Activation of Coagulation Releases Endothelial Cell Mitogens

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Abstract. Recent studies have indicated that endothelial cell function includes elaboration of growth factors and regulation of coagulation. In this paper we demonstrate that activated coagulation Factor X (Factor Xa), a product of the coagulation mechanism generated before thrombin, induces enhanced release of endothelial cell mitogens, linking these two functions. Mitogenic activity generated by cultured bovine aortic endothelial cells in response to Factor Xa included platelet-derived growth-factor-like molecules based on a radioreceptor assay. Effective induction of mitogens by Factor Xa required the integrity of the enzyme's active center and the presence of the γ-carboxyglutamic acid–containing domain of the molecule. Factor Xa-induced release of mitogens from endothelium occurred in serum-free medium and was not altered by hirudin or antibody to Factor V, indicating that it was a direct effect of Factor Xa and was not mediated by thrombin. Elaboration of mitogenic activity required only brief contact between Factor Xa and endothelium, and occurred in a time-dependent manner. Generation of enhanced mitogenic activity in response to Factor Xa was unaffected by the presence of actinomycin D and was not associated with increased hybridization of RNA from treated cells to a v-sis probe. Release of mitogenic activity was dependent on the dose of Factor Xa, being half-maximal at 0.5 nM and reaching a maximum by 5 nM. Radioligand binding studies demonstrated a class of endothelial cell sites half-maximally occupied at a Factor Xa concentration of 0.8 nM. The close correspondence between the parameters of Factor Xa-induced mitogen release and Factor Xa binding suggests these sites may be related. When Factor X was activated on the endothelial cell surface by Factors IXa and VIII, the Factor Xa formed resulted in the induction of enhanced release of mitogenic activity. These data suggest a mechanism by which the coagulation system can locally regulate endothelial cell function and vessel wall biology before thrombin-induced release of growth factors from platelets.

TRADITIONALLY, the response to vascular injury is considered to begin after the endothelium has been denuded. Exposure of subendothelium to hemostatic components would then lead to platelet deposition, which is often assumed to result in fibrin deposition (34). Furthermore, contact of cells in subendothelial layers of the vessel wall with growth factors would initiate smooth muscle proliferation (1, 5, 20, 30, 31, 39, 48, 62). However, as the cells forming the luminal vascular surface, endothelial cells are strategically located to function in the regulation of coagulation and modulation of mitogenic signals transmitted to the vessel wall. Although endothelial cell retraction and denudation may play a role in the genesis of vascular lesions (18, 19), changes in the vessel wall leading to atherosclerotic plaque formation may occur under a morphologically intact endothelium. Studies of small vessel wall injuries have shown that endothelium covers the denuded areas rapidly, perhaps maintaining continuity of the monolayer after moderate cell loss (56, 64, 65). These observations suggest an alternative view of vessel wall injury in which endothelial cell function and dysfunction are central in the pathogenesis of lesion formation.

Recent studies have indicated that endothelial cell function includes elaboration of growth factors and regulation of coagulation (11, 14–16, 21, 27, 43, 51, 60, 61, 73, 80, 83). Endothelial cells have been shown to produce growth factors, including platelet-derived growth factor (PDGF)[1]-like protein, raising the possibility that mitogens of endothelial origin could result in proliferation of vascular smooth muscle and fibroblasts (14–16, 21, 27, 80, 83). Induction of endothelial cell dysfunction by endotoxin or phorbol esters enhances release of growth factor activity (21). Endothelium has also been shown to participate in both anticoagulant and procoagulant reactions through the presence of specific binding sites that modulate coagulant activity on the cell surface (51, 61). Although anticoagulant mechanisms predominate on quiescent endothelial cells, induction of endothelial dysfunction by perturbants can shift this balance toward promotion of localized clot formation (3, 11, 43). Thus, endothelial

1. *Abbreviations used in this paper*: Factors IXa and Xa, the activated forms of Factors IX and X; Gla-domainless Factor X, Factor X modified by cleavage of the γ-carboxyglutamic acid–containing domain; PDGF, platelet-derived growth factor; PDGFc, platelet-derived growth factor–like molecules.

cell dysfunction could potentially lead to localized coagulation and growth factor release.

A link between atherogenesis and coagulation has long been suspected from clinicopathological studies. Accumulation of fibrin in atherosclerotic lesions is well known (4, 54, 68–70, 84), and a recent study indicates that an increased ratio of fibrin II to fibrinogen correlates with more advanced lesions (4). Furthermore, thrombin, the final coagulation enzyme in the pathway leading to fibrin formation (13), has been shown to induce release of PDGF-like growth factor activity from endothelial cells (32). Thrombin, however, also activates platelets (76), causing release of their intracellular stores of PDGF (82). These findings prompted us to consider the hypothesis that products of the activated coagulation system, before the formation of thrombin, could induce localized perturbation of the endothelium resulting in enhanced generation of growth factor activity. If this were true, then perturbed endothelium could play a central role in the transmission of mitogenic signals reaching subendothelial layers of the vessel wall even before fibrin deposition occurs. In addition, since the coagulation mechanism is sensitive to a wide range of stimuli and is in constant contact with endothelium, activated clotting enzymes are well suited to modulate endothelial cell function.

These considerations have led us to study products of the activated coagulation system that influence the production of growth factors by endothelial cells. The results indicate that cultured bovine aortic endothelial cells exposed to the activated form of coagulation Factor X (Factor Xa) elaborate enhanced amounts of PDGF-like activity. Enhanced release of PDGF-like activity occurred in response to relatively low concentrations of Factor Xa (half-maximal response at 0.5 nM) and after only brief exposure of the cells to the clotting enzyme, suggesting that this coagulation factor–endothelial cell interaction might occur under physiological conditions. Furthermore, activation of Factor X on the endothelial cell surface resulted in release of PDGF-like activity linking cell surface coagulant events and generation of mitogens. These data suggest a mechanism by which vessel wall biology and endothelial cell function can be locally regulated by the coagulation system before thrombin-induced release of PDGF from platelets.

Materials and Methods

Cell Culture

Primary cultures of bovine aortic endothelial cells were isolated by previously described methods (28) and grown in 10% bovine adult serum. Serum was heat-inactivated for 30 min at 56°C. Endothelial cells were subcultured in Waymouth's Medium (Gibco, Grand Island, NY) supplemented with sodium bicarbonate, nonessential amino acids, sodium pyruvate, and 10% serum as described by Schwartz (63). Endothelium was passaged in 75-cm^2 flasks (Corning Glass Works, Corning Science Products, Corning, NY) with 0.05% trypsin at a split ratio of 1:3 and used for experiments from passages 5–15. Three separate cell isolates gave comparable results. Swiss 3T3 cells were routinely subcultured every 3–4 d in Dulbecco's modified Eagle's media containing 10% bovine serum (15). Human dermal fibroblasts were derived from explants and maintained in Waymouth's medium containing 10% bovine whole blood serum.

Endothelial cells derived from human umbilical cord veins were prepared according to the method of Jaffe et al. (33). Human endothelial cell cultures contained no monocyte/macrophage-contaminating cells, as judged by morphologic criteria and by cytofluorometry using OKM2, a monoclonal antibody reactive with most human monocytes/macrophages (8).

Preparation of Conditioned Medium and Assays

Confluent cultures of endothelial cells in 35-mm Petri dishes (Falcon Labware, Oxnard, CA) were washed once with phosphate-buffered saline (PBS) (pH 7.4) (Gibco) and then placed in fresh serum-free Waymouth's medium containing 0.1% bovine serum albumin (BSA; RIA grade; Sigma Chemical Co., St. Louis, MO), 1.8 mM $CaCl_2$, and 25 mM Hepes (pH 7.4). Coagulation factors were added to dishes for the indicated times. Conditioned medium was centrifuged (4,000 *g*) for 10 min to remove debris and stored at 4°C before assay (within 5 d). Radioreceptor and mitogenic assays were carried out as described below using purified human PDGF (55) as a standard.

A radioreceptor assay measuring competition between PDGF-like molecules (PDGFc) in endothelial cell supernatants and ^{125}I-PDGF for binding to PDGF receptors was carried out as described by Bowen-Pope and Ross (7). Subconfluent human diploid fibroblasts were used 2–10 d after plating (1.5×10^4 cells/cm^2) in 2.1-cm^2 wells (Costar, Data Packaging Corp., Cambridge, MA) containing 1 ml of medium with 1% plasma-derived serum (79). The cells were cooled to 4°C on an ice tray and washed with 0.5 ml ice-cold binding medium (Ham's F-12 medium without bicarbonate containing 25 mM Hepes buffer [pH 7.2] and BSA [2 mg/ml]). The medium was replaced with an aliquot of the endothelial cell–conditioned medium plus binding medium in a total volume of 1.0 ml. The cultures were incubated at 4°C with rapid mixing for 4 h. After aspiration of the test substance and rinsing with binding medium, 0.5 ng ^{125}I-PDGF [radioiodination of PDGF was carried out as described [7]) was added in 0.5 ml binding medium, and the incubation was continued at 4°C for an additional hour. The medium was then aspirated and the cells washed three times with 1 ml of PBS (4°C) containing 1 mg/ml BSA. Bound radioactivity was determined by solubilizing the cells with 1% Triton X-100 containing BSA (1 mg/ml). Nonspecific binding, measured in the presence of at least a 100-fold excess of unlabeled PDGF, was <12% of specific binding. Standard curves, derived from wells containing purified unlabeled PDGF, were used for quantitation of PDGF-like protein. Standard deviations were generally <15%.

The mitogenic activity of endothelial cell–conditioned medium was determined by the incorporation of [^{3}H]thymidine into TCA-precipitable material by confluent cultures of 3T3 cells (15). All samples were tested at several dilutions in triplicate; standard deviations were <10% within an assay. The results of different assays carried out on different endothelial cell lines at different passages varied from 10–30%. Units of activity were determined for each assay from a standard curve using purified PDGF. One unit of mitogenic activity was defined as the [^{3}H]thymidine incorporation stimulated by 0.2 ng of PDGF compared with a control consisting of medium containing 1% plasma-derived serum (26). Stimulation of [^{3}H]thymidine incorporation (27) under these conditions directly correlates with cellular proliferation. Where indicated, the IgG fraction of goat monospecific antibody to human PDGF (6) was included in assay mixtures at a concentration sufficient to inhibit 10 ng/ml PDGF. This antibody has been previously shown to neutralize the mitogenic effect of human PDGF (6).

Endothelial cell RNA synthesis was determined by the incorporation of [^{3}H]uridine into TCA-precipitable material by endothelial cell monolayers. Monolayers in 35-mm dishes were incubated with 1.0 ml of [^{3}H]uridine (3.5 μCi; 25 Ci/mmol) in serum-free medium for 1 h at 37°C. After removing the medium, cultures were rinsed four times with 2 ml of cold 10% TCA. The precipitated material was solubilized using 0.5 M sodium hydroxide and radioactivity determined in a liquid scintillation counter. Standard deviations in triplicate determinations were <10%.

Hybridization of endothelial cell RNA with the v-sis cDNA probe was carried out by a modification of the method of Chirgwin et al. (10). In brief, endothelial cell RNA was extracted from a pellet of 5×10^6 cells using 5 M guanidinium isothiocyanate. RNA was sequentially precipitated in 4 M lithium chloride/10 mM Tris (pH 7.6) at 4°C (9), dissolved in 0.1% SDS/10 mM Tris (pH 7.6)/1 mM EDTA, phenol/chloroform extracted and precipitated in 100 mM NaCl/ethanol (1:2, vol/vol) at −70°C (45). RNA was then heated for 15 min at 60°C in 3.0 M NaCl/0.3 M sodium citrate/formaldehyde, and spotted onto nitrocellulose paper previously equilibrated with the same buffer. Hybridization was carried out for 18 h to a ^{32}P-labeled nick-translated v-sis cDNA probe (57) as described (17), washed, and exposed to film. The v-sis probe was generously provided by Dr. R. Gallo (National Institutes of Health, Bethesda, MD) and rat skin fibroblasts, which do not express v-sis hybridizable RNA, by Dr. R. Meek (University of Washington, Seattle, WA).

Preparation of Coagulation Factors

Bovine coagulation proteins were used throughout. Factor Xa, purified to

homogeneity by the method of Fujikawa et al. (25), was activated by incubation with the Factor X activator from Russell's viper venom (35) coupled to CNBr-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as described previously (70). Activation was complete by 10 min as judged by coagulant assay (100 U/mg) performed by the method of Bajaj and Mann (2) and by SDS PAGE, which showed complete cleavage of the zymogen (24). Factor Xa was inactivated by either antithrombin III (37), diisopropylfluorophosphate, or (*p*-amidinophenyl) methanesulfonyl fluoride (38), as follows. Incubation of Factor Xa (50 nM) with antithrombin III (1 μM) for 1 h at 37°C resulted in complete loss of Factor Xa coagulant activity. Antithrombin III was purified to homogeneity according to the method of Mahoney et al. (44), and the final product exhibited an inhibitory activity of 1.0 U thrombin/mg. A 10-fold molar excess of (*p*-amidinophenyl)-methanesulfonyl fluoride (Calbiochem-Behring Corp., La Jolla, CA) inactivated Factor Xa after 60 min at 37°C as described (38). Diisopropylfluorophosphate (2 mM) blocked Factor Xa (1 μM) coagulant activity after 5 min at 37°C. Unreacted (*p*-amidinophenyl) methanesulfonyl fluoride and diisopropylfluorophosphate were removed by dialysis. Factor X was radioiodinated by the solid-state lactoperoxidase method (12) using the Enzymobead Reagent (Bio-Rad Laboratories, Richmond, CA), as described previously (72). ^{125}I-Factor X was then activated as described previously for unlabeled Factor X. The iodination procedure did not affect Factor Xa coagulant activity and the final product had a specific radioactivity of 0.8–1.6 × 10^4 cpm/ng. Preparations of ^{125}I-Factor Xa were used immediately for binding studies.

Factor X modified by cleavage of the γ-carboxyglutamic acid–containing domain (the amino-terminal [1–44] peptide from the light chain) (Gla-domainless Factor X) was prepared by reacting Factor X with α-chymotrypsin (400:1, wt/wt) until <0.1% of the original coagulant activity remained as described (49, 65). Gla-domainless Factor X was then purified on QAE-Sephadex (Pharmacia Fine Chemicals) (47, 65) and activated as described for Factor X except that activation was carried out in the presence of 10 mM $CaCl_2$ and for 18 h. Gla-domainless Factor Xa and native Factor Xa were equally effective on a molar basis in the hydrolysis of Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (67) (see below), though Gla-domainless Factor Xa had considerably reduced coagulant activity as reported previously (67).

Factor IX was purified to homogeneity (250 U/mg) by the method of Fujikawa et al. (23), and was activated during incubation with Factor XIa bound to CNBr-Sepharose as described previously (72). Factor IXa (22.2 μM) was inactivated by incubation with dansyl-glu-gly-arg-chloromethylketone (600 μM) (Calbiochem-Behring Corp.) for 3 h as described by Lollar and Fass (40). Factor VIII, prepared by the method of Vehar and Davie (78), was 28-fold activatable by thrombin and had a specific coagulant activity of 4,500 U/mg. Prothrombin (13 U/mg) and α-thrombin (2.5 NIH U/μg) were prepared as described previously (42, 46). To remove any trace amounts of Factor Xa from the thrombin, thrombin preparations were chromatographed over an affigel 10 column with immobilized rabbit antibovine Factor X/Xa IgG. The pass-through fractions had thrombin concentrations of ~2 mg/ml and <5 ng/ml of Factor Xa (the limit of detection in a Factor X/Xa radioimmunoassay). These preparations were diluted appropriately and used in endothelial cell experiments. Thrombin (2.5 NIH U) was inactivated, as judged by coagulant assay, after a 1-h incubation with hirudin (6.0 U; Sigma Chemical Co.) (47).

Factor X Activation on, and Factor Xa Binding to, Endothelial Cells

Factor X activation over endothelial cell monolayers was studied by incubating confluent endothelial cells in 0.79-cm^2 well with Factors IXa (0.2 pmol/ml), VIII (1.5 U/ml), and X (300 pmol/ml) in incubation buffer (10 mM Hepes [pH 7.45] containing 137 mM NaCl, 4 mM KCl, 11 mM glucose, 2.5 mM $CaCl_2$, and 5 mg/ml BSA) (74). Where indicated, active site-blocked Factor IXa replaced native Factor IXa. At timed intervals, one aliquot (0.1 ml) was removed and added to 0.4 ml of 50 mM Tris (pH 7.9), 175 mM NaCl, 5 mM EDTA, and 0.5 mg/ml ovalbumin. Factor Xa formation was assessed using the chromogenic substrate Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (77) by adding the entire 0.5-ml sample along with 0.1 ml of substrate and measuring the change in absorbance at 405 nm. This assay was sensitive to 0.4–0.5 nM Factor Xa and the amount of Factor Xa formed was determined by comparison with a standard curve made with known amounts of Factor Xa.

Binding studies were carried out after washing confluent endothelial cells in 0.32-cm^2 wells twice with Hanks balanced salt solution. Incubation buffer was added along with ^{125}I-Factor Xa and other coagulation proteins as indicated to achieve a final volume of 0.1 ml. Binding assays were carried out for 90 min at 4°C. This amount of time was sufficient to allow maximal binding at the lowest concentrations of Factor Xa employed. Binding was terminated by five rapid washes (0.1 ml/wash) over 5 s with ice-cold incubation buffer and cells were solubilized with 0.2 N NaOH, 1% SDS, and 10 mM EDTA. Total binding was measured in wells incubated with ^{125}I-Factor Xa alone. Nonspecific binding was estimated using a 250-fold molar excess of unlabeled Factor Xa at each concentration of ^{125}I-Factor Xa. Additional amounts of unlabeled Factor Xa did not affect the results. Nonspecific binding was essentially linear with respect to radioligand concentration. Nonspecific binding was subtracted from total binding at each point in order to determine specific binding. Binding data were fit to the equilibrium binding equation of Klotz and Hunston (36), assuming a one-site model. A nonlinear least squares program (SAS Institute, Cary, NC) was used to obtain the best fit curve, to solve for the number of sites per cell and association constant, and to determine the standard error. A plot of residuals versus free radioligand concentration for the binding data shown in Fig. 5 indicated no systematic error was involved in fitting binding to the one-site model (data not shown).

Results

Previous studies have demonstrated that endothelial cells have specific binding sites for Factors IX, X, and their activated forms in addition to thrombin (41, 51). This led us to examine if incubation of confluent monolayers of bovine aortic endothelial cells with these coagulation proteins in serum-free medium would result in enhanced elaboration of mitogenic activity (Table I). The coagulation factors present normally in the plasma, Factors IX and X, and prothrombin (at their approximate plasma concentrations), did not induce generation of mitogenic activity by endothelial cells compared with controls. The activated form of Factor IX was also ineffective. Factor Xa, the central coagulation enzyme linking the intrinsic and extrinsic pathways of the clotting system (13), induced enhanced release of mitogenic activity. Factor Xa had no effect on endothelial cell viability as evidenced by lack of increased lactate dehydrogenase release during exposure of cells to the enzyme (data not shown). Furthermore, mitogenic activity shown in the figures was in all instances heat stable, since treatment of samples at 56°C for 30 min had no effect on the mitogenic activity measured. Because Factor Xa is a product of earlier stages of coagulation before thrombin formation occurs, we chose to characterize its effect on endothelial cell growth factor release. Thrombin also caused enhanced release of mitogenic activity as reported previously (32).

Table I. Effect of Coagulation Factors on Release of Growth Factor Activity from Endothelial Cells

Coagulation factor	Concentration	Mitogenic activity
	nM	*U/ml*
Factor IX	70	0.7
Factor IXa	40	1.0
Factor VIII	1 U/ml	0.7
Factor X	200	0.3
Factor Xa	4	4.5
Prothrombin	800	0.4
Thrombin	50	1.5
Thrombin	100	2.3
No addition	–	0.7

Confluent cultures of endothelial cells were incubated for 3 h at 37°C with the indicated coagulation factor and conditioned medium was assayed for mitogenic activity using 3T3 cells as described in Materials and Methods. Results shown represent the mean of triplicate determinations of units of mitogenic activity per milliliter. Standard deviations were <10%.

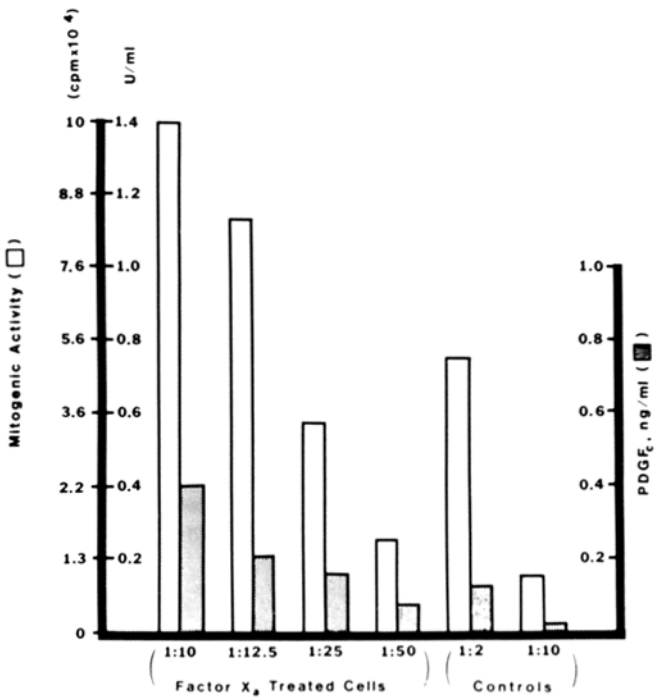

Figure 1. Factor Xa-induced release of mitogenic activity and PDGFc by endothelial cells. Confluent endothelial cell monolayers were incubated with serum-free medium in the presence or absence of Factor Xa (0.5 nM) for 16 h at 37°C. Serial dilutions of conditioned medium were then collected and tested in the [^{3}H]thymidine incorporation assay using 3T3 cells and the PDGF radioreceptor assay as described in Materials and Methods. Total [^{3}H]thymidine incorporation is shown on the left ordinate (demarcations on left side of axis) and is correlated with comparable units of mitogenic activity (demarcations on right side of axis). The concentration of PDGFc as determined from the radioreceptor assay is shown on the right ordinate. The dilution of serum-free medium from cells treated with Factor Xa or controls is shown on the abscissa. Values represent the mean of triplicate determinations. Standard deviations in the [^{3}H]thymidine incorporation and radioreceptor assays were <10 and 15%, respectively. Open bars, mitogenic activity; shaded bars; PDGFc concentration.

The mitogenic activity of conditioned medium from Factor Xa-treated endothelial cells was detectable up to a 1:50 dilution in the [^{3}H]thymidine assay using 3T3 cells (Fig. 1). Considerably lower amounts of mitogenic activity were released by untreated endothelial cells. Although this assay was sensitive for the detection of endothelial cell-derived mitogenic activity, to better characterize the molecular mitogen species in the conditioned medium, the PDGF-radioreceptor assay was carried out (Fig. 1). The results indicate that mitogenic activity released by Factor Xa-treated endothelial cells does contain molecular species that inhibit binding of purified ^{125}I-PDGF to its receptor. To further examine the contribution of PDGF and PDGF-like molecules to the mitogenic activity released by endothelium in response to Factor Xa, antiserum to PDGF was used. Because bovine PDGF has not been purified and our antiserum to human PDGF (6) does not neutralize bovine PDGF activity, it was necessary to use cultured human umbilical vein endothelial cells for these studies. When Factor Xa (0.5 nM) was incubated with cultured human endothelium (passage 2) for 14 h in serum-free medium, >90% of the mitogenic activity released could be neutralized by the IgG fraction of a monospecific goat anti-PDGF antiserum (a quantity of antiserum sufficient to neutralize 10 ng/ml of PDGF was added). These data suggest that the mitogenic species elaborated by endothelium in response to Factor Xa can be largely accounted for by PDGF or immunologically similar molecule(s).

Table II. Effect of Native Factor Xa and Modified Forms of Factor Xa on the Elaboration of Mitogenic Activity by Endothelial Cells

Coagulation factors added	Mitogenic activity
	U/ml
Factor Xa	6.75
Factor Xa–antithrombin III	0.9
DIP-Factor Xa*	1.5
pAPMS-Factor Xa	2.0
Gla-domainless Factor Xa	1.0
No addition	0.8

Confluent cultures of endothelial cells were incubated for 3 h with native or modified Factor Xa at a final enzyme concentration of 8 nM. Conditioned media were assayed for mitogenic activity using 3T3 cells as described in Materials and Methods. Results shown represent the mean of triplicate determinations. Standard deviations were <10%. Modified forms of Factor Xa were prepared as described in Materials and Methods. Antithrombin III alone had no inhibitory effect on the assay.
* DIP-Factor Xa, diisopropylfluorophosphate-treated Factor Xa; pAPMS-Factor Xa, (*p*-amidino-phenyl)-methanesulfonyl-Factor Xa.

To further study the effect of Factor Xa on release of endothelial cell mitogenic activity, modified forms of Factor Xa were studied (Table II). Native Factor Xa induced elaboration of mitogenic activity, but Factor Xa inactivated by antithrombin III was no longer effective. Since antithrombin III is a relatively large molecule (M_r ~58,000) (44) compared with Factor Xa (M_r 40,000) (24), the inhibitor could be masking multiple sites on the enzyme in addition to the active site. This led us to examine other forms of Factor Xa. Factor Xa inactivated at its active site by either diisopropylfluorophosphate or (para-amidinopheryl) methanesulfonyl fluoride was ineffective in causing release of endothelial cell mitogenic activity. Factor Xa can also be modified by removal of the amino terminal 44 residues from the light chain.

Table III. Effect of Hirudin and Antibody to Factor V on Elaboration of Mitogenic Activity by Endothelial Cells

Protein(s) added	Mitogenic activity
	U/ml
Factor Xa	6.3
Factor Xa + hirudin	6.3
Factor Xa + anti-Factor V IgG	6.0
Factor Xa + nonimmune IgG	5.9
anti-Factor V IgG	0.9
Nonimmune IgG	0.9
Hirudin	0.8
No addition	0.8

Confluent cultures of endothelial cells were incubated for 45 min with anti-Factor V or nonimmune IgG, and then Factor Xa alone or in the presence of hirudin was added for 3 h at 37°C. Conditioned medium was assayed for mitogenic activity using 3T3 cells as described in Materials and Methods. Results shown represent the mean of triplicates. Standard deviations were <10%. The final concentrations of reaction mixture components were: Factor Xa (4.4 nM), hirudin (0.5 U/ml), anti-Factor V IgG (200 μg/ml), and nonimmune IgG (300 μg/ml).

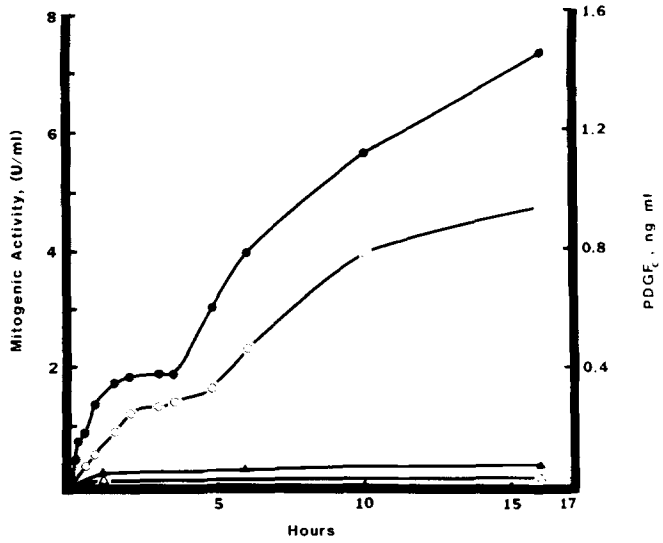

Figure 2. Time course of Factor Xa-induced release of mitogenic activity and PDGFc by endothelial cells. Confluent endothelial cells were incubated in serum-free medium with or without Factor Xa (0.5 nM) for the indicated times. Aliquots were withdrawn and tested for mitogenic activity in the [^{3}H]thymidine incorporation assay using 3T3 cells and for PDGFc in the radioreceptor assay, as described in Materials and Methods. Units of mitogenic activity are shown on the left ordinate and PDGFc is shown on the right ordinate. Symbols: supernatants from Factor Xa-treated endothelial cells assayed in the mitogenic (●) and radioreceptor (○) assays; supernatants from control endothelial cells assayed in the mitogenic (▲) and radioreceptor (△) assays. The mean of triplicate determinations is shown. Standard deviations in the [^{3}H]thymidine and radioreceptor assays were <10 and 15%, respectively.

This form of Factor Xa, Gla-domainless Factor Xa, does not interact effectively with phospholipid membrane surfaces or other coagulation factors due to lack of the γ-carboxyglutamic acid residues, though its intrinsic amidolytic activity for small substrates is unaltered (52, 67). Gla-domainless Factor Xa was not effective in the induction of endothelial cell mitogen release. Thus, for optimal release of endothelial cell mitogenic activity, both the integrity of the active site and the presence of the γ-carboxyglutamic acid–containing domain of the molecule are required. Factor Xa-induced release of mitogenic activity was not due to lipopolysaccharide in the preparation since addition of antithrombin III or treatment of the enzyme with diisopropylfluorophosphate blocked the enzyme's effect. Furthermore, treatment of lipopolysaccharide with antithrombin III or diisopropylfluorophosphate under these conditions did not affect endotoxin-induced mitogen elaboration (data not shown).

Since Factor Xa can interact with endothelial cell Factor V resulting in prothrombin activation (60, 72), it was necessary to determine if this sequence of events could account for Factor Xa-induced mitogen release (Table III). To minimize the presence of the substrate, prothrombin, all experiments were carried out under serum-free conditions after washing monolayers extensively. In previous studies, we have observed only low affinity and rapidly reversible binding of prothrombin to endothelium, which could be completely eluted under these conditions (Stern, D., and P. Nawroth, unpublished observation). Addition of the thrombin inhibitor hirudin to Factor Xa-endothelial cell incubation mixtures had no effect on mitogen elaboration. The presence of antibody to bovine Factor V at a concentration that blocks prothrombin activation on the endothelial cell surface by >90% (72) also had no effect on mitogen release in response to Factor Xa. Taken together, these findings suggest that Factor Xa-induced mitogen release is predominately due to a direct effect of Factor Xa on endothelium. Although the endothelial cell site mediating this interaction with Factor Xa is unclear, Factor V on the cell surface is probably not involved.

Endothelial cell mitogen elaboration induced by Factor Xa was dependent on the incubation time (Fig. 2). The time course of mitogen release appears to be biphasic with an initial period of release that reaches an apparent maximum by 3 h and a second phase of release evident by 4 h that continues to increase steadily. Since activated coagulation factors such as Factor Xa are rapidly inhibited under physiological conditions, release of mitogenic activity after incubation periods as short as 2 min was an important observation.

To investigate whether RNA synthesis was required for induction of mitogen release, actinomycin D (0.5 μg/ml) was added to cultures 3 h before the addition of Factor Xa (Table IV). This concentration of actinomycin D blocked total uridine incorporation by 90% and did not decrease endothelial cell viability until after 24 h of incubation. Total release of mitogenic activity after 2.5 and 24 h of exposure to Factor Xa was identical in the presence or absence of actinomycin D. Consistent with these results, cytoplasmic dot blot hybridization experiments (Fig. 3) showed no significant differences by densitometric scanning in endothelial cell RNA hybridizing with the v-sis probe whether cultures had been incubated with Factor Xa (5 nM; 16 h of incubation) or not. By this time, considerable mitogenic activity had been released (Fig. 2). These results suggest that Factor Xa-induced release of mitogenic activity was independent of new RNA synthesis and may reflect the presence of a preformed intracellular pool of mitogen. In this context, heat-treated lysates of frozen-thawed control endothelial cells did not contain significant mitogenic activity as previously reported (21, 29). Thus, Factor Xa may induce translational or post-translational processing of the molecules responsible for endothelial cell mitogenic activity. To distinguish between

Table IV. Effect of Actinomycin D on Factor Xa-induced Mitogen Release

	Incubation times			
	2.5 h		24 h	
Incubation conditions	Mitogenic activity	PDGFc	Mitogenic activity	PDGFc
	U/ml	*ng/ml*	*U/ml*	*ng/ml*
No addition	–	0.08	0.4	0.20
Actinomycin D	–	0.13	1.3	0.36
Factor Xa	2.6	0.36	10.0	2.0
Factor Xa + actinomycin D	2.8	0.44	10.5	1.85

Confluent endothelial cell monolayers were preincubated for 3 h with actinomycin D (0.5 μg/ml). Factor Xa (0.5 nM) was added next (time 0) and conditioned media were collected at 2.5 and 24 h. Conditioned media were dialyzed (to remove actinomycin D) against 1 mM ammonium bicarbonate, lyophilized, dissolved in a volume of media equal to the original sample volume, and assayed for mitogenic activity (using 3T3 cells) and PDGFc. Samples were assayed in triplicate. Standard deviations were <10%. The means are shown. Under these conditions, controls demonstrated that actinomycin D had no direct inhibitory affect on the assay.

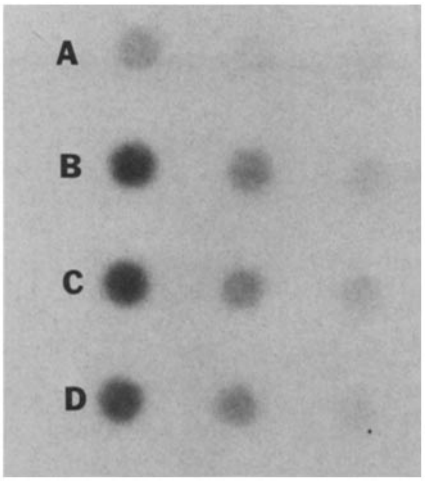

Figure 3. Hybridization of RNA to the v-sis probe from Factor Xa-treated and control endothelial cells. Confluent endothelial cell monolayers were incubated with or without Factor Xa, RNA was extracted, adsorbed to nitrocellulose paper, and hybridized with a ^{32}P-nick-translated v-sis probe as described in Materials and Methods. *A*, hybridization to RNA obtained from control rat skin fibroblasts. *B*, RNA from endothelial cells incubated in serum-free medium alone. *C* and *D*, from endothelial cells exposed for 16 h to Factor Xa, 22.2 and 4.4 nM, respectively. Samples from left to right are serial dilutions, containing 6.7, 1.7, and 0.4 μg of RNA, respectively.

these possibilities, experiments were carried out using cycloheximide. When cycloheximide was tested at 1 μg/ml, total protein synthesis was decreased by 80% and Factor Xa (5 nM)-induced mitogen release was still unaffected. Attempts to more completely block protein synthesis at higher cycloheximide concentrations were inconclusive due to poor endothelial cell viability at these levels of inhibitor. Studies at the posttranslational level using either paraformaldehyde- (1%), formaldehyde- (1%), or methanol- (50%) fixed endothelial cell monolayers indicated that Factor Xa released comparable amounts of mitogenic activity compared with untreated, viable controls (data not shown). Since the fixed cells showed no significant incorporation of radiolabeled uridine or leucine, Factor Xa-induced mitogen release appears to be independent of cellular biosynthetic processes. Factor Xa might act by directly cleaving a mitogen precursor already present in the cell or on the cell surface.

Elaboration of enhanced endothelial cell mitogenic activity was dependent on the concentration of Factor Xa added in a biphasic manner (Fig. 4). At lower levels of Factor Xa, release of mitogen increased from 0.8 to 2.7 U/ml, being maximal at a Factor Xa concentration of 5 nM (5.4 U/ml). Half-maximal release of mitogenic activity occurs at a Factor Xa concentration of ~0.5 nM. These levels of Factor Xa may be achieved under physiological conditions and are reached when Factor Xa is activated on the endothelial cell surface, as will be described below. As higher concentrations of Factor Xa are added to the endothelial cell (>11 nM), steadily increasing release of mitogenic activity was observed. Similar studies carried out with Factor X (Fig. 4) demonstrated that the zymogen was ineffective in the induction of mitogen release.

The release of mitogenic activity in response to Factor Xa appeared to saturate in the first phase of the dose-response curve (Fig. 4), suggesting that Factor Xa–endothelial cell interaction might be due to a limited number of binding sites. To better characterize the involvement of endothelium in this phenomenon, radioligand binding studies were carried out (Fig. 5). The binding of ^{125}I-Factor Xa to endothelial cells, studied at 4°C to prevent endocytosis, was saturable. Semilogarithmic plots of the binding data demonstrate half-maximal binding at a Factor Xa concentration of 0.8 ± 0.14 nM and at saturation of 2.1 ± 0.3 × 10^4 molecules bound

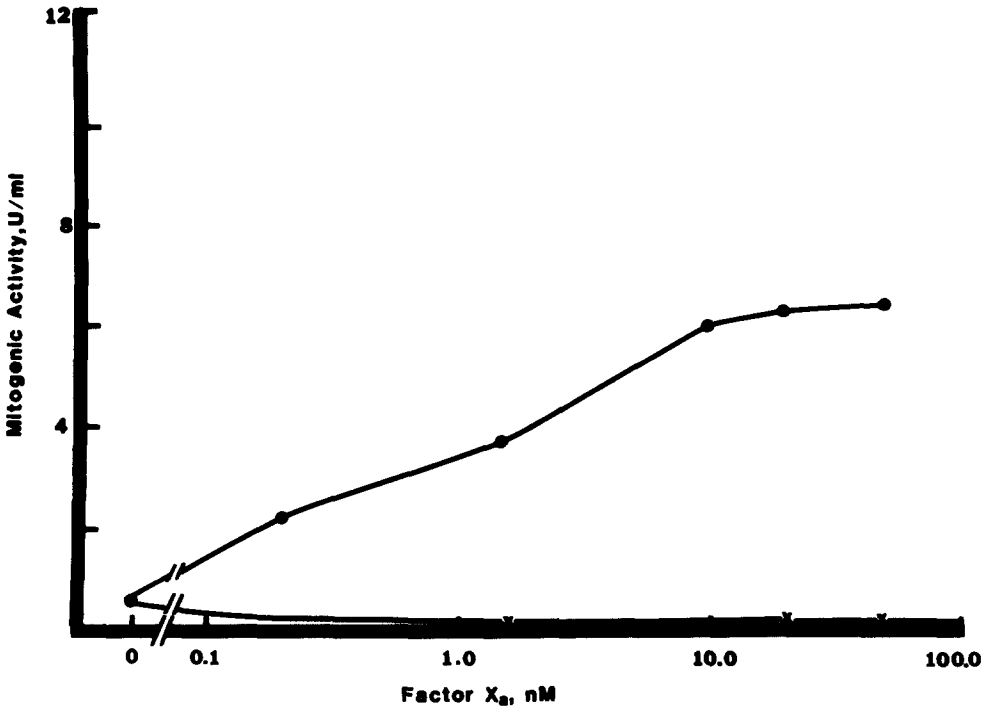

Figure 4. Dependence of released mitogenic activity on the concentration of Factor Xa incubated with endothelial cells. Confluent endothelial cell cultures in serum-free medium were incubated 3 h with increasing concentrations of Factor Xa (•) and Factor X (X). Conditioned medium was assayed for mitogenic activity in the [^{3}H]thymidine incorporation assay using 3T3 cells as described in Materials and Methods. Data shown are the means of triplicate determinations. Standard deviations were <15%.

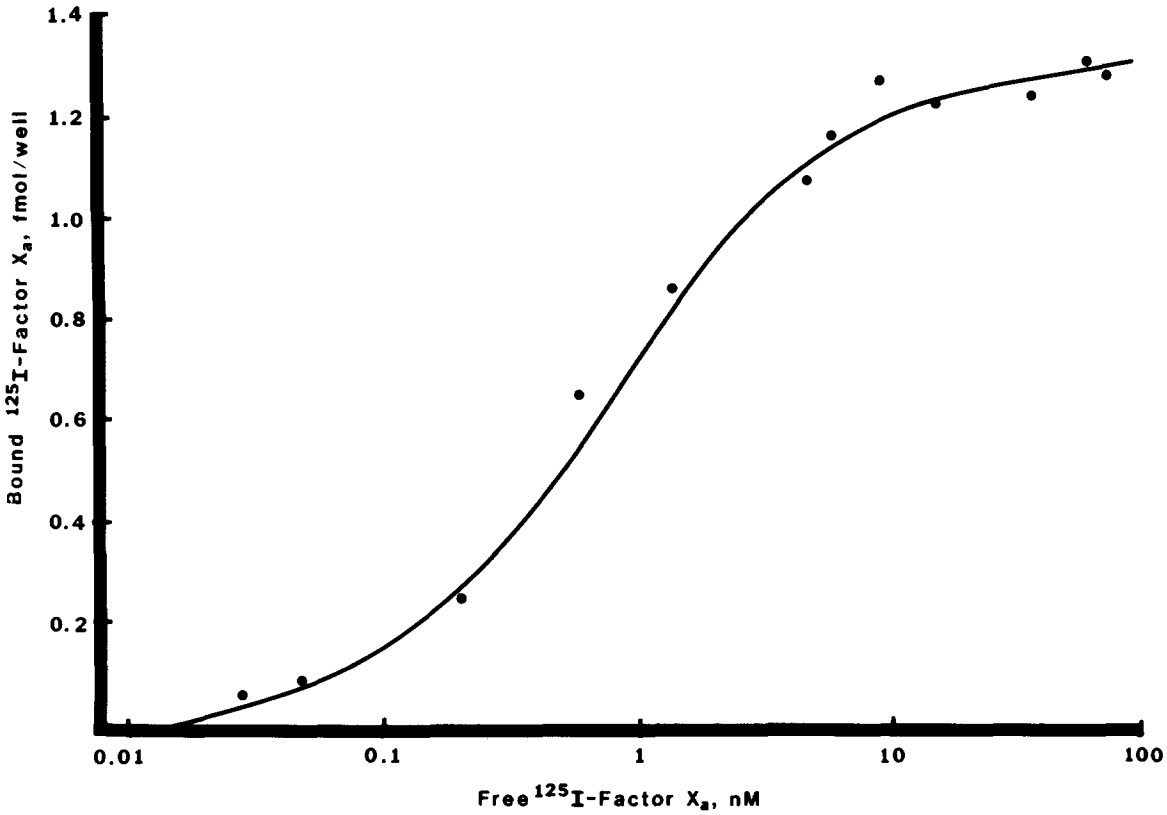

Figure 5. Binding of ^{125}I-Factor Xa to cultured bovine aortic endothelial cells. Endothelial cell monolayers were incubated with varying concentrations of ^{125}I-Factor Xa. The protocol is described in Materials and Methods. Nonspecific binding was determined from wells incubated with a 250-fold molar excess of unlabeled Factor Xa. Specifically bound ^{125}I-Factor Xa is plotted vs. the free concentration of Factor Xa. The curves indicate the best-fit line as described by nonlinear least-squares analysis.

per endothelial cell. These binding parameters are similar to the preliminary results reported by Rodgers and Shuman for Factor Xa binding to cultured bovine aortic endothelial cells (59) and a class of reversible binding sites observed by us on bovine aortic segments (72). Results of clearance studies of radiolabeled Factor Xa in vivo in the mouse are also consistent with the presence of endothelial cell binding sites (22).

Recent studies have demonstrated that endothelium can promote the activation of Factor X (74). The subsequent interaction of the Factor Xa thus formed with the endothelial cell could then provide a localized signal causing release of mitogens. If this were true, this would suggest the possibility of local regulation of mitogen generation due to interaction of the coagulation system with the vessel wall. To test this, Factor IXa-VIII–mediated activation of Factor X was examined over endothelial cell monolayers (Fig. 6). A low concentration of Factor IXa (0.2 nM) was employed along with physiological levels of Factors VIII and X. Factor Xa formation occurred (Fig 6 *A*, •) and was accompanied by the release of mitogen from the endothelium (Fig. 6 *B*, II). Release of mitogen required Factor Xa formation, since substitution of active site-blocked Factor IXa for native Factor IXa prevented Factor Xa formation (Fig. 6 A, o) and mitogen release (Fig. 6 *B*, III). Furthermore, none of the initial reaction components has the ability to cause enhanced release of mitogen from the endothelium (Table I). These results indicate that after Factor X activation has occurred on the endothelial cell surface and the product has been inactivated (Factor Xa inactivation would be complete after several minutes in the presence of plasma protease inhibitors), a circumscribed area on endothelium continues to elaborate mitogenic activity.

Discussion

The results reported here demonstrate that cultured endothelial cells elaborate increased amounts of mitogenic activity, including PDGFc, after incubation with Factor Xa. The induction of endothelial cell mitogens by Factor Xa indicates a link between activation of the coagulation system and the regulatory role of endothelium. Although quiescent endothelium does not induce activation of coagulation, perturbation of endothelial cells by agents such as endotoxin and phorbol esters can lead to the induction of tissue factor (3, 43, 60), a cofactor for the initiation of coagulation (53). These same agents have also been shown to augment mitogen release by endothelium (21). Endothelial cell tissue factor promotes Factor VII/VIIa–mediated activation of Factor X directly and via Factors VIII and IX (75). Factor Xa may then function as a coagulation enzyme causing thrombin formation and eventual fibrin deposition (73), or as a regulator of endothelial cell function by inducing the elaboration of mitogens. Thrombin results in the induction of more endothelial cell tissue factor, potentiating the activation of coagulation. This suggests a circle of interaction in which perturbation of endothelium leads to activation of coagulation, and products

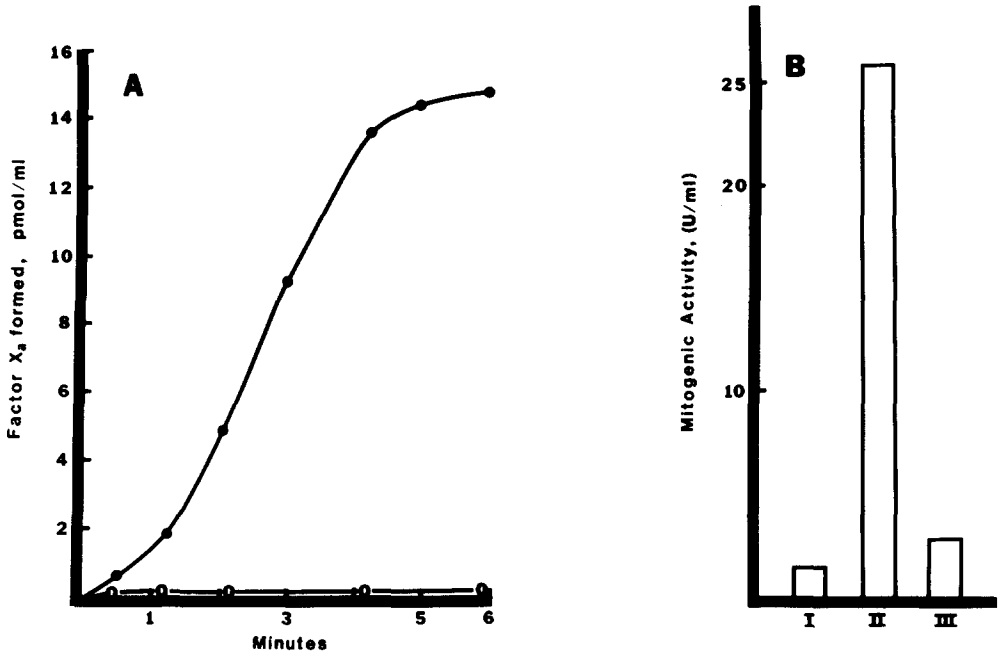

Figure 6. Activation of Factor X on the endothelial cell surface and the elaboration of mitogenic activity. (*A*) Factor Xa formation. Endothelial cell monolayers were incubated with Factor VIII (1.5 U/ml), Factor X (300 nM), Factor IXa (0.2 nM), or active site-blocked Factor IXa (0.2 nM). Aliquots of reaction mixture supernatant were withdrawn at the indicated times and assayed for Factor Xa amidolytic activity as described in Materials and Methods. The mean of duplicates is shown. Where indicated Factor IXa (●) was replaced by active site-blocked Factor IXa (○) in the reaction mixture. (*B*) Elaboration of mitogenic activity. Endothelial cell monolayers were incubated for 3 h without coagulation factors or with coagulation factors as described in *A*. Samples were assayed in the [^{3}H]thymidine incorporation assay using 3T3 cells. The mean of triplicates is shown. Standard deviations were <10%. I, no coagulation factors present; II, Factors IXa, VIII, and X present; III, active site-blocked Factors IXa, VIII, and X present.

of the activated coagulation system, Factor Xa and thrombin, perpetuate endothelial dysfunction. If this were true, then a nidus of perturbed endothelium promoting localized coagulation would also be a focus of mitogen release functioning before the development of more advanced atherosclerotic lesions characterized by endothelial cell denudation and platelet deposition. The role of endothelial cell mitogens, however, remains speculative and their ability to transmit proliferative signals within the vessel wall is unclear.

The induction of enhanced endothelial cell mitogen release by Factor Xa was specific for the enzyme. Blockade of the active site by the physiologic inhibitor antithrombin III or the low molecular agents diisopropylfluorophosphate or (*p*-amidinophenyl)methanesulfonyl fluoride rendered Factor Xa ineffective in promoting mitogen release. Although initially this suggested the possibility of a mechanism involving protease nexin, this inhibitor reacts with Factor Xa quite slowly (66). Furthermore, no higher molecular weight complexes were observed on SDS PAGE when ^{125}I-Factor Xa replaced Factor Xa in the incubation mixture. This is consistent with the results of two previous studies employing ^{125}I-Factor Xa and cultured bovine endothelial cells (50, 59). The role of the enzyme's active site is unclear in mitogen release, but may involve proteolysis of a cell surface protein or perhaps even modification of a mitogen precursor promoting its release. The lack of a requirement for de novo transcription or translation for enhanced mitogen release induced by Factor Xa is consistent with the notion that this enzyme may trigger posttranslational events leading to elaboration of growth factor activity. In this context, a recent paper has demonstrated that a portion of the v-sis gene product is localized to the plasma membrane of simian sarcoma virus transformed cells (58).

The lack of effective induction of enhanced mitogen release by Factor Xa modified by removal of the γ-carboxyglutamic acid residues, which play a role in membrane interactions (52), suggested that cell surface binding of the enzyme might be involved. In this context, radioligand studies identified a class of endothelial cell sites for Factor Xa whose binding parameters were similar to the lower range of Factor Xa concentrations causing mitogen release (half-maximal binding and enhancement of mitogen release at Factor Xa concentrations of 0.8 and 0.5 nM, respectively). Although this correlation may be fortuitous, it suggests that these sites may be related. Growth factor release at higher concentrations of Factor Xa, however, did not correspond to an identifiable class of binding sites and may represent a nonspecific protease effect rather than a specific Factor Xa-binding site interaction. Mitogen release in response to these higher concentrations of Factor Xa did not saturate and thus appears to be similar to release of PDGFc in response to thrombin over the concentration range of 10–100 nM (32).

These data reflect the close relationship between the coagulation system and endothelium. Factor Xa, a product of the activated coagulation mechanism, not only functions as a procoagulant, promoting thrombin formation on the cell surface, but can modulate endothelial cell physiology. Factor Xa can promote the release of mitogens, elevate cytosolic calcium (71), and finally be removed from the cell surface by receptor-mediated endocytosis (50). Thrombin releases PDGFc directly from the endothelium, activates platelets, recruiting them to augment the coagulant response and growth factor release, and induces endothelial cell procoagulant activity. This suggests a series of interactions in which perturbation of endothelium initiates procoagulant reactions on the vessel surface leading to Factor Xa and thrombin for-

mation. These coagulation enzymes could then potentiate the perturbed state by enhancing localized mitogen release and close the circle of interaction by further promoting the generation of procoagulants.

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