Specific Uptake of Radioiodinated Fragment E₁ by Venous Thrombi in Pigs

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ABSTRACT Fragment E₁, a product of plasmin digestion of cross-linked fibrin, binds specifically in vitro to polymerized fibrin but not to fibrinogen. Purified human Fragment E₁ was radiolabeled with ¹²⁵I or ¹³¹I by the Iodogen technique. The uptake of radioiodinated Fragment E₁ in vitro into forming or preformed clots was demonstrated. Animal biodistribution studies of radioiodinated Fragment E₁ showed its rapid removal from the circulation; radioactive catabolites did not reside long in any organ and were excreted in the urine. The uptake in vivo was evaluated in pigs with preexisting venous thrombi of various ages from 1 h up to 5 d at the time of intravenous systemic injection of the tracer. Radioiodinated fibrinogen was also injected into the same animals to compare the uptake of the two tracers. Thrombus-to-blood ratios for Fragment E₁ averaged 43:1 (range 10–108) and 29:1 (range 8–107) in thrombi 1–6 h and 1–5 d old, respectively. In contrast, mean thrombus-to-blood ratios for fibrinogen were, in the same time intervals, 26:1 (range 17–41) and 2:1 (range 0.5–3.9), respectively. It is concluded that radioiodinated Fragment E₁ is a specific marker of thrombi in vivo: its uptake by fresh thrombi is better than that of labeled fibrinogen and, in contrast to radioiodinated fibrinogen, this fragment is incorporated into old thrombi as well.

INTRODUCTION

Deep vein thrombosis (DVT)¹ is a frequent complication of many clinical syndromes, including fractured hip,

major surgical procedures, stroke, and myocardial infarction; however, clinical symptoms are evident in only ~50% of cases (1). Current methods for detecting DVT are not entirely satisfactory. Contrast venography is reliable, but it is associated with a number of serious side-effects when conventional contrast medium is used (2). Doppler ultrasound and impedance plethysmography are useful only in detecting occlusive thrombi in the thigh (2); they are of limited overall sensitivity, unless performed in combination with another test providing sensitivity in the calf, such as the ¹²⁵I-fibrinogen uptake test (FUT). Unfortunately, the FUT (3, 4) requires several days before a result is obtained, and a rapid diagnosis is desirable to decide a course of therapy. More rapid radionuclide tests, such as venography using radiolabeled particulates (5–7), may be performed to diagnose DVT, but the test is not useful below the knee and is also not reliable for providing images of thrombi, owing to the dependence upon nonspecific adhesion (7). Specific agents such as ¹¹¹Indium-labeled autologous platelets have been shown to localize in thrombi to permit imaging throughout the body (8–11), but the test is not rapid: images are usually not obtained sooner than ~24 h after injection due to the slow blood clearance of platelets. Thus, the existing noninvasive tests are either not adequately specific or are too slow.

There remains a need for a rapid, noninvasive, safe, sensitive, and specific test for the detection of DVT. Such a test is needed for the assessment not only of thrombotic disease in the lower extremities, but also thrombotic and embolic disease in the heart, brain, and lungs.

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¹Abbreviations used in this paper: DVT, deep vein thrombosis; FUT, ¹²⁵I-fibrinogen uptake test.

Fragment E₁, derived from plasmin digestion of cross-linked fibrin (12), has unique binding properties. This dimeric molecule binds only to polymers of fibrin, such as are present in a thrombus, but it does not bind to fibrinogen, fibrin monomer, nor to any soluble plasma protein (13). This specificity for the major component of venous thrombi makes Fragment E₁ particularly interesting for development as a thrombus-localizing molecular marker.

In this study, Fragment E₁ was isolated from human cross-linked fibrin, radiolabeled with isotopes of iodine, and tested for binding to fresh and aged thrombi. The specificity of Fragment E₁ binding *in vitro* was assessed and followed by measurements of target-to-background uptake in thrombi *in vivo*. The uptake of radiolabeled fibrinogen in the same model has also been measured in order to compare these two thrombus-localizing agents.

METHODS

Preparation of Fragment E₁. The isolation and purification of human Fragment E₁ was carried out as previously described (14). Briefly, fibrinogen (obtained from citrated human plasma by glycine precipitation [15]) was clotted with thrombin (bovine origin, Parke-Davis, Div. of Warner-Lambert, Inc., Morris Plains, NJ), in the presence of Factor XIIIa. The resulting cross-linked fibrin was digested with plasmin for 24 h at 37°C, after which Trasylol (aprotinin, FBA Pharmaceuticals, New York; 250 units/ml final concentration) was added to stop the digestion. Gel chromatography on a 3.5 × 170-cm column of Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) was used to separate the digestion products. The (DD)E complex, the main product of the digestion, was present in the first major protein peak. The complex was dissociated by treatment with an equal volume of 6 M urea, pH 5.5, at 37°C for 1 h. A second passage through the same column resulted in the separation of Fragment E₁ from Fragment DD. Fragment E₁ was concentrated by ultrafiltration in a stirred cell with a PM 10 membrane of 10,000 mol wt cutoff (Amicon Corp., Lexington, MA), then dialyzed against 0.05 M Tris buffer containing 0.1 M NaCl, pH 7.0, and stored at -20°C. Tests used in monitoring the progress of the isolation procedure have been described in detail (12, 14).

Polyacrylamide gel electrophoresis. Batches of Fragment E₁ were analyzed in nondissociating medium in 9% Tris-glycine gels according to the method of Davis (16). Gels were stained with Coomassie Brilliant Blue R-250 and compared visually with standards. In some experiments, radioiodinated Fragment E₁ (containing 30,000–60,000 cpm) was separated in polyacrylamide gels, which were then frozen and sliced into 1-mm sections for counting in a NaI(Tl) scintillation counter (Nuclear Chicago, Des Plaines, IL).

Radioiodination of Fragment E₁. For these studies, Fragment E₁ was labeled with ¹²⁵I or ¹³¹I. Labeling was done using the iodine monochloride technique (17) or the Iodogen technique (18). Because the efficiency of incorporation of radioiodine was <50% with the iodine monochloride method, the Iodogen method eventually replaced it in order to obtain higher labeling yields. No differences were noted in the properties of the proteins labeled by the two techniques (19). For the Iodogen method, 10 µg of Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril, Pierce Chemical Co., Rock-

ford, IL) dissolved in 10 µl of chloroform was placed into a glass serum vial and the chloroform was evaporated by vacuum while constantly rotating the vial, leaving a thin coating of Iodogen on the walls. In a separate container, 1 mg of Fragment E₁ was combined with 1–3 mCi of radioactive sodium iodide and 5 µl of 0.44 mg/ml carrier sodium iodide (starting ratio = 0.88 iodine atom/Fragment E₁ molecule) in a total volume of ~0.5 ml of a 0.05 M Tris-HCl buffer containing 0.1 M NaCl, pH 7.9. The protein and radioiodide were transferred into the Iodogen container and stirred gently with a magnetic stirring bar for 1 h at room temperature. At the end of the reaction, free radioiodide was removed by passing the reaction mixture through a column of Biogel P-2 (Bio-Rad Laboratories, Richmond, CA) packed in a 10-ml disposable pipette and eluted with a 0.05 M Tris-HCl buffer containing 0.1 M NaCl, pH 7.4. The two or three fractions (0.5 ml each) containing the protein peak were combined for further use.

Incorporation of radioiodinated Fragment E₁ into plasma clots *in vitro*. Aliquots of ¹²⁵I-Fragment E₁ were added to normal human plasma, varying the molar ratios of Fragment E to fibrinogen. Clotting was initiated by the addition of calcium and thrombin to final concentrations of 2 mM and 5 U/ml, respectively. After incubation at 37°C for 2 h, the clot was wound out onto a glass rod. The radioactivity in the clot and serum were measured with a NaI(Tl) scintillation detector coupled to a gamma spectrometer (models 810 and 530, Baird Corp. Bedford, MA). In another set of experiments, plasma clots were formed at 37°C and aged for 2 h before addition of ¹²⁵I-Fragment E₁. After 1 h of further incubation, the clots were washed in normal saline and the clot, serum, and washes were counted. All samples were tested in triplicate.

Evaluation of the stability of label *in vitro*. ¹³¹I-Fragment E₁ was diluted, either with 0.05 M Tris-HCl buffer containing 0.15 M NaCl, pH 7.4, or with human citrated plasma, pH 7.4, then incubated at 37°C for several days. At the start of the experiment and at several later intervals, aliquots of the mixtures were removed and assayed for the amount of free radioiodide by instant thin-layer chromatography (ITLC-SG media, Gelman Sciences, Inc., Ann Arbor, MI) on 1 × 9-cm strips, using 85% methanol as the eluant. The ITLC strips were cut into segments, which were counted in the scintillation counter to determine the percentage of the applied radioactivity that was protein-bound (remaining at the origin).

Thrombus uptake studies in pigs. Young pigs weighing 25–35 lb were anesthetized with halothane (Halocarbon Laboratories, Hackensack, NJ) inhalation anesthesia. This mode of anesthesia was chosen for the rapidity of induction and recovery, and for the ease in quickly altering the depth of anesthesia if necessary. The face mask for administering the gas was constructed from a plastic bottle with the bottom removed and replaced with a rubber membrane with a slit cut in it; this could be stretched over the pig's snout to provide a tight seal. The pigs were not intubated. Halothane was delivered by passing oxygen through a Verni-Trol vaporizer (Ohio Medical Products, Madison, WI) at an initial level of ~4% halothane to induce deep anesthesia, then tapering off to ~1% halothane to maintain the appropriate depth of anesthesia. An incision was made in the neck of each pig to expose the jugular vein near a bifurcation. An insulated wire lead, with insulation removed to expose 1 cm of wire at the tip, was inserted into the jugular vein through the facial branch proximally into the common vein. After insertion, the tip of the wire was located ~3 in. proximal to the point of entry. The wire was connected to a voltmeter and a power supply,

the other lead of which was connected to a metal clamp located in the tissue near the outside of the vein, above the position of the exposed tip of the internal wire. Direct current (5 mA, 2 V) was passed through the vessel wall for 30 min to induce thrombus formation. Similar electrical techniques have been used to reliably produce venous thrombi in other animals (11, 20, 21). After thrombus induction, the wires and clamp were removed and the facial branch of the vein was tied off with suture. The neck wound was closed.

A mixture of 700 μCi ^{131}I -Fragment E_1 and 70 μCi ^{125}I -human fibrinogen (Kabi, Stockholm, iodinated by the ICI method [17]) was injected intravenously into pigs in which thrombi had been induced by the electrical stimulation method. The thrombi were 1 h to 5 d old at the time of injection, in order to study the uptake by thrombi of different ages. The injection was made in a superficial ear vein, if possible in the ear on the side of the pig opposite to the side on which the thrombus had been induced. 24 h postinjection, the pigs were reanesthetized with halothane and injected with 5,000 U of heparin in 2 ml of 0.05 M Tris-HCl buffer containing 0.2 M ϵ -aminocaproic acid and 0.1 M NaCl, pH 7.6, to prevent further clotting or lysis. 3 min later, a sample of blood was drawn and the jugular vein containing the thrombus was dissected free. The segment of vein was perfused with 20 U/ml heparin in Tris/ ϵ -aminocaproic acid buffer, pH 7.6, then the segment of jugular vein containing the thrombus was removed. The thrombus was then dissected free from the vessel. The thrombus and blood were weighed, then counted in a dual-channel scintillation counter (Nuclear Chicago) with windows set for ^{131}I and ^{125}I . A sample containing ^{131}I -radioactivity alone was also counted to determine the extent of crossover of ^{131}I radiation into the ^{125}I channel. When the crossover of ^{131}I in the samples was $>10\%$ of the apparent ^{125}I counts, the samples were stored frozen until the ^{131}I had decayed to a level of crossover of $<10\%$ of the ^{125}I counts, then the samples were recounted for ^{125}I .

From these data, a thrombus-to-blood radioactivity ratio was calculated as an indication of the ability of an external detection system, such as a gamma camera, to detect the radioactivity associated with the thrombus in the face of the background radiation. The ratio was obtained using the following formula:

Thrombus/blood ratio

$$= \frac{\text{net counts in thrombus} \div \text{weight of thrombus}}{\text{net counts in blood sample} \div \text{weight of blood sample}}$$

Biodistribution of radioiodinated Fragment E_1 . The organ distribution of radioactivity following intravenous injection of radioiodinated Fragment E_1 was determined in mice and pigs in order to gain some insight into the catabolic pathway and catabolic rate of the marker. In addition, the knowledge of the biodistribution is essential for later use in estimating radiation exposure to human subjects receiving the tracer for clinical diagnosis of thromboembolic disease. 26 normal mice (40 ± 7 g body wt) each received an intravenous injection (tail vein) of 6 μCi ^{131}I -Fragment E_1 . The mice were killed at 1, 3, 5, 10, 20, and 30 min, 1, 2, 4, 6, 17, 24, and 48 h postinjection (two animals at each time period). Two normal pigs (25–35 lb) were injected intravenously with 120 μCi of ^{131}I -Fragment E_1 in an ear vein, and were killed at 24 h postinjection. Tissues and fluids were sampled for counting. The whole organs were counted after dissection from mice. With the pigs, weighed representative samples of each organ were counted, and the whole organ was weighed to estimate the radioactive content of the whole

organ. Similarly, representative samples of whole blood and bone were counted, and blood volume and whole bone mass were estimated to be 7 and 10% of body weight, respectively. For each animal, the total feces and urine excreted were counted. Because thyroid uptake of free radioiodide was so significant, an additional pair of pigs was studied after giving orally 2 drops potassium iodide solution (1 g/ml, Philips-Roxane Laboratories, Inc., Columbus, OH), diluted with 10 ml of water, to attempt to block thyroid uptake. The pigs' dose of KI per unit of body weight was equal to that for a human ($\sim 3\text{--}4$ mg KI/kg body wt).

RESULTS

Labeling yields. The efficiency of incorporation of radioiodine into Fragment E_1 using the Iodogen method was $70 \pm 7\%$ (mean $\pm$ SD, $n = 12$). It was later found that addition of carrier iodide was not necessary to achieve these yields. The specific activities of ^{125}I -Fragment E_1 and ^{131}I -Fragment E_1 were 2.0 and 1.77 mCi/mg, respectively. The iodination level for these preparations was an average of 0.65 iodine atoms per molecule.

Stability of radiolabel in vitro. During the first day of incubation, there was no significant loss of label from Fragment E_1 , whether stored in buffer or in human plasma (Table I). At the end of 96 h, there had been a slight loss of the label bound to the protein: 10% loss in buffer and 7% loss in plasma. These values were of the same magnitude as those observed upon hydrolysis of other radioiodinated proteins previously studied (22) and indicated that the radioiodine should remain attached to Fragment E_1 when injected into the blood pool of an animal.

Electrophoretic profile of radioiodinated Fragment E_1 . The distribution of radioactivity in 9% Tris-glycine polyacrylamide gels under nondissociating conditions after electrophoresis of radioiodinated Fragment E_1 is shown in Fig. 1. The radioactive profile was essentially the same as the staining pattern obtained with the unlabeled Fragment E_1 , except that the small peak in gel slice 10, corresponding to Fragment E_3 , was not stained

TABLE I
Stability of ^{131}I -Fragment E_1 In Vitro

Time	Percentage of protein-bound ^{131}I	
	Stored in Tris/NaCl pH 7.4, 37°C	Stored in citrated human plasma, pH 7.4, 37°C
h		
0	86.5	95.8
19	94.1	90.9
26	95.4	94.9
96	82.4	87.5

* Assayed by instant thin-layer chromatography using 85% methanol as eluant.

FIGURE 1 Electrophoretic pattern of radiolabeled Fragment E₁. Separation of Fragment E₁ from Fragment E₃ in a Tris-glycine nondissociating buffer in 9% polyacrylamide gels.

in the unlabeled preparation. Thus, the radioiodination procedure did not induce significant aggregation or degradation of the Fragment E₁ molecules.

Clot uptake in vitro. Radioiodinated Fragment E₁ was incorporated into forming fibrin clots (Table II). The extent of the incorporation depended upon the molar ratio of Fragment E₁ to fibrinogen. A maximum of 72.1% of the labeled Fragment E₁ became bound to the clot, at the Fragment E₁/fibrinogen ratio of 0.0016. Radioiodinated Fragment E₁ bound also to preformed clots, although to a lesser extent (Table II). Again, the extent of binding depended upon the ratio of Fragment E₁ to fibrinogen and resulted in the highest binding (14.1%) at the ratio of 0.0016. Fragment E₃ was not incorporated into forming nor into preformed clots. This is in agreement with binding studies (12) in which unlabeled Fragment E₁ was observed to bind to Fragment DD of fibrin, but Fragment E₃ did not bind.

Thrombus uptake in young pigs. At the conclusion of the electrical stimulation of the jugular vein, the exposed exterior of the vein near the region of the enclosed wire tip often appeared brown in color. The brown color was used as an indication that the stimulation had done sufficient damage to induce thrombus formation. The thrombi varied in size: thrombi <30 h old at the time of dissection were generally smaller, averaging 117 ± 107 mg, while thrombi >30 h old averaged 314 ± 208 mg. All thrombi were adherent to the vessel wall, and none appeared to completely occlude blood flow. Thrombus-to-blood ratios are plotted in Fig. 2 vs. thrombus age, separately for fibrinogen and Fragment E₁ uptake. Radioiodinated Fragment E₁ localized substantially in fresh as well as old thrombi; thrombus-

to-blood ratios ranged from 8:1 to 108:1. Radioiodinated fibrinogen, however, localized appreciably only in very fresh thrombi, those up to 5.6 h old at the time of injection of the tracer. In thrombi older than this, the thrombus-to-blood ratios for fibrinogen averaged 2.0 ± 1.0 , a degree of localization not believed to be sufficient to permit satisfactory external imaging.

Biodistribution in mice and pigs. Radioiodinated Fragment E₁ left the blood pool rapidly. The tissue distribution in mice is given in Table III. The Fragment E₁ appeared to be removed by the liver, with maximum accumulation levels achieved at 10 min. The radioiodine label may be released due to the degradation of Fragment E₁ by the liver, as the successive distribution of radioactivity resembled that of free iodide: uptake by thyroid gland, appearance in the gastrointestinal tract (especially the stomach), kidneys, and urine. In later studies in humans (Knight, L. C., unpublished observations), urine radioactivity was not precipitable with 10% trichloroacetic acid (TCA), suggesting non-protein-bound radioiodide, and the fraction of blood plasma radioactivity precipitable with TCA decreased with time, suggesting that radioiodide liberated in the liver reappears in the blood.

Metabolism of radioiodinated Fragment E₁ appeared to be slower in pigs than in mice. The 24-h pig values (Table IV) correspond to the 6-h mouse values. Partially blocking the thyroid appeared to allow excretion to proceed at a more rapid rate. This treatment was par-

TABLE II
Incorporation of Fragments E₁ and E₃ into
Fibrin Clots In Vitro

¹²⁵ I-Fragment	[Fragment E]	Incorporation* into	
		forming clots	preformed clots
	nM	%	
E ₁	7.2	72.1	14.1
	3.6	44.3	9.8
	1.8	35.4	8.4
	0.9	30.4	8.3
	0.45	32.8	7.6
	0.275	30.4	7.0
E ₃	7.2	1.6	0.13
	3.6	1.4	0.11
	1.8	1.2	0.19
	0.9	1.5	0.21
	0.45	1.1	0.21
	0.275	1.2	0.13

* The percentage of total radioactivity that remains with the compressed, washed clot; the mean of triplicate samples.

† The initial concentrations for Fragment E are presented. The initial fibrinogen concentration was 4.4 μM.

FIGURE 2 Incorporation of radiolabeled Fragment E₁ (X) and fibrinogen (O) in vivo into thrombi of various ages. Thrombi were induced in jugular veins of young pigs, aged for the time shown on the horizontal axis, then both radiolabeled Fragment E₁ and human fibrinogen were injected intravenously and the animal killed after 24 h. Each thrombus was removed along with a blood specimen and both were counted and weighed. The ratio of thrombus-to-blood radioactivities is plotted on the vertical axis.

ticularly important for lowering of the remaining blood radioactivity from 6.9 to 2.9%, which would permit improved discrimination between thrombus and background radioactivities in vivo.

DISCUSSION

Development of specific molecular markers capable of binding to thrombi in vivo and transmitting this in-

formation to an external detector is the focus of our current efforts in the diagnosis of thrombotic and thromboembolic disease. It has been demonstrated in this work that Fragment E₁ represents such a marker. Radioiodinated Fragment E₁ is incorporated in vitro into both forming and preformed clots (Table II). It is taken up in vivo by both fresh and aged thrombi (Fig. 2); this phenomenon is most likely associated with the

TABLE III
Biodistribution of ¹³¹I-Fragment E₁ in Mice

	Percent injected radioactivity per organ*												
	1 min	3 min	5 min	10 min	20 min	30 min	60 min	2 h	4 h	6 h	17 h	24 h	48 h
Liver	12.1	13.4	16.0	27.3	15.9	10.4	5.7	5.14	3.97	2.3	0.46	0.52	0.50
Spleen				0.9		0.47	0.56	0.60	0.41	0.33	0.02	0.33	0.025
Lung				1.32		1.75	1.3	1.7	1.15	0.81	0.10	0.16	
Blood	59.4	51.3	35.0	31.5	29.6	28.9	16.5	17.5	15.8	5.8	0.70	0.69	0.63
Kidneys	11.2	15.0	29.1	20.7	19.1	9.63	5.6	3.54	2.95	2.1	0.86	0.69	0.46
Stomach	1.4	1.35	0.95	2.14	9.2	14.0	17.3	15.2	12.8	7.6	0.31	0.26	0.27
Small intestine	4.4	4.9	4.8	4.0	7.1	6.1	8.5	7.3	5.6	3.0	0.35	0.32	0.19
Large intestine	1.25	1.8	2.0	2.13	2.8	2.61	3.0	3.61	5.0	3.4	0.38	0.24	0.34
Bone				5.71		8.18	6.9	9.6	6.8	3.0	8.5	0.00	0.00
Head	10.4	12.1	12.4	5.34	10.5	9.9	9.3	12.8	21.7	15.0	8.2	17.3	16.0
Urine (cumulative)				2.02		11.5	9.3	20.0	16.3	53	81	65	70
Feces				0.00		0.00	0.00	0.00	0.45	4	7.2	12.2	10.4

* Each value represents the mean based on two animals.

TABLE IV
Biodistribution of ^{131}I -Fragment E_1 in Pigs

	Radioactivity administered per organ*	
	24 h	Thyroid blocked† 24 h
Liver	2.2	0.30
Spleen	0.14	0.066
Lung	0.56	0.45
Blood	6.9	2.9
Kidneys	1.2	0.32
Stomach	3.9	0.78
Small intestine	1.7	1.8
Large intestine	2.1	2.2
Bone	1.9	1.4
Thyroid	37.8	6.1
Urine (cumulative)	40	83
Feces (cumulative)	2	0.7

* Each value represents the mean based on two animals.

† 2 drops KI solution (1 g/ml) administered orally 24 h before dosing with ^{131}I -Fragment E_1 .

unique binding affinity of Fragment E_1 for fibrin polymers (13).

The incorporation of Fragment E_1 into thrombi differs from that of fibrinogen in two aspects. First, only when thrombi are quite fresh does fibrinogen accumulate to a detectable extent as compared with the surrounding blood (Fig. 2); the FUT is known to miss old thrombi (2) and that phenomenon was confirmed in this work. The values obtained here for thrombus-to-blood ratios of iodinated fibrinogen as a function of thrombus age correlated well with similar fibrinogen uptake studies in other animal models of thrombosis (11, 20). Fragment E_1 , however, appears to bind avidly to the surface of mature thrombi as well as fresh ones, suggesting that it may be a more versatile tracer for clinical diagnosis of DVT. If a thrombus were formed from 5 ml of blood, one could predict that it would bind ~ 30 ng of Fragment E_1 , corresponding to 132,000 dpm, as calculated from the data in Table II and the specific activity of the ^{131}I -labeled preparation. This estimate of incorporation is of similar magnitude to the binding actually observed in the pig experiments. This amount of radioactivity could be easily detected externally and would be increased further by a higher specific activity preparation. It is believed that older thrombi, which are not yet endothelialized, may still propagate and/or embolize (23), and Fragment E_1 may bind to these older, possibly clinically significant thrombi, but should not bind to older thrombi that have become endothelialized and no longer pose a risk.

A second aspect in which Fragment E_1 differs from

fibrinogen is that elimination of radioiodinated Fragment E_1 from the circulation is very rapid: in mice only half of the injected radioactivity remains in the blood after 3 min and only 0.7% after 24 h (Table III); in pigs only 6.9% remained in the blood after 24 h (Table IV). This clearance quickly lowers the background radioactivity of the blood and improves the sensitivity of thrombus detection by increased thrombus-to-blood ratios. In contrast, the biologic half-time of fibrinogen in humans is 4.0–5.5 d (24), which prolongs a high blood background and delays the earliest practical thrombus imaging until ~ 1 d after injection. Although not tested in this study, it may be possible to image uptake of radioiodinated Fragment E_1 in thrombi soon after its administration.

The authors are aware of the shortcomings of a heterologous system, such as used here, in which human Fragment E_1 was tested in pigs and mice. However, attempts to purify porcine Fragment E_1 were unsuccessful since plasmic digestion patterns of porcine cross-linked fibrin were different from those of the human counterpart and the yields of the fragment were extremely low. It remains to be elucidated whether this difference resulted from the presence of agents that contribute to the slow activation of fibrinolysis in pigs. Human fibrinogen, used in pigs in this study, is known to have a structure very much like pig fibrinogen, and exhibited a thrombus uptake behavior very similar to that of other animal fibrinogens tested in their own species (11, 20). It is possible that the kinetics of removal of human Fragment E_1 and human fibrinogen may be different in pigs than in humans; this question will have to be answered in clinical trials.

Human Fragment E_1 contains 16 tyrosine residues per molecule (25), 6 of which are particularly susceptible to iodination (26). The stability of radiolabeled Fragment E_1 in vitro (Table I, Fig. 1) demonstrated that radioiodine is not being detached easily from the labeled protein. It is concluded that the fast rate of disappearance of radioactivity from the blood and appearance in the kidney and urine was dependent on rapid metabolism of Fragment E_1 rather than on the release of radioiodine in the circulation (Tables III and IV). Because the liver radioactivity is transient, rapid catabolism of the labeled protein is the probable explanation for blood disappearance rather than the uptake of small aggregates, which would also be taken up by the liver, but would reside there longer. In addition, aggregates would not penetrate the polyacrylamide gels during electrophoresis and would remain near the top of the gel. Since this was not observed (Fig. 1), the labeling procedure did not induce formation of aggregates of Fragment E_1 .

The rapid catabolism of radioiodinated Fragment E_1 and its affinity for fibrin polymers, in addition to a lack

of binding to fibrinogen or fibrin monomer, constitute requisite properties of a molecular marker of thrombosis. The data presented in this work are encouraging and suggest the applicability of radiolabeled Fragment E₁ for fast and specific detection and monitoring of thrombotic disease in humans.

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REFERENCES

- Sherry, S. 1977. The problem of thromboembolic disease. *Semin. Nucl. Med.* 7:205-212.
- Hull, R., and J. Hirsh. 1982. Diagnosis of venous thrombosis. In *Hemostasis and Thrombosis—Basic Principles and Clinical Practice*. R. W. Colman, J. Hirsh, V. J. Marder, and E. W. Salzman, editors. J. B. Lippincott Company, Philadelphia. 844-856.
- Kakkar, V. V., A. N. Nicolaides, J. T. G. Renney, J. R. Friend, and M. B. Clarke. 1970. ¹²⁵I-Labelled fibrinogen test adapted for routine screening for deep-vein thrombosis. *Lancet*. 1:540-542.
- Kakkar, V. V. 1972. The diagnosis of deep vein thrombosis using the ¹²⁵I-fibrinogen uptake test. *Arch. Surg.* 104:152-159.
- Webber, M. M., L. R. Bennett, M. Cragin, and R. Webb, Jr. 1969. Thrombophlebitis—demonstration by scintiscanning. *Radiology*. 92:620-623.
- Henkin, R. E., J. S. T. Yao, J. L. Quinn III, and J. J. Bergan. 1974. Radionuclide venography (RNV) in lower extremity venous disease. *J. Nucl. Med.* 15:171-175.
- Rosenthal, L., and N. D. Greyson. 1970. Observations on the use of ^{99m}Tc albumin macroaggregates for detection of thrombophlebitis. *Radiology*. 94:413-416.
- Goodwin, D. A., J. T. Bushberg, P. W. Doherty, M. J. Lipton, F. K. Conley, C. I. Diamanti, and C. F. Meares. 1978. Indium-111-labeled autologous platelets for location of vascular thrombi in humans. *J. Nucl. Med.* 19:626-634.
- Davis, H. H., B. A. Siegel, J. H. Joist, W. A. Heaton, C. J. Mathias, L. A. Sherman, and M. J. Welch. 1978. Scintigraphic detection of atherosclerotic lesions and venous thrombi in man by indium-111-labelled autologous platelets. *Lancet*. 1:1185-1186.
- Ezekowitz, M. D., D. A. Wilson, E. O. Smith, R. D. Burow, L. H. Harrison, D. E. Parker, R. C. Elkins, M. Peyton, and F. B. Taylor. 1982. Comparison of Indium-111 platelet scintigraphy and two-dimensional echocardiography in the diagnosis of left ventricular thrombi. *N. Engl. J. Med.* 306:1509-1513.
- Knight, L. C., J. L. Primeau, B. A. Siegel, and M. J. Welch. 1978. Comparison of In-111-labeled platelets and iodinated fibrinogen for the detection of deep vein thrombosis. *J. Nucl. Med.* 19:891-894.
- Olexa, S. A., and A. Z. Budzynski. 1979. Binding phenomena of isolated unique plasmonic degradation products of human cross-linked fibrin. *J. Biol. Chem.* 254:4925-4932.
- Olexa, S. A., and A. Z. Budzynski. 1980. Evidence for four different polymerization sites involved in human fibrin formation. *Proc. Natl. Acad. Sci. USA.* 77:1374-1378.
- Olexa, S. A., and A. Z. Budzynski. 1979. Primary soluble plasmonic degradation product of human cross-linked fibrin. Isolation and stoichiometry of the (DD)E complex. *Biochemistry*. 18:991-995.
- Kazal, L. A., S. Amsel, O. P. Miller, and L. M. Tocantins. 1963. The preparation and some properties of fibrinogen precipitated from human plasma by glycine. *Proc. Soc. Exp. Biol. Med.* 113:989-994.
- Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. NY Acad. Sci.* 121:404-427.
- McFarlane, A. S. 1963. In vivo behavior of I¹³¹ fibrinogen. *J. Clin. Invest.* 42:346-361.
- Fraker, P. M., and J. C. Speck, Jr. 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80:849-857.
- Knight, L. C., A. Z. Budzynski, and S. A. Olexa. 1981. Radiolabeling of fibrinogen using the Iodogen technique. *Thromb. Haemostasis*. 46:593-596.
- Coleman, R. E., S. S. L. Harwig, J. F. Harwig, B. A. Siegel, and M. J. Welch. 1975. Fibrinogen uptake by thrombi: effect of thrombus age. *J. Nucl. Med.* 16:370-373.
- Didisheim, P. 1972. Animal models useful in the study of thrombosis and antithrombotic agents. In *Progress in Hemostasis and Thrombosis*. T. H. Spaet, editor. Grune & Stratton, Inc., New York. 1:165-197.
- Knight, L. C., S. S. L. Harwig, and M. J. Welch. 1977. In vitro stability and in vivo clearance of fibrinogen labeled with ⁷⁷Br, ¹³¹I, or ¹²⁵I by direct or indirect synthetic methods. *J. Nucl. Med.* 18:282-288.
- Freiman, D. G. The structure of thrombi. In *Hemostasis and Thrombosis—Basic Principles and Clinical Practice*. R. W. Colman, J. Hirsh, V. J. Marder, and E. W. Salzman, editors. J. B. Lippincott Company, Philadelphia. 766-780.
- Schultze, H. E., and J. F. Heremans. 1966. Molecular biology of human proteins. Elsevier Publishing Co., New York. 1:473-481.
- Olexa, S. A., A. Z. Budzynski, R. F. Doolittle, B. A. Cottrell, and T. C. Greene. 1981. Structure of Fragment E species from human cross-linked fibrin. *Biochemistry*. 21:6139-6145.
- York, J. L., and B. Blombäck. 1979. The sites of lactoperoxidase-catalyzed iodination of human fibrinogen. *J. Biol. Chem.* 254:8786-8795.