Effect of Fibrin on Endothelial Cell Production of Prostacyclin and Tissue Plasminogen Activator

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Fibrin formed on endothelial cells has previously been shown to have deleterious effects on the cells. Additionally, substances that cause endothelial cell damage have been reported to induce cultured endothelial cells to synthesize prostacyclin and tissue plasminogen activator (t-PA). The present studies were undertaken to determine whether fibrin formed on cultured human umbilical vein endothelial cells would alter synthesis of prostacyclin and t-PA by the cells. Fibrin was found to increase synthesis of both prostacyclin and t-PA in a dose and time dependent manner. Stimulation of prostacyclin synthesis was completely inhibited by indomethacin; partially inhibited by actinomycin D, cycloheximide, and trifluoperazine; and not affected by cytochalasin D or vinblastine. In contrast, stimulation of t-PA synthesis was completely inhibited by actinomycin D and cycloheximide; partially inhibited by cytochalasin D, vinblastine, and trifluoperazine; and not affected by indomethacin. Fibrin I, formed with Reptilase, caused only slight stimulation of t-PA production, but virtually no stimulation of prostacyclin synthesis. Neither collagen polymerization on the cells nor thrombin added in concentrations that did not induce fibrin polymer formation stimulated production of either substance. Furthermore, soluble fibrin II generated in the presence of the fibrin polymerization inhibitor gly-pro-arg-pro also failed to stimulate either prostacyclin or t-PA production. The presence of platelets in the plasma from which the fibrin was formed did not affect the amount of stimulation of the cells. Fibrin-induced stimulation of endothelial cell production of prostacyclin and t-PA could act to limit vascular occlusion in vivo by inhibiting platelet function and by stimulating fibrinolysis via t-PA.

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Previous studies have demonstrated that fibrin formed on the surface of endothelial cells has deleterious effects on the cells. Fibrin has been reported to induce rapid disorganization of the endothelial cell monolayer and to induce migration of the cells. Although others reported that migration of cultured endothelial cells was inhibited by contact with fibrin, fibrin was reported to induce porcine aortic endothelial cells to become spindle-shaped and to pile up. In that study, fibrinogen, fragment D, and fragment E had no effects on the morphology of the cells, but low molecular weight fibrinogen degradation products did induce cell damage, as assessed by both morphology and 51Cr release. Fibrin was also reported to increase the level of pinocytosis in cultured bovine aortic endothelial cells and to increase the rate of DNA synthesis as reflected by 3H-thymidine incorporation. By day 4 or 5 of incubation of the cell monolayer with fibrin, the fibrin was dissolved, and pinocytosis and DNA synthesis returned to the control level. Fibrin also stimulated the proliferation of cultured rabbit aortic smooth muscle cells, and fibrin degradation products inhibited proliferation.

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Recent studies demonstrated that short-term incubation of human umbilical vein endothelial cells with fibrin led to increased release of von Willebrand factor. In contrast, intact fibrinogen induces adhesion, spreading, and microfilament organization of human endothelial cells in culture.

Endothelial cells are known to produce a number of substances in addition to von Willebrand factor that are important in the regulation of the hemostatic system. Among these are prostacyclin and tissue plasminogen activator (t-PA). Previous studies by Nawroth et al., demonstrated that perturbation of bovine aortic endothelial cells with endotoxin or phorbol ester (12-O-tetradecanoylphorbol 13-acetate) induces production of prostacyclin. Several hours were required before an effect on prostacyclin production was evident, in contrast to the rapid stimulation of prostacyclin production by substances such as thrombin, the divalent cation ionophore A23187, bradykinin, or arachidonic acid, implying a different mechanism of action. The stimulation of prostacyclin production by phorbol ester and endotoxin was inhibited by indomethacin and also by cycloheximide, actinomycin D, cytochalasin B and D, vinblastine, colchicine, and trifluoperazine, indicating that protein and RNA synthesis and an intact cytoskeleton and calmodulin system were necessary for stimulation. In addition to stimulating prostacyclin production by the cells, phorbol ester and endotoxin induced detachment of cells and loss of 51Cr and disruption of the monolayer. Studies from other laboratories have demonstrated that release of t-PA by cultured
human umbilical vein endothelial cells is stimulated by thrombin, dibutyryl cyclic AMP, platelet activating factor, and also by endotoxin. The reports of morphologic alterations and biochemical changes induced in cultured endothelial cells by fibrin, together with the previous studies on stimulation of prostacyclin production by agents that affect cell morphology, led us to hypothesize that fibrin might affect endothelial cell synthesis of prostacyclin. The t-PA production was examined as well, since stimulation of production of this enzyme by fibrin would provide a mechanism for maintaining vascular patency in the face of a stimulus to fibrin formation.

Reagents

Medium 199 was obtained from Gibco, Grand Island, NY. Collagenase was purchased from Worthington Biochemicals, Freehold, NJ. Gelatin was obtained from BBL, Cockeysville, MD. Heparin, indomethacin, cycloheximide, actinomycin D, cytochalasin D, and vinblastine were purchased from Sigma Chemical Company, St. Louis, MO. Trifluoperazine was obtained from Smith Kline & French, Philadelphia, PA. Equine tendon collagen was purchased from Hormonchemie, Munich, West Germany. Endothelial cell growth supplement was obtained from Collaborative Research, Bedford, MA. Human serum was purchased from the New York Blood Center, New York, NY. Fetal bovine serum was obtained from Hyclone, Logan, UT. Reptilase was obtained from Wellcome Diagnostics, Dartford, England. Human thrombin was obtained from John Fenton (specific activity 2523 units/mg). Reptilase was purchased from Diagnostica Stago, Asnières, France. Antibody to prostacyclin was a generous gift of J. Bryan Smith, Philadelphia, PA. 1H-6-keto-prostaglandin-F_1α (6-keto-PGF_1α) was purchased from New England Nuclear, Boston, MA. A kit for enzyme-linked immunosorbent assay (ELISA) assay of t-PA antigen was purchased from American Diagnostica, Greenwich, CT. The peptide gly-pro-arg-pro was synthesized by George Winer of the Jewish Hospital, St. Louis, MO.

Endothelial Cell Culture

Human umbilical vein endothelial cells were cultured as described previously, by using the methods of Jaffe and colleagues. Heparin and endothelial cell growth supplement were added to the culture medium as described by Thornton and colleagues. Cells were routinely grown in the presence of 15% serum.

Fibrin Formation on Endothelial Cells

Cells were grown to confluence in 35 mm dishes or 96-well plates. The medium was removed and replaced with fresh citrated plasma (platelet-poor unless noted otherwise), serum, serum-free medium, or some combination of these; fibrin formation was initiated by the addition of CaCl_2 to a final Ca^{2+} concentration of 120 mM. At the end of the incubation period for each experiment (20 hours unless otherwise stated), the supernatant fluid was aspirated from the dishes and stored frozen until assay. Fibrin I was formed on cells by addition of Reptilase to the plasma at a dilution of 1/100 after reconstitution according to the package insert and incubation for 20 hours. In one set of experiments, purified human thrombin was added to the cells with or without Ca^{2+}. In another set of experiments, acid-soluble equine tendon collagen was layered over cells in platelet-poor plasma and the cells were incubated for 20 hours.

Effect of Inhibitors on Stimulation of Prostacyclin and t-PA Synthesis

Inhibitors were added to the plasma before fibrin formation. The inhibitors used included actinomycin D (250 nM), cycloheximide D (680 nM), cytochalasin D (100 nM), vinblastine (2.2 mM), trifluoperazine (2.5 mM), and indomethacin (200 nM). The fibrin polymerization inhibitor gly-pro-arg-pro was used at a final concentration of 40 mg/ml. This concentration was required to prevent polymerization throughout the 20-hour incubation period.

Assay of 6-Keto-Prostaglandin-F_1α and t-PA

As previously described, 6-keto-prostaglandin-F_1α was measured in the culture supernatants by radioimmunoassay. The t-PA was measured by using the ELISA kit according to the manufacturer's directions.

Results

The first experiment shown in Figure 1 demonstrated that neither citrated plasma nor freshly prepared human serum caused any increase in concentration of 6-keto-PGF_1α in the culture supernatant with time, whereas fibrin that formed on the cell monolayer by addition of CaCl_2 to allow thrombin generation led to substantial production of this prostaglandin product. In the next experiments, different dilutions of citrated normal plasma (diluted with normal serum from the same donor) were added to confluent human umbilical vein
endothelial cells. CaCl₂ was added to a final concentration of 120 mM in the plasma-serum mixture, and the dishes were incubated for 18 hours. Figure 2 shows the concentrations of 6-keto-PGF₁α and t-PA in the culture supernatants as a function of milligrams of fibrin per 35 mm dish. The concentration of 6-keto-PGF₁α was maximal from 0.25 to 1.25 mg fibrin per dish in the experiment shown, whereas the t-PA concentration in the supernatant increased with increasing amounts of fibrin to the highest amount tested (5.0 mg per dish). Although the amounts of fibrin that led to maximal stimulation and to the subsequent decrease in stimulation differed in different experiments, the general shape of the curve was consistent. Similarly, the concentration at which t-PA synthesis began to be seen also varied.

The time course of appearance of 6-keto-PGF₁α and t-PA in the culture supernatant was then examined at two different fibrin concentrations (representing different points on the dose response curve), as shown in Figure 3. At both fibrin concentrations shown, there was a slight increase in concentration of both 6-keto-PGF₁α and t-PA in the supernatant over the first hour, with the major increase occurring between 1 and 6 hours or between 6 and 20 hours.

The effects of inhibitors on the appearance of 6-keto-PGF₁α and t-PA in the culture supernatant are shown in Figure 4. Panel A shows the effects of addition of inhibitors on appearance of 6-keto-PGF₁α. Both the RNA synthesis inhibitor, actinomycin D, and the protein synthesis inhibitor, cycloheximide, inhibited prostacyclin production by approximately 50%. When these inhibitors were used at one-half or one-fourth the concentration used in these experiments (not shown), there was still partial inhibition of prostacyclin production and complete inhibition of t-PA production. As expected, the cyclooxygenase inhibitor indomethacin caused nearly complete inhibition of prostacyclin production. Neither cytochalasin D (which interacts with actin) nor vinblastine (which interferes with microtubule function) inhibited production of prostacyclin. Slight inhibition was caused by trifluoperazine (an inhibitor of calcium-dependent reactions involving calmodulin, protein kinase C, and calcium-dependent protease, and of phospholipase A₂). Panel B shows the effects of these same inhibitors on the appearance of t-PA in the culture supernatant. In this case, both actinomycin D and cycloheximide caused complete inhibition, whereas indomethacin caused no inhibition. Inhibition of 60%, 80%, and 40% occurred with the addition of cytochalasin D, vinblastine, and trifluoperazine, respectively.

Fibrin formed by addition of Replase to the citrated plasma in the tissue culture dishes caused only slight stimulation of t-PA production during 20 hours incubation (Figure 5). There was an increase in t-PA concentration with both Replase and thrombin, but it was substantially greater with the Ca²⁺ (thrombin)-induced clot than with the Replase clot. Replase clots had no effect on prostacyclin production by the cells (not shown).

To further demonstrate that the stimulatory effect of fibrin was not simply due to the presence of a polymer on the surface of the cells, equine tendon collagen was layered over the cells at two different concentrations (Figure 6). At the lower concentration (10 µg/ml) no collagen fibers were grossly visible, and there was no difference in either the 6-keto-PGF₁α or the t-PA concentration as compared with the control. When the higher collagen concentration was used (100 µg/ml), collagen fibers were visible, but concentrations of both 6-keto-PGF₁α were less than the control.

Two additional sets of experiments were performed to rule out the possibility that the stimulation seen with fibrin was due to the thrombin that was generated in the dishes rather than to the fibrin. In the first, thrombin was added to dishes at different concentrations (0.01 to 1.0 units/ml),
but no stimulation of either 6-keto-PGF\textsubscript{1α} or t-PA was seen with thrombin alone. Only when Ca\textsuperscript{2+} was added along with thrombin, so that a fibrin clot formed on the cells was there significant stimulation of the cells as shown in Figure 7 for 1.0 U/ml thrombin.

Another approach to the question of whether thrombin might be responsible for the stimulation seen with the fibrin clots was to recalcify plasma in the presence of the tetrapeptide gly-pro-arg-pro, an analogue of the aminoterminal of the A\textsubscript{2} chain of the fibrin monomer, which inhibits fibrin polymerization by binding to the site complementary to the A\textsubscript{2} chain polymerization site and thus disrupting the interactions between these complementary binding sites.\textsuperscript{24} This peptide does not affect thrombin action on fibrinogen, so the fibrinopeptides are cleaved but the fibrin cannot polymerize. As shown in Figure 8, stimulation of both prostacyclin and t-PA synthesis was prevented by addition of gly-pro-arg-pro to the plasma before recalcification.

The final set of experiments compared the effects of stimulation with platelet-rich plasma with those of stimulation with platelet-poor plasma. The presence of platelets had no effect on the concentration of either 6-keto-PGF\textsubscript{1α} or t-PA in the culture supernatant (data not shown).

**Discussion**

The studies reported in this paper have shown that fibrin can have significant effects on endothelial cell function. Fibrin formed on the surface of cultured human umbilical vein endothelial cells led to increased concentrations of both 6-keto-PGF\textsubscript{1α} and t-PA in the culture supernatant over time. Stimulation of prostacyclin synthe-
previous studies were in serum-free medium rather than in plasma. The presence of thrombin inhibitors in plasma, e.g., antithrombin III and heparin cofactor II, is well-known, and these inhibitors may have decreased the effective concentration of thrombin.

The amount of stimulation of t-PA synthesis could have been underestimated in these studies since t-PA is known to bind to both fibrin\textsuperscript{25,26,27} and to endothelial cells.\textsuperscript{27} Whether the amounts of t-PA bound to either fibrin or cells in these studies was significant in relation to the amounts measured in the culture supernatants is not known.

The inhibitors studied had different effects on production of prostacyclin and t-PA. At concentrations of actinomycin D and cycloheximide that totally blocked the increased synthesis of t-PA, these inhibitors caused approximately 50% inhibition of stimulation of prostacyclin synthesis. The partial inhibition of stimulation of prostacyclin synthesis suggests that new synthesis of a protein is required for full stimulation of synthesis of this prostaglandin; possible proteins that might be involved include the phospholipase responsible for liberating arachidonic acid from cellular phospholipids, cyclooxygenase, and prostacyclin synthetase. It is unlikely that the inhibition of prostacyclin synthesis was due simply to the nonspecific toxic effects of cycloheximide or actinomycin D, since the relationship between inhibition of prostacyclin production and t-PA production remained constant over a fourfold range of inhibitor concentrations. Since t-PA is a protein, it was anticipated that stimulation of its synthesis would be completely blocked by protein and RNA synthesis inhibitors. It was anticipated that indomethacin, a cyclooxygenase inhibitor, would completely block synthesis of prostacyclin and have no effect on synthesis of t-PA. These were the results obtained.

In contrast to the findings of Nawroth et al.\textsuperscript{8} using endotoxin and phorbol ester to stimulate prostacyclin synthesis by bovine aortic endothelial cells, cytochalasin D and vinblastine, which interfere with cytoskeletal function, had no inhibitory effects on the stimulation of human umbilical vein endothelial cell prostacyclin synthesis by fibrin. These differences may be related to differences in stimulus, in cell species, or both. These agents did significantly inhibit the increase in t-PA synthesis in human umbilical vein endothelial cells exposed to fibrin, analogous to their effects on tissue factor production described by others.\textsuperscript{26,29,31} Tifluoperazine caused only about 20% inhibition of prostacyclin synthesis and 30% inhibition of t-PA synthesis in these studies, which is less than the inhibition of prostacyclin or tissue factor synthesis seen with a similar concentration of tifluoperazine in bovine aortic endothelial cells stimulated with endotoxin or phorbol ester.\textsuperscript{6,30} Which of the different effects of tifluoperazine on Ca\textsuperscript{2+}-related cell functions or on phospholipase A\textsubscript{2} activity was responsible for the inhibition of prostacyclin and t-PA synthesis is not known.

Another finding of interest was the fact that fibrin formed by addition of Reptilase to citrated plasma had much less effect on cell function than did fibrin formed by generation of thrombin. There was no stimulation of prostacyclin production and minimal stimulation of t-PA production. This difference may be attributable to the fact
that Reptilase induces formation of fibrin I, with removal only of fibrinopeptide A, whereas thrombin induces formation of fibrin II, with cleavage of both fibrinopeptides A and B. Fibrin II is a better substrate for Factor XIII-induced crosslinking, and Factor XIII would have been present in the citrated plasma used to form the fibrin. It is possible that crosslinking of the fibrin on the cells would increase its potency as a stimulator of the cells.

The data with gly-pro-arg-pro, together with the Reptilase data, demonstrate that fibrin II polymer is required for stimulation, since soluble fibrin II did not affect the cells. This experiment also provides strong evidence that the stimulation seen with fibrin cannot be attributed to thrombin, since there would be no difference in thrombin generation in the presence or absence of gly-pro-arg-pro. Similarly, it is unlikely that any other component of plasma was responsible for the stimulation, since other plasma proteins would not be expected to be affected by this peptide, except insofar as they would not be able to be incorporated into a fibrin clot when fibrin polymerization was inhibited.

The effect of fibrin on the cells did not appear to be a nonspecific effect of a polymer since collagen filaments formed on the cells did not stimulate either prostacyclin or t-PA synthesis.

The finding that platelet-rich plasma was no different from platelet-poor plasma in the degree of stimulation of either prostacyclin or t-PA production suggests that platelet secretory products do not influence human endothelial cell production of either of these substances, since secretion of granular substances would be expected during the process of fibrin formation. These studies suggest that if fibrin were formed on the surface of endothelial cells in vivo, it would induce an increase in prostacyclin synthesis by the cells, thus inhibiting platelet function in the vicinity of the fibrin and preventing platelets from contributing to further thrombin generation and fibrin formation. Increased synthesis of t-PA, if the t-PA were all active, would lead to dissolution of the fibrin on the endothelium. If, on the other hand, synthesis of the t-PA inhibitor were increased along with synthesis of t-PA, then there might be no increase in t-PA activity or even a decrease in activity, depending on the balance between stimulation of enzyme and inhibitor. Further studies will be required to resolve this issue, but earlier work discussed above suggests that the balance will favor fibrin dissolution.

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