Regulation of Factor IX_a In Vitro in Human and Mouse Plasma and In Vivo in the Mouse

Role of the Endothelium and the Plasma Proteinase Inhibitors

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Abstract. The regulation of human Factor IX_a was studied in vitro in human and mouse plasma and in vivo in the mouse. In human plasma, ~60% of the ^{125}I-Factor IX_a was bound to antithrombin III (ATIII) by 2 h, with no binding to α_2-macroglobulin or α_1-proteinase inhibitor, as assessed by gel electrophoresis and IgG-antiproteinase inhibitor-Sepharose beads. In the presence of heparin, virtually 100% of the ^{125}I-Factor IX_a was bound to ATIII by 1 min. The distribution of ^{125}I-Factor IX_a in mouse plasma was similar. The clearance of ^{125}I-Factor IX_a was rapid (50% clearance in 2 min) and biphasic and was inhibited by large molar excesses of ATIII-thrombin and α_1-proteinase inhibitor-trypsin, but not α_2-macroglobulin-trypsin; it was also inhibited by large molar excesses of diisopropylphosphoryl- (DIP-) Factor X_a, DIP-thrombin, and Factor IX, but not by prothrombin or Factor X. The clearance of Factor IX was also rapid (50% clearance in 2.5 min) and was inhibited by a large molar excess of Factor IX, but not by large molar excesses of Factor X, prothrombin, DIP-Factor X_a, or DIP-thrombin. Electrophoresis and IgG-antiproteinase inhibitor-Sepharose bead studies confirmed that by 2 min after injection into the murine circulation, 60% of the ^{125}I-Factor IX_a was bound to ATIII. Organ distribution studies with ^{125}I-Factor IX_a demonstrated that most of the radioactivity was in the liver. These studies suggest that Factor IX_a binds to at least two classes of binding sites on endothelial cells. One site apparently recognizes both Factors IX and IX_a, but not Factor X, Factor X_a, prothrombin, or thrombin. The other site recognizes thrombin, Factor X_a, and Factor IX_a, but not the zymogen forms of these clotting factors. After this binding, Factor IX_a is bound to ATIII and the complex is cleared from the circulation by hepatocytes.

Introduction

Factor IX is a vitamin K-dependent coagulation protein that can be activated by both the intrinsic and extrinsic pathways (1). Localization of the reactions of the coagulation cascade to the site of vascular injury and the regulation of the activated coagulation factors are important in thrombosis and hemostasis. Recently, Factors IX and IX_a (the activated form of Factor IX) were shown to bind to the same site on endothelial cells in tissue culture (2, 3). Factor X, prothrombin, and thrombin did not bind to this endothelial cell site. Endothelial cell binding may serve to localize clot-promoting activity, as bound Factor IX_a has at least three times the procoagulant activity of Factor IX_a in solution (3).

Relatively little is known about the regulation of Factor IX_a by the plasma proteinase inhibitors, other than antithrombin III (ATIII).[1] Bovine and human Factor IX_a have been shown to react slowly with ATIII, forming a stable 1:1 stoichiometric complex. This reaction is greatly accelerated in the presence of heparin (4–7). The reactions of Factor IX_a with α_2-macroglobulin

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1. *Abbreviations used in this paper:* ATIII, antithrombin III; α_2M, α_2-macroglobulin; α_1PI, α_1-proteinase inhibitor (α_1-antitrypsin); DIP-, diisopropylphosphoryl-.

(α_2M) and α_1-proteinase inhibitor (α_1PI) have not been described. The present studies were undertaken to examine the regulation of human Factor IX_a in vivo. The distribution of ^{125}I-Factor IX_a among the plasma proteinase inhibitors in human plasma in vitro was examined by sodium dodecyl sulfate- (SDS-) polyacrylamide gel electrophoresis and by IgG-antiproteinase inhibitor-Sepharose beads. These results were compared with those obtained from mouse plasma, and the in vivo regulation of Factor IX_a was then studied using our previously described mouse model. We have recently shown that Factor X_a binds to an endothelial cell thrombin-binding site; this binding alters the proteinase inhibitor specificity of Factor X_a such that α_2M is the primary in vivo inhibitor of Factor X_a, whereas α_1PI is the primary in vitro inhibitor of Factor X_a (8). In light of these results, the endothelial binding of Factor IX_a and the possible role of this binding in the regulation of Factor IX_a were examined in vivo in our mouse model.

Methods

Reagents. Reagents were obtained as previously described (8).

Proteins. Human Factor IX was purified as previously described (9). Human prothrombin and Factor X were prepared by the method of Miletich et al. (10). Human α-thrombin (specific activity 2,700 U/mg), DIP-thrombin, Factor X_a, and DIP-Factor X_a were prepared as previously described (8, 11, 12). Diisopropylfluorophosphate (20 mM) failed to inhibit Factor IX_a, even after reaction for 6 or 24 h; this is consistent with previous studies (6). Human Factor XI_a was prepared as described by Chung et al. (9) and used to activate Factor IX as previously described (13). Activation was 80–85% complete by 90 min, as determined by SDS-polyacrylamide gel electrophoresis under reducing conditions. α_2M, ATIII, and α_1PI were purified from human plasma as previously reported (11, 14, 15). Mouse ATIII was prepared essentially as described for human ATIII (11).

Trypsin was purchased from Worthington Biochemicals (Freehold, NJ) and was 70% active, as determined by active site titration (16). Trypsin complexes of α_2M, α_1PI, and ATIII were prepared as previously described (8).

Protein concentration. The concentrations of purified proteins were determined using the extinction coefficients and molecular weights previously reported (6, 10, 11, 17–23).

Protein radiolabeling. Human α_2M, α_1PI, and ATIII, and mouse ATIII were radiolabeled with ^{125}I by the solid state lactoperoxidase method (24). Radioactivity was measured in a gamma counter (AW14-120; Scientific Products, American Hospital Supply Corp., McGaw Park, IL). Proteins were labeled to specific radioactivities of 1,000–2,000 cpm/ng and assayed for activity. α_2M was assayed by the method of Ganrot (25). α_1PI was assayed as described by Dietz et al. by measuring the inhibition of the hydrolysis of α-*N*-benzoyl-DL-arginine-*p*-nitroanilide by trypsin (26). ATIII activity was determined as the inhibition of the thrombin-catalyzed hydrolysis of the chromogenic substrate S-2238 (27). In all cases, proteins retained at least 95% activity after radiolabeling. Human Factor IX was radiolabeled with ^{125}I using Iodobeads (Pierce Chemical Co., Rockford, IL) in 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4. Typically, 100 μg Factor IX was incubated with 1.5 mCi ^{125}I and two beads at room temperature for 30 min with mixing. Specific radioactivities of 1,000–1,500 cpm/ng were routinely obtained. The radiolabeled Factor IX displayed activity identical to that of the unlabeled Factor IX, both species being 80–85% activated by Factor XI_a in the presence of Ca^{++} ions, as assessed by SDS-polyacrylamide gel electrophoresis under reducing conditions.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis of purified protein samples was performed using 5 and 7.5% acrylamide slab gels as described by Wycoff et al. (28). SDS-polyacrylamide gel electrophoresis of plasma samples was performed on 5% slab gels according to the method of Weber and Osborn (29). 25-μl plasma samples were denatured in 75 μl 6% SDS at 95°C for 2 min. After drying, gels containing radioactivity were placed against Kodak XAR-2 film (Cardinal X-ray, Winston-Salem, NC) with an image-intensifying screen overnight at −70°C to prepare autoradiograms. Gel lanes were then sliced into sections 3 mm long in the direction of migration and the radioactivity in each slice was measured in a gamma counter. Protein content was then calculated using the previously determined specific radioactivities of the preparations. Gel profiles were plotted and relative peak areas were obtained by cutting and weighing the peaks.

Reaction of Factor IX_a with purified plasma proteinase inhibitors. Factor IX_a and ^{125}I-Factor IX_a were incubated with human α_2M, α_1PI, and ATIII, and with mouse ATIII for 2 h at 23° and 37°C in equimolar ratios and at proteinase inhibitor excess. The degree of complex formation was then determined by SDS-polyacrylamide gel electrophoresis, according to the method of Weber and Osborn (29), as described above, to assess the degree of covalent binding of ^{125}I-Factor IX_a to each inhibitor and to establish reference markers for the in vitro experiments with mouse and human plasma and for the in vivo clearance studies. In several experiments, the mixture resulting from the incubation of ^{125}I-Factor IX_a was subjected to chromatography on Ultrogel AcA22 (LKB Instruments Inc., Gaithersburg, MD) before electrophoresis.

IgG-antiproteinase inhibitor-Sepharose beads. The IgG fraction of goat antihuman α_2M, α_1PI, or ATIII was coupled to CNBr-activated Sepharose 4B CL as previously described (8). The beads were characterized by incubating 50 μl of the resultant IgG-Sepharose preparations with 1–500 μg ^{125}I-labeled human α_2M, α_1PI, and ATIII, and mouse ATIII, or with the corresponding ^{125}I-proteinase inhibitor-trypsin complex in 500 μl of 50 mM Tris-HCl, 0.15 M NaCl, 60 mg/ml bovine serum albumin, pH 7.40, at 37°C for 2 h with constant mixing in a series of saturation curves. The tubes were then centrifuged in an Eppendorf microcentrifuge. The supernatants were removed and the beads were washed three times with cold Tris buffer (1 ml each). The bottoms of the tubes were cut off and the radioactivity content in beads, supernatants, and washes was determined. Under the conditions chosen for the experiments described below, 80–90% of the applied ^{125}I-labeled proteinase inhibitors bound to the corresponding IgG-Sepharose, with ~1.5% nonspecific binding, as determined by incubating the noncorresponding inhibitors with a given IgG-Sepharose sample. The binding of the proteinase inhibitors to their corresponding IgG-Sepharoses was virtually unaffected by the formation of trypsin complexes before incubation with the beads. ^{125}I-Factor IX_a was also incubated with the IgG-Sepharoses; maximal binding of this ligand to any of the IgG-Sepharose preparations was 12%.

Mouse plasma. Mouse plasma for in vitro studies was obtained by incising anesthetized mice in the midline and cannulating the inferior vena cava. Blood was drawn into syringes containing 0.1 vol 3.8% sodium citrate and centrifuged immediately. The plasma was drawn off and used in experiments within 1 h of bleeding.

Plasma studies in vitro. Citrated human or mouse plasma (250 μl) was incubated with 1 μg ^{125}I-Factor IX_a at 37°C. Samples (25 μl) were removed at various times and either incubated with IgG-Sepharose beads in 500 μl of 50 mM Tris-HCl, 0.15 M NaCl, 60 mg/ml albumin, pH

7.40, as described above, or denatured immediately for SDS-gel electrophoresis.

Plasma elimination studies. Plasma elimination studies of ^{125}I-Factor IX and ^{125}I-Factor IX_a (0.5 to 1 μg) alone or in the presence of large molar excesses of unlabeled proteins were performed in CD-1 female mice, as previously described (8). To examine the distribution of Factor IX_a between the plasma and the cellular elements of blood, ^{125}I-Factor IX_a was injected and 800 μl of blood was collected after 5 min into 0.1 vol 3.8% sodium citrate. The sampled blood was centrifuged at 10,000 *g* for 10 min at 4°C to pellet the cellular elements and the supernatant plasma was removed with a pipette. The cellular pellet was washed three times with 1 ml cold 0.05 M Hepes, 0.15 M NaCl, pH 7.40, and the radioactivity content of the plasma, washes, and the cellular pellet was determined. In some plasma elimination studies, duplicate samples were taken at each time point. One sample was counted in a gamma counter, the other was drawn into 0.1 vol 3.8% sodium citrate and centrifuged immediately at 4°C. The plasma was then denatured immediately for SDS-gel electrophoresis. In general, studies were performed at least four times.

Tissue distribution studies. Organ distribution studies were performed as previously described after injection of 5 μg of ^{125}I-ligand (30).

Results

Reaction of Factor IX_a with purified plasma proteinase inhibitors. There was no evidence of complex formation of either Factor IX_a or ^{125}I-Factor IX_a with α_2M or α_1PI after 2 h at 23°C or 37°C, as assessed by SDS-polyacrylamide gel electrophoresis (data not shown). With human or mouse ATIII, ~50% of the Factor IX_a or ^{125}I-Factor IX_a had been converted to the ATIII complex by 2 h at 23°C or 37°C. In the presence of heparin (10 U/ml), the conversion of Factor IX_a or ^{125}I-Factor IX_a to the complex with either human or mouse ATIII was virtually 100% complete by 1 min at either 23° or 37°C (data not shown).

In vitro distribution of Factor IX_a among the proteinase inhibitors in human and mouse plasma. The time course of inactivation of ^{125}I-Factor IX_a in human and mouse plasma was studied using SDS-polyacrylamide gel electrophoresis. The results obtained with human and mouse plasma were virtually identical; the time course of inactivation of ^{125}I-Factor IX_a in mouse plasma is shown in Fig. 1, lanes *a–i*, for comparison with the in vivo results described below. Complexes of α_2M and proteinase would be seen at the top of the gel lanes if they were present. The results for both human and mouse plasma are summarized in Table I. Approximately 60% of the ^{125}I-Factor IX_a was bound to ATIII by 2 h, with no binding to α_2M in either human or mouse plasma. From these data, based on the use of 1 μg of ^{125}I-Factor IX_a and 250 μl of plasma, it is possible to calculate the rate of inhibition of Factor IX_a by ATIII. Both human and mouse ATIII inhibited ~0.02 μg of human Factor IX_a/ml plasma per min. In the presence of heparin (10 U/ml), virtually 100% of the ^{125}I-Factor IX_a was bound to ATIII by 1 min (Table I).

IgG-antiproteinase inhibitor-Sepharose bead studies. Samples of human plasma, after 2 h of incubation with ^{125}I-Factor IX_a, were mixed with goat IgG-antihuman α_2M, α_1PI, or ATIII covalently linked to Sepharose. The experiment was repeated in the presence of heparin (10 U/ml) after 1 min of incubation of ^{125}I-Factor IX_a with the heparinized plasma (Table II). The experiments were repeated with mouse plasma in the presence and absence of heparin (10 U/ml) using only the anti-ATIII-Sepharose beads. The results are essentially identical to those obtained with human plasma and are shown in Table II. These results show that ATIII is the primary inhibitor of Factor IX_a in vitro in human and mouse plasma, although in the absence of heparin the reaction is slow.

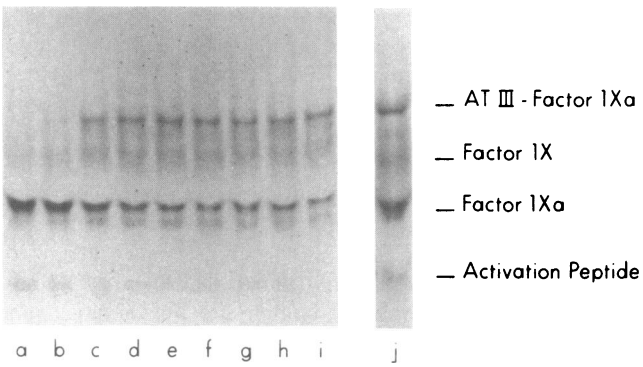

Figure 1. In vitro and in vivo distribution of ^{125}I-Factor IX_a in mouse plasma. SDS-polyacrylamide gel electrophoresis of 1 μg ^{125}I-Factor IX_a incubated with: (*a–i*) 250 μl citrated mouse plasma for various times (*a*, 0; *b*, 1; *c*, 5; *d*, 10; *e*, 20; *f*, 30; *g*, 60; *h*, 90; *i*, 120 min) or (*j*) injected into a mouse, with plasma collected after 2 min.

Plasma elimination of Factor IX. The clearance curve of ^{125}I-Factor IX is biphasic (Fig. 2 *A*), with an initial rapid dis-

Table I. In Vitro Distribution of ^{125}I-Factor IX_a in Human and Mouse Plasma Determined by SDS-Polyacrylamide Gel Electrophoresis

Plasma	Incubation conditions	Proteinase inhibitor-bound Factor IX_a (% Factor IX_a in peak)		Free Factor IX_a
		α_2M	ATIII	
Human	2 h no heparin	0	62.8	37.2
	1 min + heparin	0	99.5	0.5
Mouse	2 h no heparin	0	60.5	39.5
	1 min + heparin	0	99.0	1.0

Table II. In Vitro Distribution of ^{125}I-Factor IX_a in Human and Mouse Plasma Determined by Adsorption of IgG-Sepharose

Plasma	Incubation conditions	Antiserum to inhibitor (% total binding) α_2M	α_1PI	ATIII	Free Factor IX_a (% total)
Human	2 h no heparin	0	0	64.3	35.7
	1 min + heparin	0	0	98.7	1.3
Mouse	2 h no heparin	—	—	61.4	—
	1 min + heparin	—	—	98.1	—

appearance of protein followed by a slow phase of longer duration. Blood from one experimental animal sampled 10 min after injection of ^{125}I-Factor IX was reinjected into a second animal. The reinjected blood cleared identically (Fig. 2 *A*), thus mediating against a rapidly clearing subpopulation of modified Factor IX or contaminants. A 2,000-fold molar excess of unlabeled Factor IX inhibited the clearance of labeled Factor IX, prolonging the half-life of ^{125}I-Factor IX from 2 to 10 min (Fig. 2). These results suggest that Factor IX is rapidly cleared from the circulation, probably owing to endothelial cell binding as previously described in vitro (3). The slow late phase of clearance probably results from equilibration of the injected Factor IX with the murine Factor IX pool. To establish the specificity of this clearance process, competition experiments were performed with Factor X and prothrombin. Even at a 2,000-fold molar excess, neither species inhibited the clearance of ^{125}I-Factor IX (Fig. 2 *A*). Competition experiments were also performed with DIP-Factor X_a and DIP-thrombin, with no inhibition of the clearance of ^{125}I-Factor IX, even at 2,000-fold molar excesses (Fig. 2 *B*).

Plasma elimination of Factor IX_a. The clearance of ^{125}I-Factor IX_a was rapid and biphasic (Fig. 3 *A*). To further characterize the clearance of Factor IX_a, competition experiments were performed with unlabeled Factor IX, Factor X, prothrombin, DIP-Factor X_a, and DIP-thrombin (Fig. 3, *A* and *B*). Among the zymogens, only Factor IX inhibited the clearance of ^{125}I-Factor IX_a, indicating that Factor IX and Factor IX_a are cleared by a site that recognizes both the zymogen and the activated species. Both DIP-thrombin and DIP-Factor X_a also inhibited the clearance of ^{125}I-Factor IX_a at 2,000-fold molar excess. These results indicate that Factor IX_a binds to the high affinity endothelial cell thrombin-binding sites described by Lollar and Owen (31), which we have recently shown also bind Factor X_a (8).

In order to study directly the clearance of inactivated Factor IX_a, an attempt was made to prepare DIP-Factor IX_a. Previous studies by Davie and colleagues (6) suggested that human Factor IX_a is not inhibited by diisopropylfluorophosphate. In the present study, Factor IX_a was treated with this reagent at a concentration of 20 mM for either 6 or 24 h. There was no loss of Factor IX_a activity as compared with a control preparation of Factor IX_a incubated under similar conditions in the absence of the inhibitor. This contrasts markedly with trypsin, which in 15 min is completely inactivated at a 1 mM concentration of diisopropylfluorophosphate. Thrombin is also rapidly inhibited under these reaction conditions (31). Therefore, it was impossible to study the clearance of DIP-Factor IX_a.

The possibility that the cellular elements of blood are in-

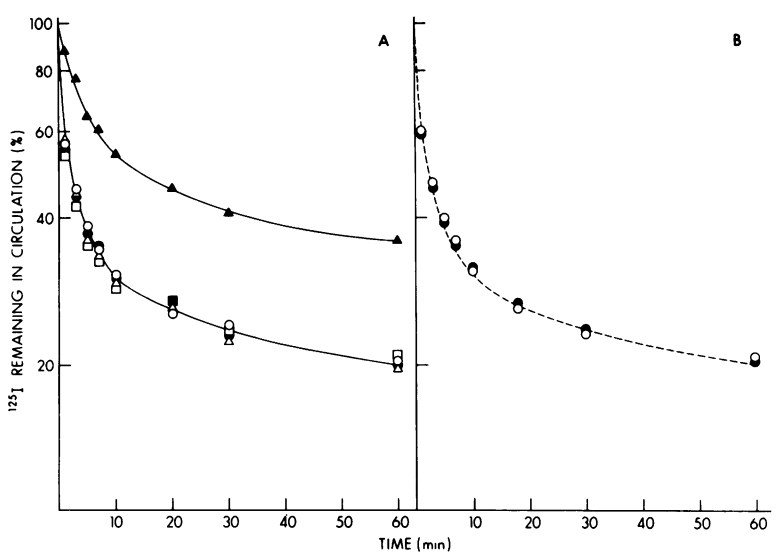

Figure 2. Clearance from the circulation of intravenously injected Factor IX. ^{125}I-labeled protein (0.5–1.0 μg) was injected into mice and blood samples were collected at intervals. (*A*) ^{125}I-Factor IX (●). Reinjection study of ^{125}I-Factor IX (○). The clearance of ^{125}I-Factor IX in the presence of 2,000-fold molar excesses of Factor IX (▲), Factor X (△), and prothrombin (□). (*B*) The clearance of ^{125}I-Factor IX in the presence of 2,000-fold molar excesses of DIP-Factor X_a (●) or DIP-thrombin (○). The clearance of Factor IX alone is shown as a dashed line for comparison.

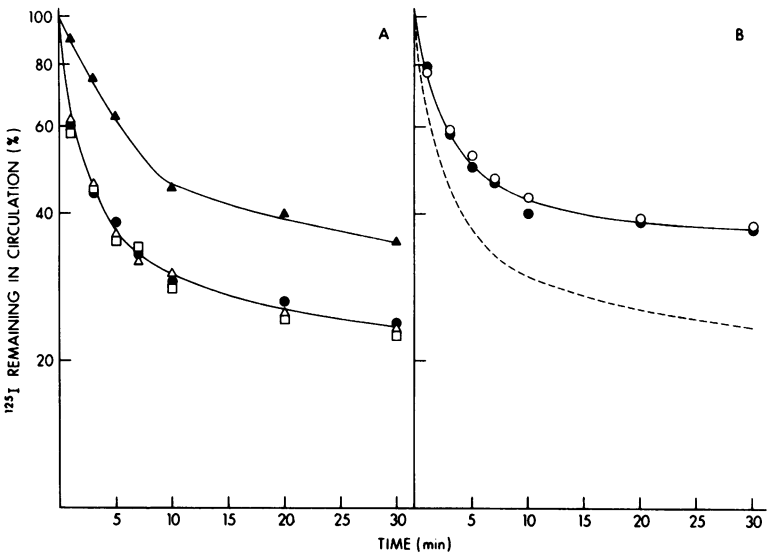

Figure 3. Clearance from the circulation of intravenously injected Factor IX_a. Clearance studies as described in Fig. 2. (*A*) ^{125}I-Factor IX_a (●). The clearance of ^{125}I-Factor IX_a in the presence of 2,000-fold molar excesses of Factor IX (▲), Factor X (△), or prothrombin (□). (*B*) The clearance of ^{125}I-Factor IX_a in the presence of 2,000-fold molar excesses of DIP-Factor X_a (●) or DIP-thrombin (○). The clearance of Factor IX_a alone is shown as a dashed line for comparison.

volved in the catabolism of Factor IX_a was investigated by injecting ^{125}I-Factor IX_a into a mouse, collecting blood into 0.1 vol sodium citrate, separating the plasma and cellular elements as described in Methods, and determining the radioactivity content of each. The cellular pellet contained 0.4% of the recovered radioactivity; the remaining 99.6% was in the plasma.

The role of the plasma proteinase inhibitors in the catabolism of Factor IX_a was investigated using trypsin complexes of α_2M and α_1PI, and ATIII-thrombin. The clearance pathways for proteinase complexes of these inhibitors have been characterized in mouse (11, 14, 15). At a 2,000-fold molar excess, α_2M-trypsin failed to inhibit the clearance of Factor IX_a (Fig. 4). At 2,000-fold molar excesses, both ATIII-thrombin and α_1PI-trypsin inhibited the clearance of ^{125}I-Factor IX_a, with α_1PI-trypsin providing somewhat poorer competition. These results are consistent with the clearance of proteinase complexes of ATIII and α_1PI by the same hepatocyte receptor (15).

To characterize further the proteinase inhibitor-dependent phase of Factor IX_a clearance, complexes of human ATIII-^{125}I-Factor IX_a were prepared and used in clearance studies (Fig. 5). The clearance of ATIII-^{125}I-Factor IX_a was extremely rapid (50% clearance in 1.5 min). The clearance of ATIII-^{125}I-Factor IX_a was inhibited by 2,000-fold molar excesses of ATIII-thrombin and to a slightly lesser degree by α_1PI-trypsin.

Organ distribution of ^{125}I-Factor IX and ^{125}I-Factor IX_a. Radiolabeled Factor IX or Factor IX_a was injected intravenously. The distributions of radioactivity at 2 and 20 min are shown in Table III. Both species are found predominantly in the liver.

In vivo distribution of ^{125}I-Factor IX_a among the plasma proteinase inhibitors. A plasma sample obtained 2 min after the injection of ^{125}I-Factor IX_a was subjected to SDS-polyacrylamide gel electrophoresis (Fig. 1, lane *j*). By this time 50% of the ligand had left the plasma compartment. The relative amounts of ^{125}I-Factor IX_a in the various species are shown in

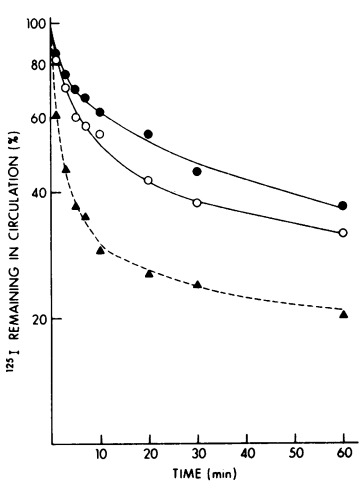

Figure 4. Clearance from the circulation of intravenously injected Factor IX_a. Clearance studies are as described in Fig. 2. The clearance of ^{125}I-Factor IX_a in the presence of 2,000-fold molar excesses of ATIII-thrombin (●), α_1PI-trypsin (○), and α_2M-trypsin (▲). The clearance of ^{125}I-Factor IX_a alone is shown as a dashed line for comparison.

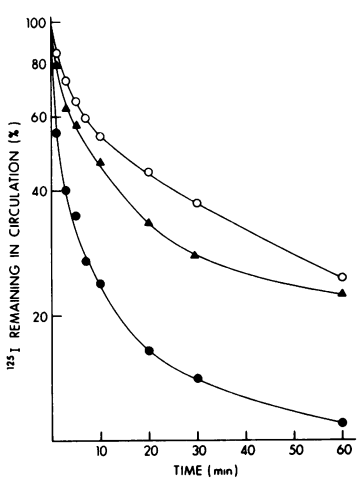

Figure 5. Clearance from the circulation of intravenously injected ATIII-^{125}I-Factor IX_a complexes. Clearance studies are as described in Fig. 2. Clearance of ATIII-^{125}I-Factor IX_a (●). Clearance of ATIII-^{125}I-Factor IX_a in the presence of 2,000-fold molar excesses of ATIII-thrombin (○) and α_1PI-trypsin (▲).

Table III. Organ Distribution of ^{125}I-Factor IX or ^{125}I-Factor IX$_a$

	% Recovered dose		
	Factor IX	Factor IX$_a$	
	2 min	2 min	20 min
Heart	0.7	0.6	0.5
Lung	2.0	2.4	1.9
Spleen	5.3	4.0	4.2
Kidneys	5.5	7.7	10.4
Liver	86.0	85.0	83.0

Table IV. A duplicate plasma sample was incubated with IgG-anti-ATIII-Sepharose beads, and the distribution of ^{125}I-Factor IX$_a$ was calculated (Table IV). These results demonstrate that nearly 60% of the recovered ^{125}I-Factor IX$_a$ is in the form of the complex with ATIII after 2 min, yielding a calculated rate of inhibition of ~0.1 μg human Factor IX$_a$/ml plasma per min, assuming a plasma volume of ~1.5 ml in the mouse (14).

Discussion

These studies were undertaken to elucidate the catabolism of Factor IX$_a$. Relatively little is known about the reaction of Factor IX$_a$ with the plasma proteinase inhibitors, except for ATIII. Factor IX$_a$ reacts slowly with ATIII in the absence of heparin (4–7). The reactions of Factor IX$_a$ with α_2M and α_1PI have not been described. These results were confirmed using Factor IX$_a$ and the purified plasma proteinase inhibitors. The inhibition of Factor IX$_a$ by ATIII was slow in the absence of heparin. In the presence of heparin (10 U/ml) the reaction was essentially 100% complete by 1 min. There was no evidence of complex formation with either α_1PI or α_2M, even after 2 h incubation with Factor IX$_a$.

In vitro studies with human and mouse plasma confirmed the results obtained with the purified inhibitors, as ATIII was shown to inhibit >60% of the Factor IX$_a$ by 2 h, with no evidence of Factor IX$_a$ bound to either α_2M or α_1PI. These results also rule out the possibility of proteinase transfer to α_2M, as described for porcine trypsin with α_1PI and α_2M (32). These results indicate that ATIII is the primary inhibitor of ATIII in vitro in human and mouse plasma. Clearance studies with mice were then undertaken to determine if this result also holds in vivo. We have recently shown that endothelial cell binding alters the proteinase inhibitor specificity of Factor X$_a$, such that α_2M becomes the primary in vivo inhibitor of Factor X$_a$, whereas α_1PI is the primary in vitro inhibitor of Factor X$_a$. Recently, it has been shown in vitro that Factor IX binds to a specific site on endothelial cells and that this site can also bind Factor IX$_a$, but not prothrombin, thrombin, or Factor X (2, 3). The clearance of ^{125}I-Factor IX was rapid and was inhibited by a large molar excess of unlabeled Factor IX, but not by large molar excesses of prothrombin, Factor X, DIP-thrombin, or DIP-Factor X$_a$. We attempted to prepare DIP-Factor IX$_a$ for study, but were unable to obtain this derivative even under extreme reaction conditions. This observation agrees with previous studies by Davie and colleagues (6).

Table IV. In Vivo Distribution of ^{125}I-Factor IX$_a$ Among the Plasma Proteinase Inhibitors by SDS-Polyacrylamide Gel Electrophoresis and IgG Sepharose

	% Total Factor IX$_a$			
	α_2M	α_1PI	ATIII	Free Factor IX$_a$
SDS-polyacrylamide gel	0	—	57.1	42.9
IgG Sepharose	—	—	59.6	40.4

The results of the present study are consistent with the in vitro endothelial cell binding experiments described above. The clearance of ^{125}I-Factor IX$_a$ was also rapid and was inhibited by a large molar excess of unlabeled Factor IX, but not by large molar excesses of prothrombin and Factor X. The clearance of ^{125}I-Factor IX$_a$ was also inhibited by large molar excesses of DIP-thrombin and DIP-Factor X$_a$, indicating that Factor IX$_a$ also binds to endothelial cell thrombin binding sites described by Lollar and Owen (31), which we have recently shown also bind Factor X$_a$ (8). This result contrasts with the in vitro endothelial cell binding studies described above, where thrombin did not inhibit the binding of Factor IX$_a$ to endothelial cells. This discrepancy may be due to cell damage or to loss of the binding site during preparation of the cells for cell culture. These results, together with the clearance and competition studies with Factor IX, suggest that there is at least one class of endothelial cell binding sites that recognizes the activated species Factors IX$_a$ and X$_a$ and thrombin, but not the zymogen forms, Factor IX, Factor X, and prothrombin. The nature of the in vivo thrombin binding site is unclear. One class of endothelial cell thrombin site is thrombomodulin. The binding of thrombin to thrombomodulin alters the substrate specificity of thrombin, since the cleavage of fibrinogen and Factor V by thrombin bound to thrombomodulin is drastically reduced (33). However, thrombin bound to thrombomodulin activates protein C at least 100-fold faster than does free thrombin (34). From the data obtained to date (2, 3, 8, 33), there is no evidence that thrombomodulin is the binding site for Factor X$_a$ or Factor IX$_a$, and it seems from the present work that there are at least two classes of Factor IX$_a$ binding sites. From all these observations it seems likely that there are several classes of endothelial binding sites that bind the vitamin K-dependent proteinases.

The possibility that the cellular elements of blood may be involved in the catabolism of Factor IX$_a$ was investigated by separating the plasma and cellular elements from an in vivo blood sample by centrifugation. The plasma fraction contained 99.6% of the recovered radioactivity, indicating that the cellular

elements of blood, such as platelets and leukocytes, are not involved in the regulation of Factor IX_a.

The role of the plasma proteinase inhibitors in the catabolism of Factor IX_a was examined by competition experiments with large molar excesses of unlabeled α_2M-trypsin, α_1PI-trypsin, and ATIII-thrombin complexes. The clearance pathways for proteinase complexes of these proteinase inhibitors have been well studied (11, 14, 15), and it has been found that proteinase complexes of ATIII and α_1PI are cleared by the same receptor on hepatocytes (15). The clearance of ^{125}I-Factor IX_a was not inhibited by a large molar excess of α_2M-trypsin, indicating that α_2M is not involved in the in vivo catabolism of Factor IX_a.

ATIII-thrombin and α_1PI-trypsin inhibited the clearance of ^{125}I-Factor IX_a, with α_1PI-trypsin providing somewhat poorer competition. These results are consistent with the clearance of α_1PI- and ATIII-proteinase complexes by the same hepatocyte receptor, with α_1PI-trypsin complexes clearing with a longer half-time of elimination, as previously described (15). To characterize further the proteinase inhibitor-dependent phase of Factor IX_a catabolism, clearance studies were performed with ATIII-^{125}I-Factor IX_a complexes. The clearance of these complexes was extremely rapid (50% clearance in 1.5 min) and was inhibited by large molar excesses of unlabeled ATIII-thrombin, and to a slightly lesser extent by α_1PI-trypsin, consistent with the results described above.

Organ distribution studies with ^{125}I-Factor IX or ^{125}I-Factor IX_a indicate that most of the recovered counts are in the liver, an organ rich in endothelium, and also the site of binding of ATIII-proteinase complexes.

The distribution of Factor IX_a among the plasma proteinase inhibitors in vivo was also examined by SDS-polyacrylamide gel electrophoresis and IgG-antiproteinase inhibitor-Sepharose beads. These experiments demonstrate that nearly 60% of the recovered ^{125}I-Factor IX_a was in the form of the ATIII complex after 2 min, whereas the in vitro inhibition of Factor IX_a is considerably slower. From the data obtained in vitro in mouse and human plasma and in vivo in the mouse, we calculated the rate of inhibition of Factor IX_a by ATIII. There was at least a fivefold increase in the rate of inhibition of human Factor IX_a in vivo.

Previous studies by Stern et al. (3) demonstrated that human Factor IX_a binds to bovine endothelium and has at least three times the procoagulant activity of Factor IX_a in solution. These investigators did not examine the effect of Factor IX_a binding on the reaction of Factor IX_a with ATIII. It is interesting that the present data suggest a fivefold increase in binding of human Factor IX_a to murine ATIII in the presence of murine endothelium. While such data may not be directly comparable, both coagulant activity and binding to ATIII involve similar active site-substrate interactions, with ATIII functioning as a suicide substrate. It appears, therefore, that there is considerable conservation of functional and binding properties of the vitamin K-dependent proteinases, despite the different species employed for study. These data are consistent with previous studies from this laboratory that examined the reaction of human α-thrombin with both human and murine ATIII (11, 15), as well as with studies that compared the reactivity of human Factor X_a to that of ATIII from both species (8).

In summary, our data suggest that there are at least two classes of Factor IX binding sites in vivo. One class of sites apparently binds both Factor IX and Factor IX_a, while the other class binds the activated forms of prothrombin, Factor X, and Factor IX.

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