Fibronectin: Blood Turnover in Normal Animals and During Intravascular Coagulation

By Laurence A. Sherman and Joan Lee

Plasma fibronectin (FN) binds fibrin in vitro by both noncovalent and covalent bonds and is decreased in DIC. In rabbits, conventionally purified 125I-FN had a complex blood clearance with a late t½ of 71 hr. A large portion was apparently altered, as evinced by rapid clearance and an intravascular/total body ratio (C1) of 0.28–0.51. 3H-labeled FN, made in vivo by injection of 3H-hydroxybutyric acid, had a t½ of 73 hr. Crosstransfusion of 125I-FN and 3H-FN into a second set of animals gave similar t½s and C1s of 0.74–0.82, indicating the altered 125I-FN was biologically screened in the first animals. Other animals were gavaged 125I-fibrinogen and "screened" 125I-FN. Intravenous thrombin (50–60 U/kg/1 hr) caused a 25%–50% decrease in both 125I-fibrinogen and 125I-FN. Ancrod injection reduced fibrinogen by >90% but had no effect on 125I-FN. 125I-FN levels did not change when thrombin was given after ancrod. No crosslinked FN-fibrinogen α-chain was found in the plasma, nor was the thrombin-induced fall in FN affected by spermidine blockade. These experiments demonstrate that FN and fibrin bind in vivo during defibrination and are rapidly cleared from the blood. The abnormal fibrin resulting from ancrod either does not bind FN in vivo or does so reversibly.

Covalent and Noncovalent Binding of FN to Fibrin

In Vitro Methods

Covalent and noncovalent FN-fibrinogen reaction mixtures were subjected to 3%-7% gradient slab gel electrophoresis. Noncrosslinked FN/fibrinogen reaction mixtures were subjected to noncovalent binding assay to fibrinogen/fibrin in vivo, in the same manner that it does in vitro. To investigate further the in vivo pathobiologic role of FN, studies were performed in animals to elucidate the normal clearance of FN, and in addition, to examine its turnover in experimental intravascular coagulation. This forms the basis of the present report.

MATERIALS AND METHODS

Homologous fibrinogen fraction I-4 was purified from citrated rabbit plasma by the method of Blomback and Blomback. 1 Rabbit FN was purified by modification of the methods of Mosesson and Umfleet, 4 Mosher, 2 and also by the gelatin affinity technique of Rousslahti et al., 3 including use of arginine as an eluant. 10

In Vitro Methods

Covalent and Noncovalent Binding of FN to Fibrin

Fifty microliters (5 μg) of factor XIIIa was activated with 25 μl (0.5 U) of thrombin at 37°C for 5 min. Then 25 μl of CaCl2 (50 μM) was added and incubated for another 10 min. After that, a solution containing 150 μg of fibrinogen (675 μg), 50 μg of FN (150 μg), 25 μl dithiothreitol (DTT) (50 μM), and 175 μl of 0.14M sodium chloride 0.01M Tris-chloride, pH 7.4, buffer was added. The reaction mixture was incubated at 37°C for 2 hr. An equal volume of 2% SDS and 2% DTT in 10M urea was added to stop the reaction. After the clot was dissolved, 10–20 μl of the reaction mixture was subjected to 3%-7% gradient slab gel electrophoresis. Noncrosslinked FN/fibrinogen was prepared by the same method as the covalizing reaction, but without factor XIII and calcium chloride in the reaction mixture.

Gradient Slab Gel Electrophoresis

Polyacrylamide gradient slab gel electrophoresis was performed by the modified methods of Laemmlil 12 and Studier. 13 The covalent and noncovalent FN-fibrinogen reaction mixtures were subjected to a 3%-7.5% gradient and 0.75 mm thickness slab gel electrophoresis at 0.025M Tris, 0.192M NaCl, and 0.1% SDS electrode buffer, pH 8.3, with a current of 15 mA per gel for 3 hr. After the run, the gel was stained with Coomasie blue solution overnight, and destained with 7.5% acetic acid solution. For storage or autoradiography, the destained slab gel was placed on filter paper and air bubbles trapped beneath the gel were removed. For autoradiography, the gel was covered with a piece of Saran wrap and laid on the gel dryer for 60 min. After drying, the sheet of gel and 5 × 7 inches of Kodak X-Omat R film were placed in stainless steel cassettes overnight, and the film was developed in an automatic X-Omat processor.

Animal and Sample Collection

New Zealand white male rabbits, weighing from 2.5 to 3.5 kg, were used. Blood samples were drawn from the median ear artery using 1/100 volume of 38% sodium citrate as the anticoagulant.

Protein Radiolabeling In Vitro and Sample Measurement

(A) Both FN and fibrinogen were radioiodinated with 131I or 125I by the ICI method of McFarlane. 14 The final iodine content was ±0.5 atom/molecule of protein.
FN was radiolabeled by a solid phase lactoperoxidase method using Enzymobeads (Bio-Rad Laboratories). One-hundred micrograms (200 μl) FN, 100 μl 1% enzymebeads, 10 μl 125I-Na (1.0 mCi), 50 μl 1% beta-glucone, and 40 μl 1 M potassium phosphate (pH 7.0) were added together. The mixture was allowed to sit at room temperature for 20 min, then applied to a Sephadex G-25 superfine gel filtration column. Prior to radiolabeling, the column was first washed with water and then washed 3 times with Dulbecco phosphate-buffered saline (without calcium and magnesium) with 0.1% BSA (heat denatured). The labeling efficiency was 85%-90%.

Clearance of In Vivo Labeled (3H) FN Versus In Vitro Labeled 125I-FN

Twenty-five to 50 μCi of tritiated, mixed amino acids (New England Nuclear, Boston, Mass.) were injected into the rabbits. Following injection, serial blood samples were obtained. The separated plasma samples were passed over gelatin affinity columns and the bound FN subsequently was eluted from the column with 8M urea. This was treated with TCA as described above. The radioactivity of the material was then determined in a beta scintillation counter. For purposes of comparing the clearance of in vitro labeled FN versus in vivo H-incorporated material, 125I-labeled purified FN was injected into the same animals at varying times before or after injection of the H amino acids. Radioactivity of the 125I and the 3H-labeled material was separately determined on the same samples from the affinity columns. As a precaution against artefactual readings of the H measurements because of the presence of 125I, the 3H counts were repeated 1 mo (4 half-lives for 125I) later.

Normal In Vivo Distribution of FN

Purified 125I-FN was injected and serial plasma samples obtained. The clearance curves were analyzed by the method of Matthews to obtain blood t1/2 and C1 (intravascular/total body distribution).

Clearance of In Vivo Labeled (3H) FN Versus In Vitro Labeled 125I-FN

To elucidate any potential effect of thrombin on FN other than through fibrin formation, the following experiment was performed. Animals were defibrinated with ancrod (1–2 U/kg). When defibrination and clearance of fragments were effectively complete, 50–70 U/kg of thrombin were injected over 1 hr.

Crosslinking Blocking

Animals were injected with 10–15 μCi 125I-FN and 125I fibrinogen. After 24 hr, the first 3.0-mI blood sample was collected. The animals were then injected with 7 μmole spermidine in 3.0 ml normal saline calculated to give a plasma concentration of 50–70 μM. Subsequently, 70 μmole spermidine with 65 U thrombin/kg body weight in 18 ml of normal saline were injected over a period of 1 hr using an infusion pump. These doses were calculated to maintain concentrations well above the in vitro concentration (10−3 M) of spermidine, which give 100% blocking of crosslinking. Once the injection was completed, 1-hr, 24-hr, and 48-hr samples were drawn. The radioactivity of 12% trichloroacetic acid (TCA) precipitable and nonprecipitable proteins and the specific activity of clottable fibrinogen were determined as previously described.

RESULTS

Normal Clearance

131I-FN was cleared from the blood with a large, rapidly cleared, early component (Fig. 1). In this and succeeding experiments, the cpm/ml of the initial blood sample is considered 100% radioactivity. The late component had a t1/2 of 72 hr. Examination of the disappearance curves indicated a large portion of the radioactivity disappearing from the blood stream was not removed by the techniques.
within the first 24 hr. While a portion of this disappearance represents intravascular/extravascular redistribution, the $C_t$ of 0.45 is lower than would be expected for a protein of the size of fibronectin (mol wt ~440,000). Although $C_t$ cannot be determined with the $^3$H in vivo incorporated FN, a similarly large early fall in blood radioactivity was not seen. This suggested the early component seen with $^{131}$I-FN was in part due to altered or denatured $^{125}$I-FN.

Normal Distribution

Because of the aforenoted question of partial denaturation, the cross-injection studies were performed. When plasma containing $^3$H-FN and $^{131}$I-FN was given to a second animal, the same late curve was noted for $^{131}$I-FN (Fig. 2) as with $^{125}$I-FN without cross-injection (Fig. 1). However, a smaller early rapid component was found with a $C_t$ of 0.80, presumably representing only intravascular/extravascular redistribution. After cross-injection, the $^3$H-FN and $^{131}$I-FN had the same clearance, indicating the apparently denatured $^{131}$I-FN had been removed in the first (donor) animal, and the remaining $^{131}$I-FN behaved in vivo the same as native ($^3$H) FN. The $^3$H-FN and $^{131}$I-FN had the same SDS-acrylamide gel mobility, with only a small portion being in a monomer form. As these samples had been subjected to a gelatin-urea column, no conclusions could be drawn concerning the natural occurrence of monomer.

Several different purification procedures for $^{125}$I-FN were tried (Table 1). Using the $^3$H-FN as a standard, all the $^{125}$I-FN preparations exhibited partial alteration as assessed by a low $C_t$. In all instances, the late $t_1/2$ was comparable to that noted with $^3$H-FN. All screened (crosstransfused) preparations had $C_t$ values of 0.74–0.82. In two experiments with gelatin column preparations of FN, radioiodination was done with lactoperoxidase. Partial alteration was noted as with the ICl-labeled preparations.

### Table 1. Clearance of FN and Fibrinogen

<table>
<thead>
<tr>
<th>Method</th>
<th>No.</th>
<th>$C_t$</th>
<th>Late $t_1/2$</th>
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<tbody>
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<td>$^3$H in vivo incorporation</td>
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</tr>
<tr>
<td>FN</td>
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<td>0.81</td>
<td>73</td>
</tr>
<tr>
<td>Fibrinogen</td>
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<td>0.81</td>
<td>61</td>
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<tr>
<td>Radioiodination in vitro</td>
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</tr>
<tr>
<td>FN</td>
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<td></td>
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<td>DEAE, sepharose</td>
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<tr>
<td>Fibrinogen</td>
<td>4</td>
<td>0.77</td>
<td>62</td>
</tr>
</tbody>
</table>

Several different purification procedures for $^{125}$I-FN were tried (Table 1). Using the $^3$H-FN as a standard, all the $^{125}$I-FN preparations exhibited partial alteration as assessed by a low $C_t$. In all instances, the late $t_1/2$ was comparable to that noted with $^3$H-FN. All screened (crosstransfused) preparations had $C_t$ values of 0.74–0.82. In two experiments with gelatin column preparations of FN, radioiodination was done with lactoperoxidase. Partial alteration was noted as with the ICl-labeled preparations.
Crosslinking Studies

Plasma samples drawn immediately after completion of thrombin injection (see above) were examined for covalently bound FN-fibrin α-chain by reduced gel electrophoresis. Gels were compared with in vitro crosslinked standards by both gel slicing and autoradiography. No crosslinked FN α-chain species could be found in the plasma samples, although γ-γ dimers and α polymers were present.

Indirect Evidence for FN-Fibrin Crosslinking

Inhibition of possible FN-fibrin crosslinking was performed by injection with spermidine during thrombin injection. The fall in fibrinogen and FN was the same as in control thrombin experiments without spermidine injection, e.g., no evidence for spermidine blocking factor XIII crosslinking of FN to fibrin could be found.

DISCUSSION

The observed intra/extravascular distribution (C1) of 3H-FN is in keeping with its molecular weight and

Thrombin Injection

Injection of thrombin (60–70 U/kg) after administration of 125I-FN and 131I-fibrinogen resulted in a rapid fall in both FN and fibrinogen during the period of infusion (Fig. 3). Thereafter, FN and fibrinogen disappearance approximated that seen in the normal studies. Higher doses of thrombin frequently resulted in the death of the animals.

Ancrod

Infusions of ancrod caused virtually complete defibrination, with clottable fibrinogens of 2%–5% of the starting values. In contrast to the thrombin studies, FN levels did not abruptly change, either during or after the infusions (Fig. 4).

Ancrod/Thrombin

Eighteen hours after defibrination with ancrod, thrombin (60–70 U/kg) was injected. No change in the FN occurred (Fig. 5), indicating intact fibrinogen had to be present for thrombin to affect FN clearance.
the distribution ratio of other large plasma proteins. Its blood t1/2 is relatively short but on the same order as fibrinogen. The fibrinogen t1/2 found here is in keeping with previous data in rabbits here and elsewhere.14,18 Calculation of fractional catabolic rates values yields of 29.2%/24 hr for FN and 34.9%/day for fibrinogen. Other proteins of immunologic importance have a wide range of fractional catabolic rates ranging from 6.8%/24 hr for IgG19 to 56.4% for C320 in man. Coagulation proteins t1/2 in man vary from 6 hr for factor VII21 to 6 days for factor XIII.22

The lower C1 found with in vitro labeled purified FN presumably resulted from alteration, possibly denaturation of part of the 125I-FN. The agreement of the t1/2s of the late components of 125I-FN and 3H-FN demonstrates that part of the purified 125I-FN was not altered and behaved normally. Presumably, the change of FN occurred during purification rather than during radiolabeling. Both the ICl and lactosuperoxidase methods are known to result in negligible denaturation.14 This partial alteration of FN by a variety of purification methods would suggest that caution is advisable in interpreting in vivo findings using non-screened purified FN.

Two types of interactions between FN and fibrin have been described. The first is a noncovalent form, enhanced in the cold and by heparin.8 The second is covalent crosslinking by activated coagulation factor XIII (transaminidase).3 In these in vitro studies, the a-chain of fibrin has been shown to be essential for the binding.2 Limited data exist to indicate that fibrin/FN interaction occurs in vivo. Bang et al.23 show comigration of immunoreactive fibrinogen and FN on gel filtration after experimental DIC. The elution point was earlier than fibrinogen or FN controls. Mosher has demonstrated decreased levels of FN in patients with DIC.8 In the present work, the parallel fall in 131I-fibrinogen and 125I-FN after thrombin administration clearly indicate in vivo interaction between fibrin and FN.

A question exists as to the mechanism. The in vitro binding of fibrin to FN described above could have occurred in vivo after thrombin converted the fibrinogen to fibrin. An alternative is a direct or indirect action of thrombin, altering or denaturing FN. The denatured FN would then be rapidly “cleared” by the reticuloendothelial system. In the experiment with sequential administration of ancrod and thrombin, fibrinogen was virtually completely removed from the blood by the venom. Thus, subsequent thrombin injection could not produce fibrin. Direct or indirect action of thrombin on FN could still have occurred. The lack of change in FN clearance found in this experiment leads to the conclusion that with intravascular coagulation, changes in FN clearance only result from interaction with fibrin.

Ancrod-derived fibrin probably does not interact with FN because of both the lack of an intact fibrin a-chain and the fact that ancrod does not activate factor XIII. The nature of the in vivo FN/fibrin binding cannot be totally resolved. In a series of experiments we were unable to find evidence for circulating covalently bound FN/fibrin. In vitro FN/fibrin crosslinking proceeds more slowly than fibrin γ-γ crosslinking.5 It is conceivable that FN/fibrin is cleared from the blood before FN/fibrin crosslinking can occur. FN/fibrin crosslinking at cell surfaces also cannot be excluded. Oh et al.24 have described injected plasma fibronectin being later found on cell surfaces.24 This suggests that plasma and tissue fibronectin are not mutually exclusive in vivo. Nothing in our results would negate this view.

Noncovalent FN/fibrinogen binding has chiefly been demonstrated at low temperatures, where it is more pronounced. However, Kaplan and Snedeker25 demonstrated that fibronectin increased fibrin solubility at 37°C, under conditions thought to exclude factor XIII crosslinking. Mosher17 has also postulated weak 37°C binding as a means of permitting factor XIII crosslinking. Kaplan and Snedeker25 hypothesized that the FN/fibrin complex serves to solubilize and carry fibrin to phagocytic sites. Kaplan et al.26 have demonstrated that increased fibronectin limits the reduction in fibrinogen found in experimental defibrination. It is conceivable that enhanced solubilization may facilitate the known thrombin neutralizing effect of fibrin. Divergent reports exist as to the necessity for FN in fibrin uptake by macrophages.27,28 Additionally as noted, fibrin may interact with cell surface or tissue FN as well.

We have established change in FN turnover in experimental intravascular coagulation and strongly implicated direct in vivo FN/fibrin interaction. However, the foregoing other reports would suggest that the dynamics and functions of FN/fibrin complex in vivo are complex and will require much further delineation.

REFERENCES
2. Blumenstock FA, Saba TM, Weber P, Laffin R: Biochemical and immunological characterization of human opsonic α-SB glyco-
FIBRONECTIN

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