Structure and *In Vivo* Behavior of Human Fibrinogen Fragment-D[1] (36372)

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(Introduced by W. O. Weigle)

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The cleavage of fibrinogen and fibrin by plasmin in an orderly stepwise sequence into a series of progressively smaller fragments is well recognized (1). The cleavage fragments, which appear pathophysiologically important in mediation and modulation of the inflammatory response (2), differ in their biochemical (3), pathophysiological (4), and antigenic properties (5, 6). Cleavage culminates in the generation of two stable protein fragments generally referred to as fragment-D (fg-D) and fragment-E (fg-E) (1). The Fg-D has significant demonstrable biologic activity *in vitro*, including inhibition of fibrin monomer polymerization, antithrombin properties, and platelet aggregation (7, 8). In order to evaluate the potential pathophysiologic significance of fg-D *in vivo*, determination of plasma half-life, stability, and binding to other proteins was evaluated in this study.

Physicochemical properties of fg-D have been described (9); and molecular weights of 66,000 to 85,000 daltons have been reported (10). Homogeneity of fg-D has been questioned because of hyperfine banding on polyacrylamide disc electrophoresis and a broad elution profile from ion exchange columns (11). Evidence regarding the molecular weight, polypeptide chain substructure, and the possible molecular basis for the apparent heterogeneity of this molecular fragment are presented.

Materials and Methods. Preparation of fibrinogen and cleavage. Fibrinogen was prepared from two pools of frozen human plasma by cold ethanol fractionation (12). It was further purified by precipitation at 26% saturation with ammonium sulfate at 4°, dialyzed and clarified by ultracentrifugation in 5% sucrose at 74,000*g* for 30 min. Enzymatic cleavage of fibrinogen at 2.4 mg/ml in 0.14 *M* NaCl, 0.01 *M* sodium phosphate (pH 7.3) (PBS) was conducted at 37° for 18 hr with 2 μg of plasmin[3]/mg of fibrinogen.

Isolation methods. Preparative molecular exclusion chromatography was conducted on Sephadex G-200 (2.5 × 70 cm) or G-100 (2.5 × 85 cm) in 0.1 *M* NaCl, 0.01 *M* Tris–HCl, pH 8.0. Anion exchange chromatography utilized 2.5 × 30 cm columns of DEAE-cellulose[4] equilibrated with 0.01 *M* Na_2HPO_4 starting buffer. Elution was accomplished with a combined salt-pH gradient from 0.01 *M* Na_2HPO_4 to 0.3 *M* KH_2PO_4 (9).

Immunoelectrophoretic analysis (IEP). Characterization by IEP was performed in 1% Difco Noble agar, 0.025 *M* Veronal (pH 8.6), 0.01 *M* EDTA on 3 × 1 in. microslides using a potential difference of approximately 5.5 V/cm.

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[3] Urokinase activated purified human plasminogen, Cutter Labs.

[4] Schleicher & Schuell, standard grade.

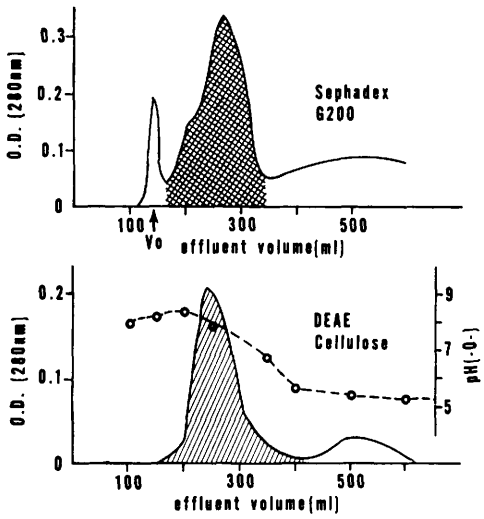

FIG. 1. Purification of fg-D: (a) Following plasmin digestion of fibrinogen, the combined fg-D:E core is recovered in a relatively clean state in the retarded (shaded) peak from Sephadex G-200. (b) Fg-D and fg-E are separately resolved and recovered from DEAE-cellulose utilizing a salt-pH gradient. The first peak (shaded) is fg-D and the second is the fg-E. Residual trace contamination of the tail of fg-D with fg-E is removed by repeat chromatography.

Polyacrylamide gel analytical methods. Polyacrylamide disc electrophoresis was performed in 7.5% polyacrylamide gel at pH 8.6 (13). Hyperfine banding was analyzed by the disc electrophoretic variation of Hedrick and Smith (14) employing 6, 7, 8, 9, and 10% polyacrylamide gels but in Tris–glycine buffer, pH 9.3. Relative mobility (R_m) was plotted against acrylamide concentration. Molecular weight estimates of fg-D were performed in 8, 10, and 13.6% polyacrylamide disc gels in 0.1 *M* phosphate (pH 7.0), 0.10% sodium dodecyl sulfate (SDS) (15). Standard protein markers were hemoglobin chains (15,500), β-lactoglobulin (18,400), ovalbumin (44,000), human albumin (65,000), bovine albumin (68,000), *E. coli* tryptophan synthetase (44,000 with polymers of 88,000; 132,000; and 176,000) (16). For estimation of fg-D subunit molecular weights, 0.5% SDS was included in the gels.

Reduction of fg-D. Reduction of fg-D to its subunit chains was accomplished in the presence of 0.2 *M* 2-mercaptoethanol and 0.1% SDS for 2 hr at 37°. The products were analyzed directly and also after reduction followed by alkylation with 0.21 *M* iodoacetic acid.

In vivo *behavior of fg-D.* Thirteen normal rabbits (2–4 kg) were employed for *in vivo* studies. All were provided with 1% KI in their drinking water. Fg-D was radioiodinated with ^{125}I utilizing the modified chloramine T method (17). Rabbits were given ^{125}I-fg-D intravenously, the dose varied from 160 to 1200 μg of protein in 1 ml of PBS. Plasma was sampled after intravascular equilibration (5 min) and serially thereafter. Rabbits utilized for study of catabolism and urinary excretion of ^{125}I or ^{125}I-fg-D were hydrated with 250 ml of 5% dextrose in 0.45% saline given intravenously and fluids were continued to maintain urinary output. Urine was collected from the bladder by catheterization. Whole plasma samples (200 μl) were counted in a sodium iodide crystal well counter and expressed as a percentage of postequilibration counts. Serial plasma samples were further studied by molecular exclusion chromatography on 2.5 × 25 cm columns of Sephadex G-200 or G-150 at 4°. The elution profile was continuously monitored at 254 nm, and the radioactivity of fractions was assayed. The fg-D void volume index ($V_{fg\text{-}D}$) was determined by comparison of the effluent volume at which peak radioactivity was eluted with the void volume ($V0$) of the column. Urine was collected during the control preinjection period and at 30 min, 1 hr, 2 hr, and periodically to 150 hr. Samples were counted for ^{125}I and precipitability by 10% TCA; and antiserum to fg-D was assessed.

Results. Following prolonged plasmin digestion of fibrinogen, only fg-D and fg-E were found on IEP. Following dialysis to remove small peptides, the fg-D:E core (18) was recovered in a single peak on Sephadex G-200 chromatography (Fig. 1a). This bimolecular polymer was again isolated in the exclusion peak of Sephadex G-100 and subjected to ion exchange chromatography (Fig. 1b). The fg-D was recovered in a rather highly purified state as shown by IEP and

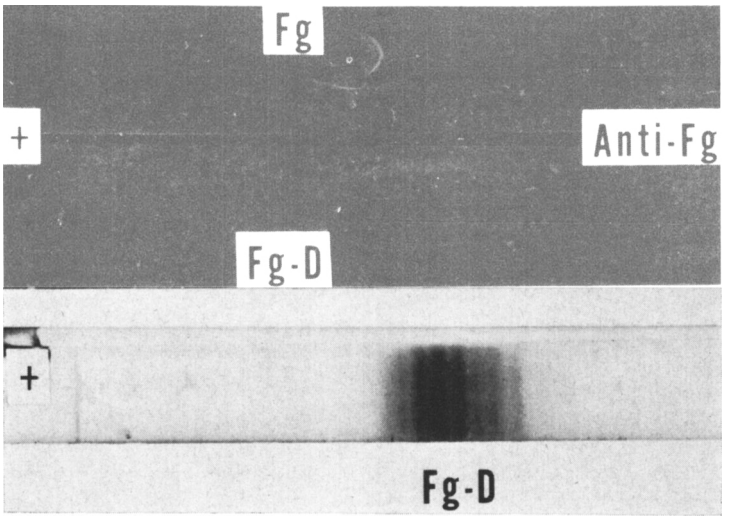

FIG. 2. Purified fg-D produces a single arc of characteristic mobility by IEP employing anti-fibrinogen: Migration on polyacrylamide disc gel is characteristic for fg-D and contains no detectable contaminants. Hyperfine banding, suggestive of heterogeneity, is also clearly observed.

standard polyacrylamide gel disc electrophoresis (Fig. 2).

Structural studies. Polyacrylamide gel disc electrophoresis of fg-D by the method of Hedrick and Smith gave an estimated molecular weight of 78,000 ± 3900 calculated from the slope of relative mobility (R_m), using least square analysis (Fig. 3). Following reduction or reduction followed by alkylation of fg-D, three polypeptide chains were resolved in SDS polyacrylamide gels. Estimated molecular weight of these polypeptide subunits were 44,000 ± 2700, 25,350 ± 900, and 10,400 ± 720.

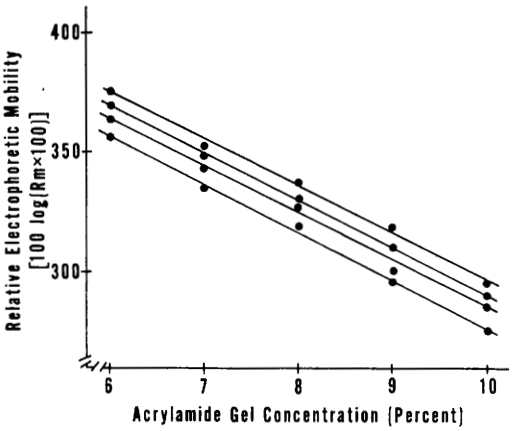

FIG. 3. The Hedrick and Smith (14) method, as demonstrated in Fig. 5, is applied to fg-D: The four major hyperfine bands give identical slopes ($p = .05$). This indicates absolute R_m differences independent of size differences and is characteristic of charge isomers.

Polyacrylamide gel disc electrophoresis of fg-D in 7% gel at pH 8.6 gave a broad band of characteristic mobility with eight hyperfine bands (Fig. 2). The physicochemical basis for this hyperfine banding was studied using the technique of Hedrick and Smith (14). The R_m of four fg-D bands, of sufficient density to permit identification and precise measurement, was assayed following electrophoresis in sequentially increasing concentrations of acrylamide gel. The log of the R_m of each of the four major hyperfine bands decreased in a linear fashion with increasing acrylamide concentration (Fig. 3). The slope of each line was identical, suggesting a similar molecular weight, within ±1000 daltons (19), for the protein in each hyperfine band.

In vivo *behavior.* Molecular exclusion chromatographic behavior of ^{125}I-fg-D *in vitro* and serial plasma samples from rabbits given ^{125}I-fg-D was assessed (Table I). The specific molecular exclusion volume ($V_{fg\text{-}D}$) was reasonably constant and in agreement with anticipated exclusion of a molecule of approximately 78,000, whether analyzed in

TABLE I. Molecular Exclusion Behavior of ^{125}I-fg-D *in Vitro* and *in Vivo*.[a]

^{125}I-fg-D sample	Relative molecular exclusion vol of ^{125}I-fg-D: Sephadex G-150	Sephadex G-200
In vivo		
Plasma, 15 min		2.06
1 hr		2.16
2 hr		2.18
4 hr		1.86
23 hr	1.56	
In vitro		
Normal plasma	1.36	2.36
Saline	1.43	

[a] ^{125}I-fg-D was either mixed with saline or plasma for 30 min at 37° for *in vitro* studies. ^{125}I-fg-D was given intravenously to rabbits and plasma samples obtained at indicated intervals for the evaluation of intermolecular interactions *in vivo*. The relative elution volume (V_{fg-D}) is determined by the elution volume of peak ^{125}I radioactivity compared with a dextran blue internal standard for void volume (V_0). V_{fg-D} = (elution vol ^{125}I-fg-D)/V_0.

saline, mixed with plasma *in vitro*, or injected *in vivo*. No association with other proteins was detectable by this method. There was no evidence for fg-D polymerization or dissociation into any of the three characterized polypeptide chains. Small amounts of non-TCA precipitable radioactivity were present in the plasma and had an apparent molecule size of less than 4000 by molecular exclusion chromatography.

Clearance of ^{125}I-fg-D from the plasma compartment was very rapid, with an intravascular half-life of 2 hr ± 12 min (Fig. 4). The clearance rate was independent of plasma concentration throughout the dose range 50–500 μg of fg-D/kg. The radioactivity in plasma was 89% precipitable by 10% TCA and 91% precipitable by antisera to fg-D. Excretion in urine was delayed. No increase in radioactivity over base line was observed at 30 min. At 1 hr, excretion of radioactivity was initially detected and increased exponentially thereafter. None of this radioactive label was precipitable by 10% TCA.

Discussion. Although there is discussion of the exact molecular weight of fibrinogen, estimates of the molecular weights of the fragments are less well established. Fg-D has been estimated at 75-88,000 daltons by sedimentation coefficient but only 68,000 by molecular exclusion chromatography (10). SDS polyacrylamide gel electrophoresis appears to be a rather reliable method for molecular weight estimation (19). Utilizing this method we have arrived at a molecular weight estimate of 78,800 ± 3900 for fg-D. This is within the range suggested by some reported studies (10). No systematic investigation of fg-D substructure has been presented, although Budzynski *et al.* (18) noted three smaller fragments following sulfitolysis of fg-D. The presence of three subunits or polypeptide chains was confirmed in our study, and molecular weights of 44,000, 25,350, and 10,400 are proposed. The sum of molecular weights of these chains, assuming one chain of each per intact fg-D, is 79,800 which further substantiates the molecular weight of 78,800 separately estimated for fg-D. This subunit structure may be useful in reconstruction and conformational mapping of the fibrinogen molecule. The possible role of these fg-D subunits might also be considered in respect to the pathophysiological properties of fg-D, particularly with regard to the effect of fibrinolytic products on platelets

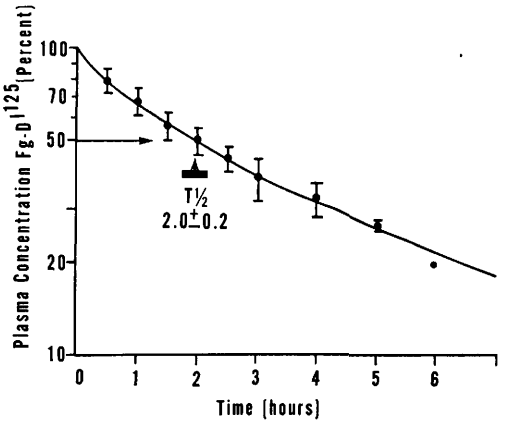

FIG. 4. ^{125}I-labeled fg-D was cleared from the plasma of rabbits at a simple exponential rate with mean half-life ($T_{½}$) of 2.0 ± 0.2 hr: The slope, or rate of clearance, was not significantly influenced by concentration over a 10-fold range.

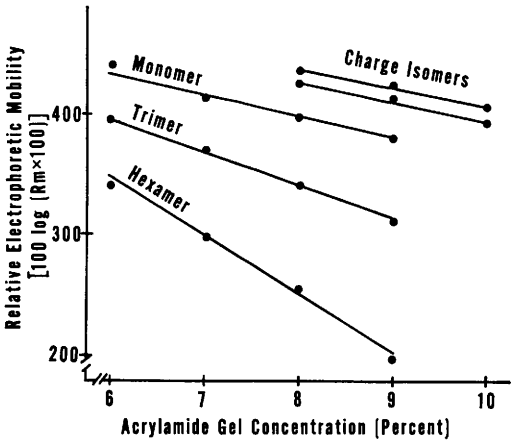

FIG. 5. An example of discrimination between polymers or size variants and charge isomers by the method of Hedrick and Smith (14) is shown: The differences in slopes observed with *E. coli* tryptophan synthetase monomer, trimer, and hexamer are characteristic for a polymer system. The charge isomers of ovalbumin yield identical slopes, by least square analysis, but differing in absolute R_m.

where conflicting reports of platelet aggregation and decreased platelet aggregation have been reported (8, 20). The localization of the cleavage-associated neoantigen of fibrinogen (6) to a specific fg-D subchain has not yet been accomplished, but is currently being attempted.

Although fg-D behaves as a discrete protein by a variety of immunophysicochemical characteristics, microheterogeneity has been noted in our study, as well as by others (9, 11). The basis for apparent heterogeneity has not been defined, but the possibility that fg-D might not be a single homogeneous submolecular entity has been entertained. The effects of charge can be distinguished from those due to differences in molecular weight by analyzing the effects of sieving on the R_m of a protein according to Hedrick and Smith (Fig. 5). Utilizing this approach, fg-D appears to be a molecular fragment of rather homogenous size with eight charge isomers.

The fg-D is a potent inhibitor of fibrin polymerization (7). This effect is thought to be a result of association with fibrin monomers and resultant interference with normal association forces between fibrin monomers. This phenomenon is of some additional relevance in light of observations by Fletcher *et al.* (21) of "macrofibrinogen" in plasma from patients with intravascular coagulation. The possibility that not only circulating fibrin monomers, but also fibrinogen or fibrin cleavage fragments may bind to plasma fibrinogen and generate macromolecular complexes should be considered. We observed fg-D in plasma only in a free state, thus suggesting that this fragment does not directly give rise to such complexes or directly bind to fibrinogen. No interaction between fibrinogen or other plasma proteins was detectable *in vivo* or *in vitro* in this study. The plasma half-life of fg-D is brief, with an observed value of 2 hr ± 12 min. Identification of significant quantities of fg-D in plasma should thus signify recent active fibrinogenolysis; and rapid clearance from the circulation should significantly limit the *in vivo* pathophysiological effects.

The fg-D was not cleared intact in urine. This is as expected from the size of fg-D and the observation that other molecules of similar size are restricted at the glomerular filtration barrier (22). This observation supports the concept that observation of fg-D in urine represents direct entry into the postglomerular urinary space rather than normal excretion via the glomerular filtrate.

Summary. Polyacrylamide gel disc electrophoresis has been used to determine molecular weights of fg-D and its three subunits. A molecular weight of 78,800 ± 3900 was determined for fg-D. Three subunits, apparently representing individual polypeptide chains with molecular weights of 44,000 ± 2700; 25,350 ± 900; and 10,400 ± 720 have been demonstrated. The sum of the molecular weights of these polypeptide chains agrees closely with the estimated size of the intact fg-D fragment. We have confirmed microheterogeneity of fg-D on disc acrylamide gel electrophoresis and have demonstrated that this heterogeneity is a reflection of charge isomerization and does not represent detectable differences in size. *In vivo* studies demonstrated that fg-D does not interact with plasma proteins but circulates in a free state. It is cleared rapidly from plasma with

a half-life of 2 hr ± 12 min, and excreted in urine following catabolic degradation.

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