

## CELLULAR PROCESSING OF BOVINE FACTORS X AND X<sub>a</sub> BY CULTURED BOVINE AORTIC ENDOTHELIAL CELLS

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Recent studies (1–4) have shown that the endothelial cell has multiple hemostatic properties. The presence of thrombomodulin (1) and anticoagulant heparin-like molecules on the cell surface (2), and the elaboration of prostacyclin (3) and plasminogen activators (4) provide powerful mechanisms for inhibition of the coagulation system. In addition to these and other anticoagulant properties, recent studies (5, 6) have suggested potential procoagulant activities of the endothelial cell. Endothelial cells can bind Factors IX and IX<sub>a</sub> (5, 6). Cell-bound Factor IX can be activated, and cell-bound Factor IX<sub>a</sub>, in the presence of Factor VIII, can promote Factor X activation (7, 8). Factor X also binds to endothelium (5, 7), and Factor X<sub>a</sub> incubated with endothelial cells promotes prothrombin activation (8, 9). This reaction is dependent on Factor V, which is expressed by the endothelium (8–10). Furthermore, perturbation of endothelial cells by agents such as endotoxin results in the induction of tissue factor activity (7, 11–13). These studies suggest that endothelial cells can potentially play a role in procoagulant (14), in addition to anticoagulant, reactions.

An important determinant of the coagulant activity of cell-associated coagulation proteins concerns their cellular processing. If an enzyme remains localized on the cell surface, its activity can be modulated by the cells while it remains in contact with its substrates and inhibitors in the plasma. In addition, cellular binding can initiate clearance via endocytosis and degradation. Previous studies have indicated that coagulation Factor X (5, 7) and its activated form, Factor X<sub>a</sub> (8, 15), bind specifically to distinct sites on the endothelial cell surface. In this study, we have examined the cellular processing of bound Factors X and X<sub>a</sub>. The results indicate that cell-bound Factor X is internalized slowly compared with its dissociation from the cell surface. In contrast, Factor X<sub>a</sub> is internalized more rapidly, and is subsequently degraded, presumably via a lysosomal-dependent pathway. Activation of Factor X on the endothelial cell surface leads to formation of bound Factor X<sub>a</sub>, which is subject to cellular processing. Thus,

D. Stern completed this work during his tenure as recipient of a Clinician Scientist Award from the American Heart Association (with funds contributed in part by the New York affiliate). This work was also supported by grants HL-15486, HL-21006, and HL-16919 from the National Institutes of Health, Bethesda, MD.

internalization and degradation of Factor X<sub>a</sub> may be important mechanisms regulating participation of cell-bound Factor X<sub>a</sub> in coagulation.

### Materials and Methods

**Coagulation Factors.** Bovine coagulation factors were used throughout these studies. Bovine Factor X was purified to homogeneity (100 U/mg) by the method of Fujikawa et al. (16). Factor X was radioiodinated by the method of Bolton and Hunter (17), using *N*-succinimidyl 3-(4-hydroxy, 5-[<sup>125</sup>I]iodophenyl) propionate (Amersham Corp., Arlington Heights, IL), with a specific activity of 1,950 Ci/mmol. After evaporation to dryness, Bolton and Hunter reagent (2 mCi) was incubated with Factor X (10 μg) in borate buffer (0.1 M, pH 8.8) for 35 min at 4°C. The reaction mixture was chromatographed on a Sephadex G25 column (0.9 × 20 cm) to remove excess Bolton and Hunter reagent. The specific radioactivity of <sup>125</sup>I-Factor X was 2,500–8,500 cpm/ng over seven iodinations, and Factor X coagulant activity was not affected by the radiolabelling procedure. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)<sup>1</sup> of <sup>125</sup>I-Factor X (Fig. 1, open circles) demonstrated that the heavy chain was labelled. Radioiodinated Factor X was activated immediately after labelling by incubation with the coagulant protein from Russell's viper venom (18) coupled to CNBr-Sepharose in the presence of 5 mM CaCl<sub>2</sub>, as described (8). SDS-PAGE of <sup>125</sup>I-Factor X<sub>a</sub> (Fig. 1, filled circles) showed the radiolabel remained in the heavy chain after activation.

Bovine Factor IX was purified to homogeneity (260 U/mg) by the method of Fujikawa et al. (19). Factor IX was activated by incubation with Factor XI<sub>a</sub> bound to CNBr-Sepharose in the presence of 5 mM CaCl<sub>2</sub> as described previously (6). Factor IX<sub>a</sub> (22.2 μM) was inactivated by incubation with dansyl-glutamine-glycine-arginine-chloromethylketone (600 μM) for 3 h at 25°C, as described by Lollar and Fass (20). The product had no residual coagulant activity. Bovine Factor VIII–von Willebrand Factor (vWF) was prepared by the method of Newman et al. (21). Subsequently, it was chromatographed on an antifibronectin and antifibrinogen affinity column. The final material was homogeneous on reduced SDS-PAGE (5%), showing one band of mol wt 200,000, corresponding to vWF. The Factor VIII/vWF preparation used in this work had a protein concentration of 1.1 mg/ml and a Factor VIII coagulant activity of 60 U/ml. Factor VIII coagulant activity in this preparation was stable over 6 mo of storage at –80°C, and no other coagulant activities (including factors VII, IX, X, XI, and XII) were detected by clotting assay (22). Bovine prothrombin was purified to homogeneity (13 U/mg), as described (23).

SDS-PAGE was carried out by the method of Weber and Osborn (24) using tube gels 6 cm in length. Gels were sliced by hand into 1-mm sections, and counted in an LKG

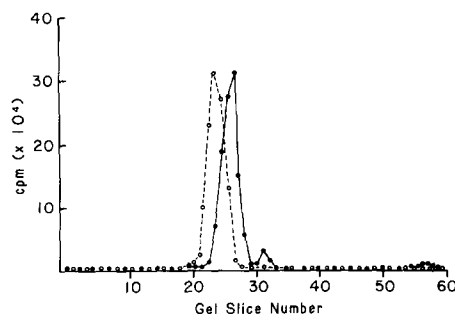


FIGURE 1. Reduced SDS-PAGE (7.5%) of <sup>125</sup>I-Factor X (○) and X<sub>a</sub> (●). SDS-PAGE was carried out, and gels were sliced and counted as described in Materials and Methods.

<sup>1</sup> Abbreviations used in this paper: SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; vWF, von Willebrand Factor.

(Rockland, MD) Rackgamma counter. Electrophoresis molecular weight-calibration proteins were purchased from Pharmacia Fine Chemicals (Piscataway, NJ).

**Cell Culture.** Bovine aortic endothelial cells were isolated from calf aortas (a gift of the Great American Veal Co., Newark, NJ), as described by Schwartz (25), and were grown in Dulbecco's modified Eagle's medium with penicillin-streptomycin (50 U/ml, 50  $\mu$ g/ml, respectively) and 10% fetal calf serum (lot 100413; Hyclone, Sterile Systems, Logan, UT). The cultures were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. The cells were separated for subculture with 0.25% trypsin with 0.05% EDTA (Gibco, Grand Island, NY). Cells (passages 3–22) from different aortas were grown to confluence in 35 mm dishes (Corning Glass Works, Corning, NY), and at confluence, there were 1.1–1.5  $\times 10^5$  cells/cm<sup>2</sup>. Experiments were carried out 1 d after the cells achieved confluence. The cells were characterized morphologically by contact inhibition of growth, and cobblestone appearance in the monolayer (25), the presence of vWF antigen using indirect immunofluorescence (26), and angiotensin-converting enzyme activity (27). Viability during the experiments was assessed by trypan blue exclusion and was >90% at all times during these experiments.

**Binding and Internalization Studies.** Before an experiment, monolayers were washed three times over 20 min with incubation buffer (10 mM HEPES, pH 7.45, containing 137 mM NaCl, 4 mM KCl, 11 mM glucose, 2.5 mM CaCl<sub>2</sub>, and 0.5 mg/ml bovine serum albumin-fatty acid free). Then fresh incubation buffer (1.0 ml) was added, along with radiolabelled Factor X or X<sub>a</sub>, and other proteins as indicated. At the specified times, unbound ligand was removed by washing six times with ice-cold incubation buffer. Cell-associated radioactivity was characterized as surface bound, based on elution during a 3-min incubation at 4°C with incubation buffer containing dextran sulfate (10 mg/ml, 1 ml) (see Results), or internalized, if it remained cell-associated after dextran sulfate treatment and could then be eluted with Tris-buffered saline (0.05 M Tris, 0.10 M NaCl, pH 7.45) containing Nonidet P-40 (1%; 1 ml). Elution of radioactivity with Nonidet P-40 was complete after 10 min at 37°C. Cellular degradation of <sup>125</sup>I-Factor X and <sup>125</sup>I-Factor X<sub>a</sub> was assessed by trichloroacetic acid precipitation and immunoreactivity (28) determinations. Tracer precipitability was tested in 5% trichloroacetic acid, since this concentration of trichloroacetic acid precipitated >95% of the <sup>125</sup>I-Factor X or <sup>125</sup>I-Factor X<sub>a</sub> before incubation with the cells. The immunoreactivity of radiolabelled Factors X and X<sub>a</sub> was tested using a monospecific rabbit antibody against Factor X (16) (generously provided by Dr. K. Fujikawa, Department of Biochemistry, University of Washington, Seattle), which also reacts with Factor X<sub>a</sub>. Antibody (1/5,000 dilution; 50  $\mu$ l) was incubated with the tracer (10  $\mu$ l) for 12 h at 4°C, then staphylococcal protein A (10% suspension; 50  $\mu$ l) (Enzyme Center, Boston, MA) was added for 30 min at room temperature, and the tubes were centrifuged at 10,000 g for 3 min. The supernatants were aspirated, and the pellets were counted. Monospecific antibody to bovine antithrombin III was generously provided by Dr. Kurachi (Department of Biochemistry, University of Washington).

Dissociation of bound <sup>125</sup>I-Factor X or <sup>125</sup>I-Factor X<sub>a</sub> was measured as described by Lollar et al. (29): after the monolayers were incubated with tracer and washed free of unbound material, incubation buffer (1.5 ml) was added. At the indicated times, cells were washed with incubation buffer, and the remaining cell-associated radioactivity was determined using Tris-buffered saline (0.05 M Tris, 0.1 M NaCl, pH 7.45) containing Nonidet P-40 (1%). Increasing the volume of incubation buffer to 10 ml during the dissociation period had no effect on the rate or extent of dissociation.

Chloroquine treatment of endothelium was carried out by preincubating the cells with chloroquine (80  $\mu$ M) in incubation buffer for 2 h at 37°C, then adding radiolabelled proteins.

In other experiments (Fig. 6), cells were incubated with radioligand for 4 h at 4°C to allow surface binding. Monolayers were then washed six times, and incubation buffer (1.0 ml), prewarmed to 37°C, was added. At the indicated times, radioactivity in the supernatant and associated with the cells was determined as described above.

During the course of these experiments, monolayers were assessed for detachment and release of lactate dehydrogenase (Sigma Chemical Co., St. Louis, MO) into the medium.

Cell detachment was negligible throughout the experiments, and there was no increase in the lactate dehydrogenase activity (30) in the supernatant.

*Activation Studies.* Endothelial cell monolayers were washed three times with incubation buffer over 20 min. Fresh incubation buffer (1.0 ml at 37°C) was then added, along with Factor IX<sub>a</sub> and Factor VIII/vWF. 30 s later, <sup>125</sup>I-Factor X was added. Formation of Factor X<sub>a</sub> was monitored by the generation of amidolytic activity in the Bz-Ile-Glu-Gly-Arg-pNA synthetic substrate assay (Helena Laboratories, Beaumont, TX) (31) and cleavage on SDS-PAGE (32). The Factor X<sub>a</sub> amidolytic assay was carried out by withdrawing one aliquot (0.1 ml) from each well at the indicated time, and adding it to 0.5 ml of 50 mM Tris-HCl (pH 7.8), 175 mM NaCl, 10 mM EDTA, and 0.5 mg/ml ovalbumin. The entire sample was then placed in a cuvette along with synthetic substrate (0.1 ml; 2.0 mM), and the change in optical density at 405 nm was monitored. The amount of Factor X<sub>a</sub> formed was determined by comparison with the linear portion of a standard curve in which known amounts of Factor X<sub>a</sub> were assayed under identical conditions.

*Colloidal Gold Labelling and Electron Microscopy.* Suspensions of colloidal gold were prepared (33) with an average diameter of  $18.6 \pm 2.7$  nm (mean  $\pm$  SD) and at a concentration of  $1.72 \times 10^{12}$  particles/ml (34). Colloidal gold-protein conjugates were prepared at pH 5.8, and complete surface labelling was achieved, as determined by the serial electrolyte test (35). Labelled colloids were evaluated for stability as previously described (36). Bovine serum albumin-colloidal gold conjugates were prepared at pH 5.0, as a control for nonspecific binding. Experiments were then carried out as described for radioligands. Internalization was evaluated by direct gold counting, and results are expressed as gold particles per cell.

For electron microscopic examination, monolayers were fixed with buffered glutaraldehyde (3%), postfixed in osmium tetroxide (37), and stained in tannic acid (0.25%). Samples were then dehydrated, and embedded. For scanning electron microscopy, monolayers were fixed, stained, dehydrated, and critical-point dried. Samples were sputter-coated with gold-palladium and 4–5 micrographs were taken of each sample for cell area analysis. Cell area was  $1,000 \mu\text{m}^2/\text{cell}$ . Quantitation of gold binding (38) was carried out by counting only single particles within 8 nm from the outer leaflet of the plasma membrane. 20 micrographs were counted from each sample, duplicate samples were obtained at each time point, and the experiments were performed twice.

## Results

Incubation of cultured endothelium with <sup>125</sup>I-Factor X and <sup>125</sup>I-Factor X<sub>a</sub> resulted in a time-dependent association of radioligands with the cells (Fig. 2). Simultaneous addition of a hundred-fold molar excess of unlabelled Factor X blocked <sup>125</sup>I-Factor X-endothelial cell binding, but similar concentrations of Factors IX, IX<sub>a</sub>, X<sub>a</sub>, and prothrombin had no effect, as was reported previously (5, 7). Similarly, excess unlabelled Factor X<sub>a</sub> blocked <sup>125</sup>I-Factor X<sub>a</sub>-endothelial cell binding, but Factors IX, IX<sub>a</sub>, X, and prothrombin had no effect (see also 8). Thus, the binding of Factors X and X<sub>a</sub> involve specific interactions of each ligand with distinct cellular binding sites. Although binding reached an apparent maximum for both proteins by 30 min at 4°C, at higher temperatures, 21°C and 37°C, there was a steady increase in the cellular association of both ligands. Dissociation studies showed that specific binding was completely reversible at 4°C, but not at 37°C (Fig. 2). This suggested that at 37°C, either irreversible surface binding had occurred or, alternatively, the cell-bound ligand had been internalized. To distinguish between these possibilities, methods to quantitate surface-bound ligand were studied. Dextran sulfate completely eluted all <sup>125</sup>I-Factor X or X<sub>a</sub> bound at 4°C after a 3-min incubation. Dextran sulfate did not result in endothelial cell detachment. Gentle treatment of the monolayers with

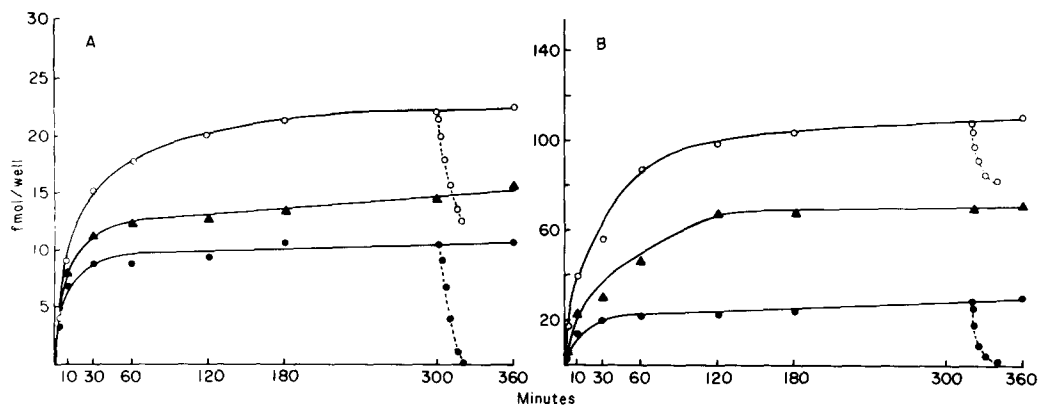


FIGURE 2. Binding and uptake of  $^{125}\text{I}$ -Factor X and  $\text{X}_a$ . Endothelial cell monolayers ( $1.2 \times 10^6$  cells/well) were incubated at  $4^\circ\text{C}$  ( $\bullet$ ),  $21^\circ\text{C}$  ( $\blacktriangle$ ) or  $37^\circ\text{C}$  ( $\circ$ ) with 1 ml of incubation buffer containing either  $^{125}\text{I}$ -Factor X (1 nM) (A) or  $\text{X}_a$  (1 nM) (B). A duplicate set of wells was incubated with the same tracer in the presence of a 100-fold molar excess of unlabelled ligand (nonspecific binding). At the indicated times, the cells were washed six times with incubation buffer at  $4^\circ\text{C}$ , then solubilized using incubation buffer containing Nonidet P-40 (1%) as described in Materials and Methods. Specific binding (total - nonspecific binding) is plotted against incubation time. Dotted lines indicate dissociation studies employing the method of infinite dilution were carried out in parallel. Each point is the mean of duplicates and the experiments were repeated three times.

trypsin (1 mg/ml for 5 min) also dissociated cell-bound radioactivity when binding was carried out at  $4^\circ\text{C}$ . This exposure of cells to trypsin did not disrupt the monolayer or affect cell viability, as determined by trypan blue exclusion. In contrast, when binding was carried out at  $37^\circ\text{C}$ , both dextran sulfate and trypsin effected only partial dissociation of cell-associated  $^{125}\text{I}$ -Factor X and  $\text{X}_a$ . This was analogous to the results depicted in Fig. 2, where dissociation studies were carried out by the infinite dilution (29) method. These results suggested that internalization of both ligands (see below) could be responsible for formation of the pool of cell-associated radioligands at  $37^\circ\text{C}$  resistant to dissociation by these multiple methods. This pool of radioligand was elutable by solubilizing the cells using nonionic detergent (1% Nonidet P-40), and is referred to as the internalized pool. In contrast to previous results (8) demonstrating covalent linkage of Factor  $\text{X}_a$  to the surface of bovine aortic vessel segments, no similar complex was detectable in SDS-PAGE of the cultured endothelial cells (data not shown). Cell-bound  $^{125}\text{I}$ -Factor X and  $\text{X}_a$  migrated similarly to the unbound proteins on SDS-PAGE. Pilot studies with an antibody to antithrombin III, which can block formation of Factor  $\text{X}_a$ -antithrombin III complexes (8), showed no effect on Factor  $\text{X}_a$  binding and internalization.

Prompted by these findings, internalization studies were carried out with  $^{125}\text{I}$ -Factor X and  $\text{X}_a$  (Fig. 3). In both cases, surface binding reached an apparent maximum by 30 min, and a pool of internalized radioligand formed and increased steadily up to 180 min. A greater proportion of the cell-bound  $^{125}\text{I}$ -Factor  $\text{X}_a$  is internalized and degraded compared with  $^{125}\text{I}$ -Factor X. Degradation of  $^{125}\text{I}$ -Factor  $\text{X}_a$  was progressive during incubation with the cells, based on decreased precipitation of radiolabelled material in trichloroacetic acid (Fig. 3), and loss of immunoreactivity (Fig. 4). Degradation occurred only at  $37^\circ\text{C}$ , and in the

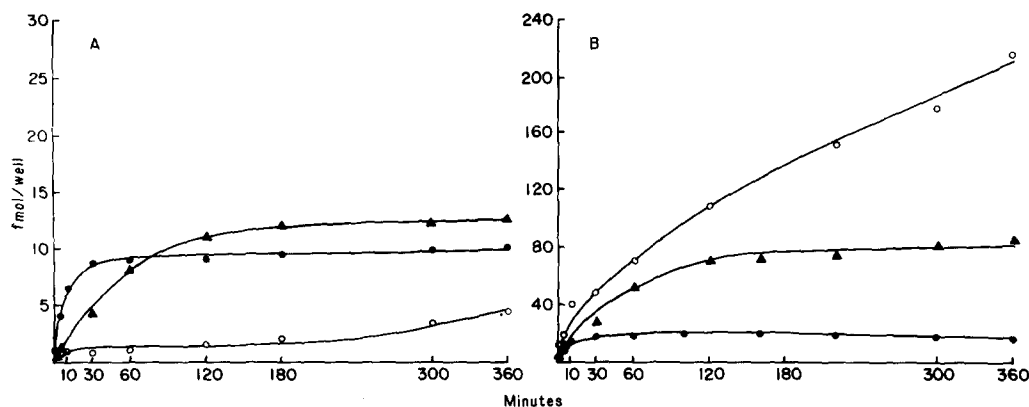


FIGURE 3. Uptake and degradation of  $^{125}\text{I}$ -Factor X and X<sub>a</sub>. Endothelial cell monolayers ( $1.3 \times 10^6$  cells/well) were incubated with 1 ml of incubation buffer at 37°C for the indicated times with  $^{125}\text{I}$ -Factor X (1 nM) (A) or X<sub>a</sub> (1 nM) (B). The amount of surface bound (●), internalized (▲), and degraded (○) ligand was determined as described in Materials and Methods. Each point is the mean of duplicate wells, and the experiment was repeated five times. Data is normalized to  $10^6$  cells.

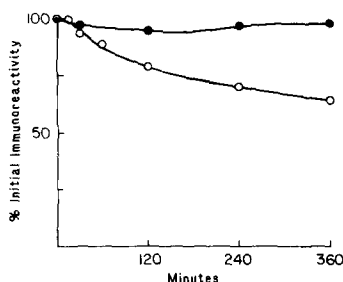


FIGURE 4. Immunoreactivity of  $^{125}\text{I}$ -Factor X (●) and X<sub>a</sub> (○) during incubation with endothelial cells ( $1.4 \times 10^6$  cells/well). Endothelial cells were incubated with 1 ml of incubation buffer containing  $^{125}\text{I}$ -Factor X<sub>a</sub> (1 nM) or X (1 nM) at 37°C. Immunoreactivity was determined as described in Materials and Methods. Data shown are the mean of duplicates and the experiment was repeated three times.

presence of the endothelial cells. Addition of chloroquine (an agent that blocks intracellular degradation of a variety of radioligands [39]) to the cultures prevented degradation of Factor X<sub>a</sub> (Fig. 5), and resulted in a marked increase in the apparent size of the internalized pool. In contrast, Factor X was internalized, but not significantly degraded (Figs. 3 a and 4). Over eight independent experiments, the overall rate of  $^{125}\text{I}$ -Factor X and X<sub>a</sub> uptake (the sum of the radioligand bound, internalized, and degraded) at a concentration of 1 nM, assessed over the first 30 min, averaged 0.5 and 3.2 fmol/min/ $10^6$  cells, respectively. The rate of  $^{125}\text{I}$ -Factor X<sub>a</sub> degradation averaged 0.9 fmol/min/ $10^6$  cells over 120 min, whereas the rate of Factor X degradation was negligible up to 140 min. Specificity of the cellular uptake of  $^{125}\text{I}$ -Factor X and X<sub>a</sub> was indicated by the 80–90% inhibition of binding and internalization in the presence of a 100-fold molar excess of unlabelled protein.

These results suggested that Factor X<sub>a</sub> cellular processing consisted of surface binding followed by internalization and degradation. Factor X was internalized,

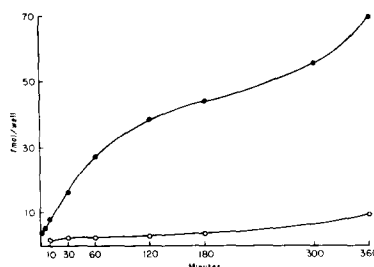


FIGURE 5. Effect of chloroquine on degradation of  $^{125}\text{I}$ -Factor  $X_a$  by endothelial cells ( $1.3 \times 10^6$  cells/well). The protocol was identical to that in Fig. 3, except that the  $^{125}\text{I}$ -Factor  $X_a$  concentration is 0.2 nM, and chloroquine ( $80 \mu\text{M}$ ) was added where indicated. Symbols represent the amount of degraded  $^{125}\text{I}$ -Factor  $X_a$  per  $10^6$  cells in the presence (○) and absence (●) of chloroquine. Data shown are the mean of duplicates and the experiment was repeated four times.

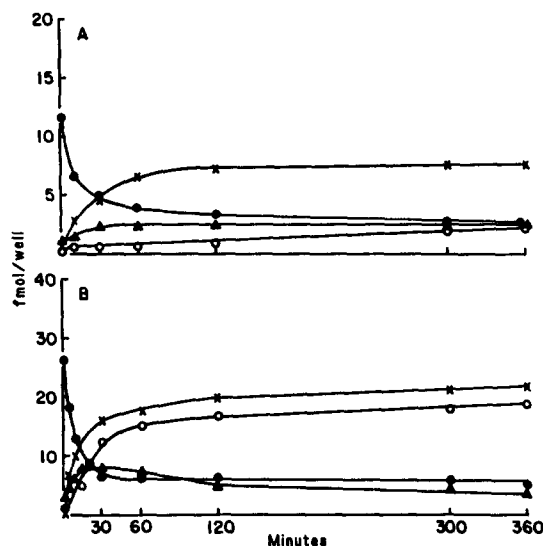


FIGURE 6. Endocytosis of surface-bound  $^{125}\text{I}$ -Factor X and  $X_a$ . Endothelial cell monolayers ( $1.3 \times 10^6$  cells/well) were preincubated for 4 h at  $4^\circ\text{C}$  with  $^{125}\text{I}$ -Factor X (1 nM) (A) or  $X_a$  (1 nM) (B). Monolayers were washed six times with incubation buffer at  $4^\circ\text{C}$ , then rapidly warmed to  $37^\circ\text{C}$  by adding 0.9 ml incubation buffer at  $37^\circ\text{C}$ . At the indicated times, an aliquot of supernatant was obtained and counted (x). Formation of trichloroacetic acid-soluble radioactivity in the supernatant was determined (○). Then the cells were washed three times with incubation buffer, and surface-bound (●) and internalized (▲) ligand were quantitated. The mean of duplicates is shown and the experiment was repeated four times. Data is normalized to  $10^6$  cells.

though at a considerably slower rate, and was not appreciably degraded. Consistent with these hypotheses, pulse-chase experiments (Fig. 6) demonstrated that Factor  $X_a$  bound to the cell surface at  $4^\circ\text{C}$  was subsequently internalized at an initial rate of  $0.38 \text{ fmol}/\text{min}/10^6$  cells, and degraded at  $0.40 \text{ fmol}/\text{min}/10^6$  cells. Similar experiments with Factor X demonstrated a considerably slower rate of internalization ( $0.07 \text{ fmol}/\text{min}/10^6$  cells), and a negligible rate of degradation over the first 60 min. Cellular processing of Factor X thus occurs slowly compared with Factor  $X_a$ . Internalized Factor X is returned to the cell surface and medium

undegraded. Another difference in Factor X<sub>a</sub> and Factor X cellular handling is the accelerated dissociation of the enzyme at low pH (Fig. 7). Thus, Factor X<sub>a</sub>-binding site complexes can dissociate in acidic intracellular compartments, allowing Factor X<sub>a</sub> to enter lysosomes, where degradation proceeds. Factor X binding is relatively more constant over the pH range of 5–8, thus preventing its accumulation in acidic intracellular structures.

Morphologic studies using colloidal gold conjugates prepared with Factor X and Factor X<sub>a</sub> (Fig. 8), confirmed the results of radioligand binding studies. At 4°C the gold particles were evenly distributed and closely attached to the cell surface in each case (Fig. 8, *a* and *b*). After warming the cells to 37°C, clustering of gold particles and uptake at coated pit regions was observed (Fig. 8, *c* and *d*). Finally, Factor X<sub>a</sub>-gold particles accumulated in lysosome-like structures, whereas internalized Factor X accumulated to only a limited extent intracellularly in lysosomes or elsewhere (Fig. 8, *e-f*). The close association of the Factor X-gold probes to the inner lysosomal membrane suggests continued receptor interaction, and may account for the apparent externalization of Factor X. Factor X<sub>a</sub>-gold probes are randomly distributed in the lysosomal lumen, suggesting receptor-ligand dissociation. Factor X-gold conjugates were shuttled back to the cell surface. In each case, addition of a 100-fold molar excess of Factor X or Factor X<sub>a</sub> not conjugated to the gold inhibited binding and internalization by >85%.

The importance of endocytosis as a clearance pathway for cell-bound Factor X<sub>a</sub> was studied by activating Factor X on endothelial cell monolayers (Fig. 9). Previous studies (7, 8) have demonstrated endothelial cell-dependent activation of Factor X by Factors IX<sub>a</sub>-VIII. When <sup>125</sup>I-Factor X, Factor IX<sub>a</sub>, and Factor VIII/vWF were incubated with endothelial monolayers, Factor X<sub>a</sub> amidolytic activity was detectable in the supernatant in parallel with the appearance of <sup>125</sup>I-Factor X<sub>a</sub> on SDS-PAGE. <sup>125</sup>I-Factor X<sub>a</sub> was also present on the endothelial cell surface and in the internalized pool as shown by SDS-PAGE (data not shown). During the incubation period, the precipitability of the tracer in trichloroacetic

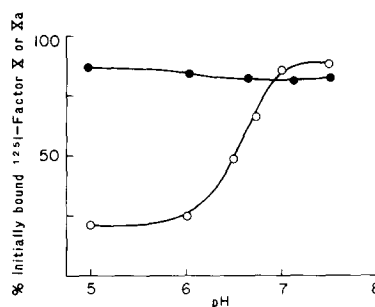


FIGURE 7. pH dependence of <sup>125</sup>I-Factor X and X<sub>a</sub> dissociation from the endothelial cell surface. Endothelial cell monolayers ( $1.3 \times 10^6$  cells/well) were equilibrated with incubation buffer at pH 7.45. Then <sup>125</sup>I-Factor X (●) or X<sub>a</sub> (○) was added for 20 min at 4°C. Monolayers were washed, incubated with incubation buffer at the indicated pH (time 0), and ligand dissociated after 60 sec was determined by the method of infinite dilution, as discussed in Materials and Methods. The <sup>125</sup>I-Factor X or X<sub>a</sub> bound at 3 min is reported as a percent of the ligand bound at time 0. Data shown represent the mean of duplicates and the experiment was repeated three times.



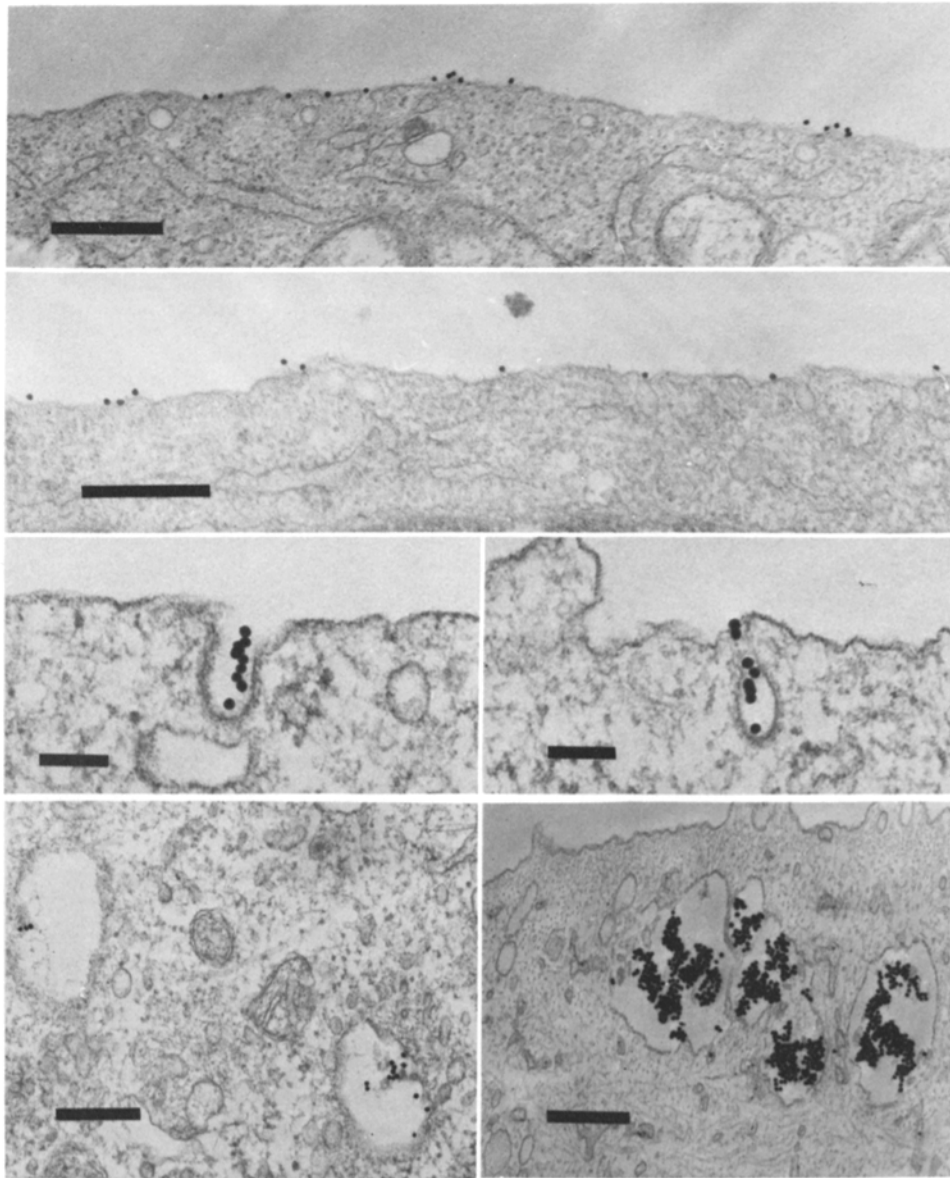


FIGURE 8. Binding and internalization of Factor X and  $X_a$  conjugated to colloidal gold. Surface labelling of Factor X-gold (A) and Factor  $X_a$ -gold (B) after incubation with endothelial cells for 2 h at 4°C, followed by rinsing and fixation at 4°C. In both cases, gold particles are randomly distributed at the outer membrane surface. Marker bar represents 0.5 nm. After incubation at 4°C for 2 h, cells were rapidly warmed to 37°C to initiate endocytosis of surface-bound Factor X-gold (C) and Factor  $X_a$ -gold (D). Endocytosis proceeds at coated pit regions involving clustering of the gold probes. Marker bar represents 0.1 nm. After 2 h at 37°C, there is limited accumulation of Factor X-gold (E) in structures resembling lysosomes, whereas greater accumulation in these lysosomal-like structures is seen in cells labelled with Factor  $X_a$ -gold (F). Marker bar represents 0.5 nm.

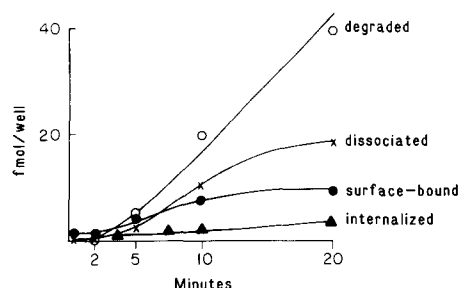


FIGURE 9. Factor IX<sub>a</sub>-VIII mediated Factor X activation on the endothelial cell surface: cellular processing of the Factor X<sub>a</sub> formed. Endothelial cell monolayers ( $1.4 \times 10^6$  cells/well) were incubated with Factors IX<sub>a</sub> (60 pM), VIII/vWF (0.5 U/ml), and  $^{125}\text{I}$ -Factor X (100 nM). At the indicated times, samples were removed to determine Factor X<sub>a</sub> amidolytic activity (x), and cell surface-bound (●), internalized (▲), and degraded (○) radioligand.

acid steadily decreased (Fig. 9). This was due to degradation of cell-associated radioligands rather than proteolytic cleavage of Factor X by Factor IX<sub>a</sub>, since activation of Factor X does not decrease the trichloroacetic acid-precipitability of  $^{125}\text{I}$ -Factor X prepared by the method of Bolton and Hunter (Fig. 1). The radioligand being degraded was probably Factor X<sub>a</sub> initially bound to the cell surface, since omission of Factor IX<sub>a</sub> or addition of active site-blocked Factor IX<sub>a</sub> (dansyl-glu-gly-arg-Factor IX<sub>a</sub>) in its place prevented Factor X activation and cellular degradation of radioligands (data not shown). The total amount of Factor X<sub>a</sub> formed thus includes Factor X<sub>a</sub> bound to the cell surface, released into the supernatant (1.6 fmol/min/ $10^6$  cells over the first 10 min), and degraded by the cells (2 fmol/min/ $10^6$  cells over the first 10 min). Thus, only 44% of the Factor X<sub>a</sub> formed is released into the supernatant. The remainder is subject to endocytosis and degradation.

### Discussion

The results reported here indicate that Factors X and X<sub>a</sub> are internalized after association with their specific endothelial cell binding sites. The Factor X<sub>a</sub>-binding site complex then probably dissociates in acidic prelysosomal compartments, allowing Factor X<sub>a</sub> to enter lysosomes, where degradation occurs. Factor X<sub>a</sub> cellular processing is thus analogous to that described for low density lipoprotein (40), asialoglycoproteins (41), and a variety of other ligands (39). In contrast, Factor X is slowly internalized, and principally shuttled back to the cell surface in a manner similar to the transferrin pathway (reviewed in 39). This cellular processing of cell-bound coagulation factors indicates that the endothelial cell surface is not a passive template for assembly of coagulation factor complexes, but is a dynamic surface with its own regulatory mechanisms.

Several mechanisms have been described by which endothelial cells can regulate the coagulant activity of serine proteases. In each case, these mechanisms are distinct from the pathway described in this work, involving specific binding sites for Factor X and Factor X<sub>a</sub>. Anticoagulant heparin-like molecules have been isolated from vascular tissues (42), and their functional activity has been demonstrated in animal infusion studies (2). Antithrombin III, a plasma glycoprotein

that inhibits a number of serine proteases, including those involved in blood coagulation (43), binds to these heparin-like molecules on the surface of native endothelium, resulting in enhanced anticoagulant activity (44). The cell surface thus provides heparin-like cofactor activity enhancing antithrombin III-mediated inactivation of multiple coagulation enzymes. Protease nexins, especially type I, are secretory proteins that covalently complex with the active site of serine proteases (45). The enzyme-protease nexin complex then binds to the cell surface, with subsequent internalization and degradation (45). Formation of the enzyme-protease nexin complex is the signal for cell-surface binding followed by cellular processing. In addition, covalent (46, 47) and noncovalent (1, 48) thrombin-endothelial cell interactions have been shown to modulate coagulant activity of the cell-associated enzyme. In each of these cases, endothelial cells inactivate or modulate serine protease activity, though only in the case of thrombin (1) and the Factor X/X<sub>a</sub> studies reported here are the cellular binding sites specific for only one protease.

When Factor X<sub>a</sub> is added to cultured endothelial cells, only a small fraction, ~1–8%, binds to the monolayers allowing for cellular processing. This suggests that Factor X<sub>a</sub> generated in the fluid phase would predominately remain in the supernatant, even in the presence of cells. Mechanisms such as the heparin-antithrombin III system may be the principal inactivators under these conditions. Alternatively, when the surface/volume ratio is increased, as in the microvasculature, and Factor X activation occurs on the cell surface, then mechanisms involving clearance of cell-associated Factor X<sub>a</sub> may become important. Factor X activation on the endothelial cell surface (Fig. 9) results in the formation of Factor X<sub>a</sub> amidolytic activity in the supernatant, but this represents only 44% of the total Factor X<sub>a</sub> generated. The remainder becomes internalized and degraded, and thus does not enter the fluid phase as intact Factor X<sub>a</sub>.

Recent studies (49) have shown that endothelial cells can participate in a series of procoagulant reactions terminating in the formation of a fibrin clot closely associated with the cell surface. These reactions can be initiated by activation of cell-bound Factor IX by the intrinsic or extrinsic pathway (7). Once Factor IX<sub>a</sub> is formed, an endothelial cell-localized Factor IX<sub>a</sub>-VIII-X complex assembles, and results in the activation of Factor X (50). When Factor X<sub>a</sub> is formed on the cell surface, it can associate with endothelial cell Factor V (10), promoting activation of prothrombin (8, 9), or it can be removed from the cell surface by endocytosis. This prompts the hypothesis that endocytosis and degradation of cell-bound Factor X<sub>a</sub>, the physiologic prothrombin activator, provides a pathway for regulation of procoagulant events on the vessel surface.

### Summary

Previous studies have shown that Factor X and Factor X<sub>a</sub> bind specifically to distinct sites on the endothelial cell surface. Since the coagulant activity of a cell-bound clotting protein is dependent on its remaining on the cell surface, endocytosis and degradation studies have been carried out. Cell-bound Factor X was internalized at 0.07 fmol/min/10<sup>6</sup> cells, a rate slower than its dissociation from the cell surface. Endocytosed Factor X was not degraded, but was returned to the cell surface. In contrast, Factor X<sub>a</sub> was internalized at an initial rate of 0.38

fmol/min/10<sup>6</sup> cells and subsequently degraded at about the same rate. The degradation of Factor X<sub>a</sub> was prevented by chloroquine. These results suggest that Factor X<sub>a</sub> is internalized and degraded by a lysosomal-dependent pathway. Studies with Factor X- and X<sub>a</sub>-colloidal gold conjugates showed endocytosis proceeding at coated pit regions, and accumulation of Factor X<sub>a</sub>-gold particles in lysosome-like structures. Endocytosis was studied as a clearance pathway for cell-bound Factor X<sub>a</sub> by activating Factor X with Factors IX<sub>a</sub> and VIII on the endothelial cell surface. Endocytosis of the Factor X<sub>a</sub> formed was significant, as only 44% of the Factor X<sub>a</sub> formed was released into the supernatant, whereas the remainder was internalized and degraded. Thus, endocytosis of Factor X<sub>a</sub> bound to its specific endothelial cell sites may be an important factor in the balance of vessel wall hemostatic mechanisms.

We thank Dr. S. Silverstein for advice and encouragement in the completion of this work.

*Received for publication 5 April 1985.*

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