu\text{g}/\text{ml}$ ; Pfizer, Inc., New York). Experiments using bovine aortic and capillary endothelial cells were carried out by the same protocols as outlined above.

Rabbit anti-human IL-1 antibody was prepared and coupled to Sepharose 4B as described previously (22). IgG from nonimmune animals was prepared similarly and also coupled to Sepharose 4B. The IgG-Sepharose columns were equilibrated with medium

199 containing bovine serum albumin (fatty acid free) (0.5%) and polymyxin B (1  $\mu\text{g}/\text{ml}$ ) before use.

*IL-1 and IL-2 Assays.* IL-1 activity was measured in a costimulator assay using murine thymocytes as described previously (23). In brief, thymocytes ( $1.5 \times 10^6/\text{well}$ ) from 4–8-wk-old C3H/HEJ mice (The Jackson Laboratory, Bar Harbor, ME) were cultured for 72 h in Iscoves modified Dulbecco's medium (Gibco Laboratories) containing fetal calf serum (10%), glutamine (1%),  $\beta$ -mercaptoethanol (50  $\mu\text{M}$ ), and phytohemagglutinin (1%). Supernatant (50  $\mu\text{l}$ ) from stimulated endothelial cells was added to each well at the start of a 72 h assay, and [ $^3\text{H}$ ]thymidine (1  $\mu\text{Ci}/\text{well}$ ; New England Nuclear, Boston, MA) was added 18 h before harvesting. Before assaying supernatants of endothelial cells containing added cycloheximide, samples were extensively dialyzed vs. saline (dialysis tubing had a molecular weight cut-off of 3,500). Control samples containing cycloheximide and medium not exposed to cells were also dialyzed and assayed. Under these conditions cycloheximide had no effect on thymocyte proliferation in response to added IL-1 (Genzyme, Boston, MA). In addition to studying culture supernatants, a lysate of endothelial cells was also assayed. The lysate was prepared by three freeze-thaw cycles ( $-80$  to  $37^\circ\text{C}$ ) and cellular debris was removed by centrifugation (10,000  $g$  for 20 min). Samples were assayed in triplicate. Lipopolysaccharide, up to 100  $\mu\text{g}/\text{ml}$ , did not induce thymocyte proliferation above background in this assay.

Culture supernatants containing added thrombin were treated with a 100-fold molar excess of purified bovine antithrombin III (see below) to inactivate any residual thrombin activity. Thrombin, however, even at the highest doses used (40–50 U/ml) had only a minimal effect on the thymocyte proliferation assay. The latter effect was abrogated by the addition of antithrombin III. Antithrombin III alone did not induce proliferation of murine thymocytes or affect proliferation of thymocytes induced by purified IL-1 (Genzyme). Antithrombin III and diisopropyl fluorophosphate had no effect on endotoxin-induced IL-1 elaboration by endothelial cells (see below). DIP- $\alpha$ -thrombin,  $\gamma$ -thrombin, and D-Phe-Pro-Arg-CH<sub>2</sub>- $\alpha$ -thrombin (see below) had no direct effect on the thymocyte proliferation assay at the concentrations used in these experiments.

IL-2 activity was assessed by measuring proliferation of the murine IL-2-dependent CTLL-2 line (the characteristics of this cell line have been previously described) (24–25) induced by addition of supernatants from stimulated and control endothelial cells. The assay was carried out by incubating CTLL-2 cells ( $10^4$  cells per well) in the presence or absence of endothelial cell supernatants for 48 h, then adding [ $^3\text{H}$ ]thymidine (1  $\mu\text{Ci}/\text{well}$ ) and harvesting 18 h later. No IL-2 activity was detectable in control or stimulated endothelial cell supernatants.

*Preparation of Coagulation Proteins and Tissue Factor Assay.* Human factors IX (250 U/mg) and X (130 U/mg) were purified to homogeneity from plasma by the method of DiScipio et al. (26). Factor IX was activated, as previously described (27), during incubation with factor XI<sub>a</sub> coupled to Sepharose in the presence of  $\text{CaCl}_2$  (5 mM) for 45 min at  $37^\circ\text{C}$ . Factor X was activated during incubation with the coagulant protein from Russell's viper venom coupled to Sepharose in the presence of  $\text{CaCl}_2$  (5 mM) for 30 min at  $37^\circ\text{C}$  as previously described (28). In each case activation appeared complete by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Human prothrombin and human factor VII<sub>a</sub> were purified to homogeneity, as described previously (29–30). Bovine antithrombin III (inhibitory activity of 1.0 U thrombin/ $\mu\text{g}$ ) was purified to homogeneity by the method of Mahoney et al. (31). Human  $\alpha$ -thrombin (2.5 NIH U/ $\mu\text{g}$ ) was purified as described (32).  $\alpha$ -Thrombin was inactivated by incubation with diisopropylfluorophosphate (33) (2 mM at pH 7.5; Calbiochem-Behring Corp., San Diego, CA) for 20 min at  $37^\circ\text{C}$ . The reaction mixture was then dialyzed extensively vs. Hepes-saline [10 mM (pH 7.4)/0.14 M]. A coagulant assay (34) indicated no residual thrombin activity. Thrombin (0.15  $\mu\text{g}/\text{ml}$ ) was also completely inactivated by antithrombin III (250  $\mu\text{g}/\text{ml}$ ) after 10 min at  $37^\circ\text{C}$  (35).  $\gamma$ -Thrombin and D-Phe-Pro-Arg-CH<sub>2</sub>- $\alpha$ -thrombin were prepared as described (36).

Tissue factor activity of endothelial cell monolayers was assessed after washing the cells three times in Hepes-saline by adding factor VII<sub>a</sub> (0.5 nM) and factor X (300 nM) in 10 mM Hepes (pH 7.4), 137 mM NaCl, 4 mM KCl, 11 mM glucose, 2.5 mM  $\text{CaCl}_2$ , 2 mg/

ml bovine serum albumin at 37°C. Aliquots of the reaction mixture supernatant (0.1 ml) were withdrawn at 7 min, diluted in 0.5 ml of 50 mM Tris-HCl (pH 7.8), 175 mM NaCl, 0.5 mg/ml ovalbumin, 10 mM EDTA, and assayed for factor X<sub>a</sub> amidolytic activity. Amidolytic activity was measured using the synthetic substrate Bz-Ile-Glu-Gly-Arg-pNA (0.5 mM) by monitoring the increase in absorbance at 405 nM (37). The concentration of factor X<sub>a</sub> formed was determined by comparison with a factor X<sub>a</sub> standard as described previously (28). Under these conditions, factor X<sub>a</sub> formation was linear and limited only by the amount of tissue factor present. Before assaying cells treated with thrombin, the added thrombin was neutralized by excess antithrombin III and monolayers were then washed to remove antithrombin III. Similar results were observed when dansylarginine *N*-(3-ethyl-1,5-pentanediy)amide, a specific thrombin inhibitor (38), replaced antithrombin III.

## Results

Incubation of cultured human umbilical vein endothelial cells with lipopolysaccharide resulted in the elaboration of IL-1 activity (Fig. 1). The IL-1 activity of supernatants from lipopolysaccharide-treated endothelial cells was easily detectable up to a 1:32 dilution in the murine thymocyte costimulation assay. Although the thymocyte assay was sensitive for the detection of endothelial cell IL-1 activity, to better characterize the molecular species in the supernatants causing the proliferative response, we used an affinity column with anti-IL-1 antibody coupled to Sepharose (Fig. 2). Chromatography of lipopolysaccharide-treated endothelial cell supernatants on the anti-IL-1 column removed their proliferative activity in the thymocyte assay. A control column, with nonimmune IgG coupled to the resin, had no effect on IL-1-like activity in the culture supernatants.

Generation of IL-1 activity was dependent on the concentration of lipopolysaccharide added, increasing steadily from 10 ng/ml to 10 µg/ml and, soon thereafter, reaching an apparent maximum (Fig. 3A). IL-1 found in the super-

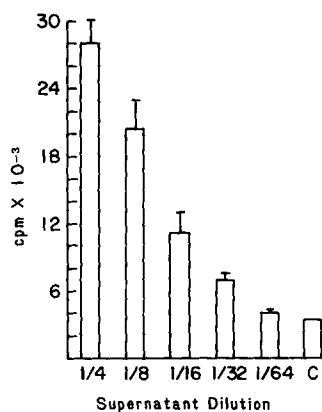


FIGURE 1. Elaboration of IL-1 activity by lipopolysaccharide-treated endothelial cells. Endothelial cell monolayers (passage 3) were incubated with medium 199 (1 ml) containing fetal calf serum (2%) in the presence or absence (C) of lipopolysaccharide (20 µg/ml) for 17 h at 37°C. Samples from lipopolysaccharide-treated cells were diluted in medium as indicated and assayed for IL-1 activity in the thymocyte proliferation assay. Samples from untreated cells (C) were assayed at a 1:4 dilution (identical results were observed at 1:1 and 1:8 dilutions). Sample dilution is plotted vs. [<sup>3</sup>H]thymidine incorporation. The mean ± 1 SD of triplicate determinations is shown.

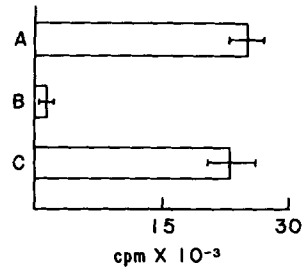


FIGURE 2. Effect of anti-IL-1 column on the IL-1 activity of lipopolysaccharide-treated endothelial cell supernatants. Endothelial cell monolayers (passage 4) were incubated with medium 199 (1 ml) containing fetal calf serum (2%) in the presence of endotoxin (10  $\mu\text{g}/\text{ml}$ ) for 18 h at 37°C. Aliquots of culture supernatants (0.2 ml) were then chromatographed on an anti-IL-1 column (bed volume, 0.8 ml) or a column made by coupling nonimmune rabbit IgG to the resin (bed volume, 0.8 ml). The initial culture supernatant (A), pass-through fraction from the anti-IL-1 column (B), and pass-through fraction from the control IgG column (C) were assayed in the thymocyte proliferation assay. The mean  $\pm$  1 SD of triplicate determinations is shown.

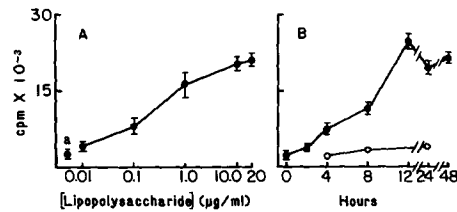


FIGURE 3. Dose dependence and time course of lipopolysaccharide-induced elaboration of IL-1 activity by endothelial cells. (A) Dose dependence. Endothelial cell monolayers (passage 2) were incubated for 16 h at 37°C with medium 199 containing fetal calf serum (2%) in the presence of the indicated concentrations of lipopolysaccharide. Samples were then assayed in the thymocyte proliferation assay. The direct effect of lipopolysaccharide (20  $\mu\text{g}/\text{ml}$ ) added to the thymocyte assay in the absence of endothelial cell supernatant is shown (a). (B) Time course. Endothelial cell monolayers were incubated in the presence (●) or absence (○) of lipopolysaccharide (10  $\mu\text{g}/\text{ml}$ ) for the indicated times at 37°C. The supernatants were then assayed for IL-1 activity in the thymocyte proliferation assay as described. The mean of triplicate determinations is shown in A and B above.

nant was not due to release from a preformed intracellular pool since lysates of endothelial cells not treated with lipopolysaccharide did not have significant IL-1 activity. To investigate whether protein synthesis was required for induction of IL-1 release, we added cycloheximide to cultures during lipopolysaccharide stimulation (Fig. 4). Inhibition of IL-1 production was observed in the presence of cycloheximide (1  $\mu\text{g}/\text{ml}$ ), indicating that new protein synthesis was required.

Comparable lipopolysaccharide-induced generation of IL-1 activity was observed with endothelial cells from four different vessels, using both primary cultures and serially passaged cells (passages 1–18). Furthermore, endotoxin-treated bovine aortic and capillary endothelial cells also generated IL-1 activity, though the amounts were lower (~30%) than that observed with the human umbilical vein endothelial cells (data not shown). Elaboration of IL-1 activity occurred in a time-dependent manner after the addition of lipopolysaccharide to endothelial cultures (Fig. 3B). IL-1 activity steadily increased up to 16 h and

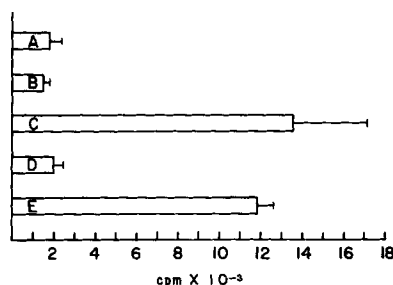


FIGURE 4. Effect of cycloheximide on lipopolysaccharide-induced IL-1 elaboration. Endothelial cells (passage 6) were incubated with medium 199–fetal calf serum (2%) alone (A), containing lipopolysaccharide (10  $\mu\text{g}/\text{ml}$ ) in the presence of cycloheximide (1  $\mu\text{g}/\text{ml}$ ) (B), or containing only endotoxin (10  $\mu\text{g}/\text{ml}$ ) (C). This concentration of cycloheximide did not reduce cell viability. Culture supernatants were dialyzed extensively vs. saline and assayed for IL-1 as described. Addition of lipopolysaccharide (10  $\mu\text{g}/\text{ml}$ ) directly to the IL-1 assay (D) resulted in little thymocyte proliferation. Cycloheximide (1  $\mu\text{g}/\text{ml}$ ), after dialysis, had no effect on the thymocyte proliferation assay (E). The mean  $\pm$  1 SD is shown.

then reached a plateau. In contrast, control endothelial cell cultures in media alone maintained a low level of IL-1 activity.

Since endothelial cells do not usually interact with lipopolysaccharide, we asked if any more physiological molecules could induce endothelial cell IL-1 elaboration. Prompted by recent studies demonstrating specific interactions between endothelium and coagulation factors (9, 28, 40–45), we examined the effect of a variety of coagulation proteins on endothelial cell IL-1 elaboration (Table I). These studies were carried out in serum-free medium to prevent inactivation of activated coagulation factors by inhibitors present in serum, such as antithrombin III. Coagulation factors normally present in plasma, i.e., the zymogens, factors IX, X, and prothrombin (added at plasma concentrations), did not induce generation of IL-1 activity by endothelial cells. Although the activated form of factors IX (IX<sub>a</sub>) and X (X<sub>a</sub>), induced no or only small amounts of IL-1 release, respectively, thrombin induced elaboration of significant amounts of IL-1. To further study the effect of thrombin on endothelial cell elaboration of IL-1, we used thrombins modified at specific sites (Table II). Native  $\alpha$ -thrombin induced elaboration of IL-1 endothelium, but thrombin inactivated by antithrombin III was no longer effective. Since antithrombin III is a relatively large molecule ( $M_r$  ~58,000) (31, 35) compared with thrombin ( $M_r$  ~36,500) (32), the inhibitor could be masking multiple sites on the enzyme in addition to the active site. This led us to examine the effect of other forms of thrombin.  $\gamma$ -thrombin (36), a form of thrombin deficient in procoagulant activity but which retains esterolytic activity, was less effective than  $\alpha$ -thrombin in the induction of IL-1. D-Phe-Pro-Arg-CH<sub>2</sub>- $\alpha$ -thrombin (36), which is modified just adjacent to the catalytic site within the fibrinopeptide groove (39), did not induce endothelial cell IL-1 elaboration. Consistent with this data, which suggests the importance of the active site region of  $\alpha$ -thrombin, we found that DIP- $\alpha$ -thrombin, which has a blocked catalytic center, was ineffective in the induction of endothelial cell IL-1.

Endothelial cell IL-1 elaboration induced by  $\alpha$ -thrombin was dependent on the concentration of enzyme added and the incubation time (Fig. 5). The time course of IL-1 elaboration was comparable to that observed with lipopolysaccha-

TABLE I  
Effect of Coagulation Factors on the Generation of IL-1 Activity by Endothelial Cells

| Coagulation factor      | Concentration    | [ <sup>3</sup> H]Thymidine incorporation |
|-------------------------|------------------|--|
|                         | $\mu\text{g/ml}$ | <i>cpm</i>                               |
| Factor IX               | 5                | 1,389 $\pm$ 76                           |
| Factor IX <sub>a</sub>  | 1                | 1,312 $\pm$ 111                          |
|                         | 10               | 1,294 $\pm$ 68                           |
| Factor X                | 10               | 1,517 $\pm$ 163                          |
| Factor X <sub>a</sub> * | 1                | 3,781 $\pm$ 265                          |
|                         | 10               | 5,088 $\pm$ 460                          |
| Prothrombin             | 100              |  |
| $\alpha$ -Thrombin      | 0.2              | 11,483 $\pm$ 1,083                       |
|                         | 2                | 13,382 $\pm$ 1,165                       |
| No addition             |                  | 1,301 $\pm$ 91                           |

Endothelial cells (passage 3) were incubated with medium 199 containing bovine serum albumin (0.5%) and polymyxin B (1  $\mu\text{g/ml}$ ). Then coagulation factors at the indicated concentrations were added for 16 h at 37°C. Cultures containing factor X<sub>a</sub> and thrombin were incubated with antithrombin III (400  $\mu\text{g/ml}$ ) for 10 min at 37°C and then all samples were assayed in the thymocyte proliferation assay. The mean  $\pm$  1 SD is shown.

\* Factors IX<sub>a</sub> and X<sub>a</sub> are the activated forms of factors IX and X, respectively.

TABLE II  
Effect of  $\alpha$ -Thrombin and Modified Thrombins on the Induction of IL-1 Activity in Endothelial Cells

| Coagulation factors added                          | [ <sup>3</sup> H]Thymidine incorporation |
|--|--|
|  | <i>cpm</i>                               |
| $\alpha$ -Thrombin                                 | 11,688 $\pm$ 971                         |
| $\alpha$ -Thrombin-antithrombin III                | 1,264 $\pm$ 131                          |
| $\gamma$ -Thrombin                                 | 4,298 $\pm$ 385                          |
| D-Phe-Pro-Arg-CH <sub>2</sub> - $\alpha$ -thrombin | 1,460 $\pm$ 78                           |
| DIP- $\alpha$ -thrombin                            | 1,300 $\pm$ 35                           |

Endothelial cells (passage 6) were incubated with medium 199 containing fetal calf serum (2%) and polymyxin B (1 mg/ml). One of the following thrombins was added for 16 h at 37°C:  $\alpha$ -Thrombin (0.3 U/ml or 0.12  $\mu\text{g/ml}$ ),  $\alpha$ -thrombin (0.15  $\mu\text{g/ml}$ ) inactivated by antithrombin III (250  $\mu\text{g/ml}$ ),  $\gamma$ -thrombin (0.2  $\mu\text{g/ml}$ ), D-Phe-Pro-Arg-CH<sub>2</sub>- $\alpha$ -thrombin (0.18  $\mu\text{g/ml}$ ), and DIP- $\alpha$ -thrombin (0.11  $\mu\text{g/ml}$ ). Samples were assayed in a thymocyte proliferation assay. Control endothelial cell monolayers not incubated with any form of thrombin incorporated 1,400  $\pm$  350 cpm of [<sup>3</sup>H]-thymidine. Modified forms of thrombin were prepared as described in Materials and Methods.

ride. Although the amount of thrombin causing optimal IL-1 elaboration is relatively high, IL-1 is generated at lower concentrations, which may occur as prothrombin is generated on the endothelial cell surface (9, 28, 43). IL-1 activity

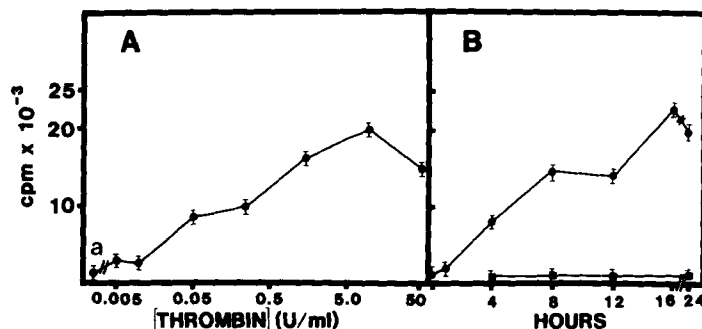


FIGURE 5. Elaboration of IL-1 activity by endothelial cells treated with  $\alpha$ -thrombin. (A) Dependence on dose of  $\alpha$ -thrombin. Endothelial cell monolayers (passage 5) were incubated for 16 h at 37°C with medium 199 (1 ml) containing 0.5% bovine serum albumin, polymyxin B (1  $\mu$ g/ml), and the indicated concentrations of thrombin. A 100-fold molar excess of bovine antithrombin III was then added to each culture dish for 10 min at 37°C and supernatants were assayed in the thymocyte proliferation assay. The direct effect of thrombin (50 U/ml), in the absence of endothelial cell supernatants, on proliferation in the thymocyte assay is shown (a). (B) Time course of  $\alpha$ -thrombin-induced elaboration of IL-1. Endothelial cell monolayers were incubated for the indicated times at 37°C with medium 199 (1 ml) containing 0.5% bovine serum albumin (fatty acid free) and polymyxin B (1  $\mu$ g/ml) in the presence (●) or absence (■) of thrombin (5 U/ml). Antithrombin III (0.8 mM) was added for 10 min and then supernatants were assayed in the thymocyte proliferation assay. The mean  $\pm$  SD is shown.

produced by endothelial cells in response to thrombin could be removed from culture supernatants by the anti-IL-1 column, as described previously for lipopolysaccharide-induced IL-1 activity (Fig. 2). Thrombin-induced IL-1 generation was not due to lipopolysaccharide in the preparation since addition of antithrombin III or treatment of the enzyme with diisopropyl fluorophosphate blocked the enzyme's effect. Furthermore, treatment of lipopolysaccharide with antithrombin III or diisopropyl fluorophosphate under these conditions did not affect endotoxin-induced IL-1 elaboration.

These results indicate that a potent procoagulant enzyme, thrombin, can induce IL-1 in a highly specific manner. Since IL-1 is known to induce tissue factor activity in cultured endothelial cells (7), this suggested the possibility of self-regulation of procoagulant events on the endothelial cell surface. To test this hypothesis, IL-1-containing supernatants from endothelial cells treated with thrombin were incubated with fresh cultures, after the residual thrombin was inactivated, and induction of tissue factor activity was tested (Fig. 6). Procoagulant activity, characterized as tissue factor based on the factor VII<sub>a</sub> dependence of factor X activation (8), was induced in endothelium. Similar induction of tissue factor activity was observed when cultures were incubated with exogenous IL-1 (Fig. 6). The ability of both  $\alpha$ -thrombin-treated endothelial cell supernatants and exogenous IL-1 to induce tissue factor in endothelial cells was prevented by chromatography of the samples on the anti-IL-1-Sepharose column.

### Discussion

The results reported here indicate that cultured endothelial cells elaborate IL-1 in response to perturbation. Generation of IL-1 activity in response to endotoxin suggests that endothelium participates in the regulation of inflammatory/



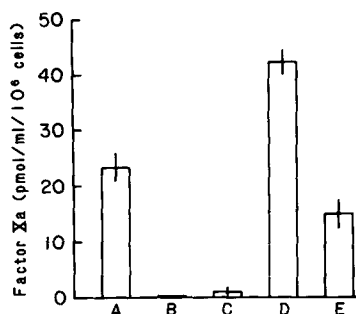


FIGURE 6. Effect of culture supernatants from thrombin-treated endothelial cells on the procoagulant activity of fresh endothelial cell cultures. Human endothelial cell monolayers (passage 3) were treated with  $\alpha$ -thrombin (10 U/ml) in medium 199 containing bovine serum albumin (0.5%) and polymyxin B (1  $\mu$ g/ml) for 8 h at 37°C. After this incubation period the supernatant (endothelial cell-conditioned medium) was incubated with antithrombin III (2  $\mu$ M) (10 min at 37°C) to inactivate residual thrombin, filtered (0.22  $\mu$ m), and added to fresh endothelial cells for 6 h. Monolayers were washed and then 1 ml of 10 mM HEPES (pH 7.4) containing 137 mM NaCl, 4 mM KCl, 11 mM glucose, 2.5 ml CaCl<sub>2</sub>, 2 mg/ml bovine serum albumin, along with human factor VII<sub>a</sub> (0.5 nM) and/or factor X (300 nM), was added for 7 min at 23°C. The reaction mixture was assayed for factor X<sub>a</sub> amidolytic activity using the synthetic peptide substrate Bz-Ile-Glu-Gly-Arg-p-nitroanilide (0.5 mM). The amount of factor X<sub>a</sub> formed after 7 min (mean  $\pm$  SEM) is shown. (A) Endothelial cell-conditioned medium was incubated with the cells and factor X<sub>a</sub> formation was assessed in the presence of factors VII<sub>a</sub> and X. (B) Endothelial cell-conditioned medium was incubated with the cells and factor X<sub>a</sub> formation was assessed in the presence of factor X alone. (C) Medium 199 containing thrombin (10 U/ml) was incubated for 8 h in a glass tube and then antithrombin III (2  $\mu$ M) was added to inactivate the thrombin. This medium (1 ml) was then incubated with the cells for 6 h at 37°C and factor X<sub>a</sub> formation was assessed in the presence of factors VII<sub>a</sub> and X. (D, E) Medium 199 containing purified exogenous IL-1 (5 U/ml, D; 1 U/ml, E) (supplied in 5% fetal calf serum) was incubated with endothelial cells, and factor X<sub>a</sub> formation was assessed in the presence of factors VII<sub>a</sub> and X. The mean and SEM of triplicates are shown.

immunologic phenomena. The induction of endothelial cell IL-1 activity by thrombin indicates a link between activation of the coagulation system and the regulatory role of the endothelial cell. Although quiescent endothelium does not induce activation of coagulation, perturbation of endothelial cells *in vitro* by a variety of agents, including IL-1, can lead to the induction of tissue factor, a cofactor for the initiation of coagulation (8). Endothelial cell tissue factor, in the presence of factor VII/VII<sub>a</sub>, results in the activation of factors IX and X, propagating a procoagulant pathway on the cell surface that leads to the generation of thrombin (9, 28). Thrombin may then function as a coagulation enzyme or as a regulator of endothelial cell function, including the elaboration of IL-1. IL-1, finally, promotes the synthesis and expression of additional endothelial cell tissue factor. Although multiple pathways are operative in inhibiting thrombin procoagulant activity in the plasma and on the endothelial cell surface (28, 33, 35, 44–47), thrombin-induced IL-1 generation represents a cellular mechanism by which this enzyme can potentially promote procoagulant events.

The induction of IL-1 activity by thrombin was specific for the enzyme (Tables I and II) and required an intact catalytic center. Thus, DIP- $\alpha$ -thrombin and D-Phe-Pro-Arg- $\alpha$ -thrombin (36) did not induce IL-1 elaboration but  $\alpha$ -thrombin did (Table II).  $\gamma$ -thrombin, in which the fibrin exosite region is blocked (39), was able to induce IL-1, though less effectively than the intact enzyme. This is

consistent with many of thrombin's cellular actions, such as stimulation of mitogenesis (48) and prostaglandin synthesis (49), but differs from thrombin's role as a chemotactic agent (39) in which DIP- $\alpha$ -thrombin is even more effective than the active enzyme. Thrombin-induced IL-1 generation may be related to the previously described (45) class of irreversible endothelial cell binding sites specific for the enzyme. Other mechanisms may be involved, such as thrombin-protease nexin complexes (50) or thrombin-induced proteolysis of the IL-1 translation product (51, 52) to potentially more active forms. The role of thrombin as an inducer of IL-1 suggests that activation of coagulation can play a role in priming the immune and inflammatory response. Thus, in addition to the well-known activation of coagulation leading to fibrin deposition, observed in a range of inflammatory lesions, a product of the coagulation system, thrombin, can play a role in the regulation of the inflammatory response.

The present data reflect the close relationship between the hemostatic and inflammatory systems. Perturbation of endothelium by stimuli such as IL-1 leads to induction of tissue factor procoagulant activity. Tissue factor promotes initiation of coagulation by priming the reactions leading to thrombin formation. Thrombin, by inducing endothelial cell IL-1 production, closes a circle of interactions between the inflammatory and coagulation systems.

### Summary

Interleukin 1 (IL-1) is a potent mediator of inflammatory and immunologic phenomena. In addition, IL-1 may be intimately involved in the regulation of hemostasis, since interaction of IL-1 with endothelial cells has been reported to induce tissue factor activity. We demonstrate that perturbation of the endothelial cell induces augmented IL-1 release. Human umbilical vein endothelial cells perturbed by treatment with lipopolysaccharide produced enhanced amounts of IL-1 activity. IL-1 activity from lipopolysaccharide-treated endothelial cell supernatants could be absorbed by an antibody to IL-1 coupled to Sepharose. Elaboration of IL-1 activity was dependent on the dose of lipopolysaccharide and occurred in a time-dependent manner. Addition of cycloheximide blocked generation of IL-1 activity. A physiological vessel wall perturbant, the coagulation enzyme thrombin, induced comparable amounts of IL-1 activity in endothelial cell cultures. This effect was specific for the enzyme, since active site-blocked thrombin and prothrombin had no effect on IL-1. In addition, IL-1-containing supernatants from thrombin-stimulated endothelial cells induced tissue factor procoagulant activity in fresh endothelial cell cultures. Thus, in contrast to the multiple, known inhibitory mechanisms that block thrombin procoagulant activity, these data suggest a circle of interaction in which thrombin induces endothelial cell elaboration of IL-1, a mediator of endothelial cell procoagulant activity. Endothelial cell production of IL-1 in response to perturbation allows these cells to play an integral role in the regulation of the inflammatory and coagulation systems.

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