Discriminating Neoantigenic Differences Between Fibrinogen and Fibrin Derivatives
(disseminated intravascular coagulation/fibrinolysis/fibrinogenolysis/molecular immunology/neoantigens)

EDWARD F. PLOW AND THOMAS S. EDGINGTON

Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037

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ABSTRACT Discrimination between the physiological cleavage fragments of fibrinogen and fibrin offers an approach to differentiation between fibrinogenolytic processes and fibrinolysis after coagulation. By use of the cleavage-associated neoantigen of fibrinogen (fg-Dneo) as a molecular marker, characteristic differences between the D regions of fibrinogen derivatives and fibrin derivatives can be demonstrated. The expression of fg-Dneo by X, Y, D:E complex, and D-fragments of fibrinogen or fibrin is shown to be quantitative and unitary. Characteristic differences between fg-Dneo sites present on fibrinogen cleavage fragments, as contrasted to fibrin cleavage fragments, are indicated by different competitive inhibition slopes, and appear to reflect differential binding affinity of selected anti-fg-Dneo antibodies for the specific molecular site. There is a linear relationship between the slope of quantitative competitive inhibition and the relative molar ratio of fibrinogen and fibrin derivatives. Identical immunochemical expressions are observed in vitro and in vivo, and support the thesis that cleavage in vivo is produced by plasmin. The differential immunochemical features of fg-Dneo expression may be the result of stable conformational and/or subtle structural differences between the D region of fibrinogen and fibrin cleavage fragments and suggest that precise changes in the D region are associated with the fibrin transition. These molecular features not only provide additional insight into the molecular immunology and structure of fibrinogen, but also appear to offer a new molecular approach to discrimination between fibrinogenolytic mechanisms as contrasted to fibrinolysis secondary to coagulation.

Conversion of fibrinogen to fibrin by thrombin (EC 3.4.4.13), the fibrin transition, is the culmination of the coagulation system, a central theme in hemostatic balance, and a commonly invoked pathogenetic mechanism (1). Cleavage of fibrin by plasmin (EC 3.4.4.14), fibrinolysis, is intrinsic to maintenance of the hemostatic balance and leads to a generic series of polypeptide fragments possessing multiple pathophysiologic activities (2, 3). Plasmin may also cleave fibrinogen directly (fibrinogenolysis), leading to a series of fragments generally similar to those generated from fibrin (4). Although fibrinogen and fibrin differ strikingly in physicochemical behavior, the fibrin transition is associated with only a 2% loss of mass. This finding suggests that the physicochemical and pathophysiological properties of fibrin may result from conformational changes in the molecule. Such conformational differences may lead to both structural and conformational differences between the plasmin cleavage derivatives of fibrinogen and fibrin. Although only minor or equivalent biochemical differences between fibrin and fibrinogen cleavage fragments have been suggested (5, 6), specific and systematic major differences have not been defined.

In this work, we have compared the quantitative and qualitative molecular expressions of fibrin and fibrinogen cleavage fragments with respect to a specific marker, the cleavage-associated neoantigen, fg-Dneo (7, 8). We have shown (9) that this antigenically recognizable molecular site is expressed in an equimolar fashion by X, Y, D:E complex, and D-fragments of fibrinogen, but not by fibrinogen itself. We now demonstrated that the fg-Dneo site is also expressed by the comparable fibrin derivatives in vitro and in vivo, and this molecular site is expressed in a characteristically different fashion by fibrin derivatives. This immunochemical difference in fg-Dneo expression suggests characteristic and stable conformational or minor structural differences between the D regions of fibrinogen and fibrin derivatives, and offers a molecular approach to the recognition and differentiation of fibrinogenolytic from fibrinolytic processes and their associated disease states.

MATERIALS AND METHODS

Fibrinogen was purified from human plasma by sequential fractionation with cold ethanol and with ammonium sulfate (7, 10). Fibrin was prepared from purified fibrinogen (98% coagulable); 10 units of bovine thrombin (Parke, Davis & Co., Detroit, Mich.) was added per mg of fibrinogen (2 mg/ml), in the presence of 0.02 M EDTA to prevent crosslinking by Factor XIII. Coagulation was done at 4°C to facilitate uniform inclusion of plasmin directly into the fibrin clot, while inhibiting plasmin activity. Both fibrin and fibrinogen were digested at 37°C with urokinase-activated plasmin (Cutter Laboratories, Berkeley, Calif.). At various intervals, aliquots were withdrawn, 0.02 M e-aminocaproic acid was added, and the digests were applied to a 2.5 X 90 cm A-1.5 Agarose column equilibrated with 1.0 M NaCl-0.04 M e-aminocaproic acid-0.01 M Tris·HCl (pH 8.0). Protein peaks corresponding to the X- and Y-fragments, and the D:E complex were recovered in the column effluent of both fibrinogen and fibrin digests (9). These species were then recycled separately on the same column to insure purity. D:E complex fractions were separated into constituent D- and E-fragments by DEAE-cellulose chromatography (11). Each of the purified fragments appeared

Abbreviations: X, Y, D, E, and D:E complex, defined plasmin cleavage fragments of fibrinogen/fibrin; fg-, fibrinogen-derived plasmin cleavage fragments; fb-, fibrin-derived plasmin cleavage fragments; fg-Dneo, cleavage-associated neoantigen of fibrinogen.

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to be homogeneous and free of other fragments as judged by: (i) analytical molecular exclusion chromatography; (ii) immunoelectrophoresis in agar gel; and (iii) analytical polyacrylamide gel electrophoresis (9, 12). Sensitivity of each of these techniques permits detection of contamination at the 2–5% level (9). Each of the cleavage fragments of fibrinogen derivation was indistinguishable from its homologous fibrinogen cleavage fragment by the same three analytical methods.

The cleavage-associated neoantigen, fg-Dneo, was assayed by competitive inhibition of the primary binding radioimmunoassay. The four-compartment double-antibody system (7) used specific anti-fg-Dneo antiserum harvested 3 weeks after initial immunization (lot no. R6127-2) and absorbed as described (9). The protein concentration of the various preparations were measured by microkjeldahl nitrogen determinations, and molar concentrations were calculated from published molecular weights (5, 13). Competitive inhibition slopes were determined: Slope = Δ % inhibition / Δ log (competitive antigen concentration [M]) × 100.

Fibrin was solubilized in the chaotropic agent, KI, by coagulation of 2.0 ml of fibrinogen (2.0 mg/ml) with 40 units of thrombin for 30 min at room temperature, followed by the addition of 1.0 ml of 2.0 M KI in 0.1 M Tris·HCl (pH 8.6).

**TABLE 1. Expression of the cleavage-associated neoantigen, fg-Dneo, by cleavage fragments of fibrinogen**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Relative binding affinity*</th>
<th>50% competitive inhibition</th>
<th>Fg-Dneo per molecule†</th>
</tr>
</thead>
<tbody>
<tr>
<td>fg</td>
<td>0.008</td>
<td>&gt;10 μM</td>
<td>0.00</td>
</tr>
<tr>
<td>fg-X</td>
<td>0.204</td>
<td>5.86 nM</td>
<td>0.98</td>
</tr>
<tr>
<td>fg-Y</td>
<td>0.210</td>
<td>6.99 nM</td>
<td>0.83</td>
</tr>
<tr>
<td>fg-D:E</td>
<td>0.208</td>
<td>6.62 nM</td>
<td>0.89</td>
</tr>
<tr>
<td>fg-D</td>
<td>0.205</td>
<td>5.80 nM</td>
<td>1.00</td>
</tr>
<tr>
<td>fg-E</td>
<td>0.000</td>
<td>&gt;10 μM</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Competitive inhibition slopes.
† Calculated from the relative molar concentration of fg-D required for 50% competitive inhibition, divided by the concentration of the given test fragment required for similar competitive inhibition.

After complete dissolution of the clot (usually 2 hr), the solution was dialyzed against various concentrations of KI in Tris buffer. Solubility of the fibrin persisted at concentrations of KI greater than 0.05 M, but when it was dialyzed against Tris buffer alone, more than 90% of the material absorbing at 280 nm was coagulable. Radioimmunoassays in the presence or absence of 0.05 M KI demonstrated that the chaotropic agent did not alter fg-Dneo expression by fb-D or fg-D, and that the neoantigenic sites present in the intact fibrinogen molecule were not exposed by iodide. Patient plasmas used in this study and the criteria for diagnosis of disseminated intravascular coagulation have been described (9).

**RESULTS**

Differential expression of the fg-Dneo site by fibrin, as contrasted with fibrinogen, cleavage derivatives was explored with D-fragments of fibrinogen (fg-D) and fibrin (fb-D). Both fg-D and fb-D express fg-Dneo (Fig. 1) and apparently possess the full complement of fg-Dneo determinants, since complete inhibition of 125I-labeled fg-D binding by anti-fg-Dneo is attained at equilibrium concentrations of fg-D or fb-D. However, qualitative and quantitative differences in the presentation of fg-Dneo by the two different D-fragments to the specific antibody are apparent. 7.43 nM fb-D produces the same 50% competitive inhibition as 3.98 nM fg-D; thus, 1.87 times more fb-D is required to achieve the same competitive inhibition as fg-D. Qualitative differences in fg-Dneo expression by fg-D and fb-D are indicated by the different slopes of the competitive inhibition profiles (Fig. 1). The slope of the linear portion of the fg-D curve is 0.203, compared to 0.344 for fb-D. This observation suggests that the relative binding affinity of the anti-fg-Dneo antibody for the two D-fragments is markedly different, and provides the first evidence for the discriminating immunoochemical differences between fibrin and fibrinogen cleavage fragments.

The results in Tables 1 and 2 extend the comparison of fg-Dneo expression to the other discrete isolated fibrinogen and fibrin cleavage fragments. Fg-X, fg-Y, and fg-D:E exhibit similar expression of fg-Dneo as the D-fragment of fibrinogen.

**TABLE 2. Expression of the cleavage-associated neoantigen, fg-Dneo, by cleavage fragments of fibrin**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Relative binding affinity†</th>
<th>50% competitive inhibition</th>
<th>Fg-Dneo per molecule†</th>
</tr>
</thead>
<tbody>
<tr>
<td>fb†</td>
<td>0.006</td>
<td>&gt;10 μM</td>
<td>0.00</td>
</tr>
<tr>
<td>fb-X</td>
<td>0.341</td>
<td>12.2 nM</td>
<td>0.98</td>
</tr>
<tr>
<td>fb-Y</td>
<td>0.339</td>
<td>15.5 nM</td>
<td>0.77</td>
</tr>
<tr>
<td>fb-D:E</td>
<td>0.342</td>
<td>13.5 nM</td>
<td>0.89</td>
</tr>
<tr>
<td>fb-D</td>
<td>0.339</td>
<td>12.0 nM</td>
<td>1.00</td>
</tr>
<tr>
<td>fb-E</td>
<td>0.000</td>
<td>&gt;10 μM</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Mean for fb fragments 0.340 ± 0.001 13.3 nM ± 1.25 0.91 ± 0.10
Mean for fg fragments 0.207 ± 0.003 6.38 nM ± 0.21 0.92 ± 0.16

* Competitive inhibition slopes.
† Calculated from relative molar concentration of fb-D required for 50% competitive inhibition, divided by the concentration of a given test fragment required for similar competitive inhibition.
‡ Solubilized in potassium iodide.
The relative binding affinity of these fragments, as reflected by their competitive inhibition slopes, are nearly identical. When a value of 1 is assigned for fg-Dneo expression to the primary standard for the fibrinogen derivatives, fg-D, about the same value of 1 for expression of fg-Dneo is calculated for the other fragments, except for fg-E. These results are similar to those found with a different anti-fg-Dneo antiserum (no. R6127-4; ref. 9).

From Table 2 it is observed that fb-X, fb-Y, and fb-D:E express fg-Dneo in a qualitatively and quantitatively similar manner as fb-D. Competitive inhibition slopes are comparable, quantitative inhibitions are similar, and fg-Dneo expression, as compared with fb-D, is about equal to 1. The parent fibrin molecule solubilized in KI and fb-E exhibited no fg-Dneo expression. The mean slope for all fibrinogen-derived fragments is 0.207 ± 0.003, as contrasted to 0.340 ± 0.001 for all fibrin-derived fragments. While the slope variations within each family of fragments are minimal, the difference between the two sets of fragments appears to be highly significant. The relative molar expression of fg-Dneo by fibrin fragments of 0.91 ± 0.10 is comparable to the value of 0.92 ± 0.16 for all fibrinogen fragments. Variations from unity are noted specifically for the Y-fragments and the D:E complexes, with both the fibrin and fibrinogen analogues exhibiting similar deviations from the anticipated value. This result may be due to the complete lack of fg-Dneo expression by minor subpopulations of these fragments, to consistent errors in the determination of protein concentrations, or to minor inaccuracies in the published molecular weights.

Fig. 2 demonstrates that these differences in competitive binding affinity of fibrin and fibrinogen cleavage fragments do not arise artifactually during isolation of the fragments. Fresh plasma samples were used to simulate: (a) fibrinolysis by clotting with thrombin and cleaving with plasmin; and (b) fibrinogenolysis by cleaving directly with plasmin. Immunochemical expression of fg-Dneo by fibrinolytic, fibrinogenolytic, and control plasmas was assayed. The fibrinolytic plasma is clearly distinguished from the fibrinogenolytic plasma on the basis of their inhibition profiles (Fig. 2). The slope of 0.206 for the fibrinogenolytic plasma and 0.342 for the fibrinolytic plasma are consistent with the slopes obtained with the isolated and purified fibrinogen derivatives (0.207) and fibrin derivatives (0.340), respectively.

Although fibrinogenolysis and fibrinolysis are conceptually distinct pathological processes, one event does not preclude the other, and both might occur simultaneously in vivo. If differences in competitive binding affinity are to provide an effective means for discriminating fibrin from fibrinogen cleavage products, fragments of either molecular origin should contribute to the observed competitive inhibition slope in proportion to their concentration. Mixtures of fg-D and fb-D at known molar concentrations were assayed and the slopes of the competitive inhibition profiles were measured. Fig. 3 illustrates that the competitive inhibition slopes exhibit a linear dependence on the relative proportion of fb-D and fg-D. This result not only indicates that the proportion of fibrin and fibrinogen fragments in a mixture of unknown molecular origin may be determined, but also that the molar concentration of fibrin and fibrinogen-derived fragments may be calculated (see Appendix).

The differential expressions of fg-Dneo characteristic of fibrinogen and fibrin cleavage fragments are also observed in vivo, as shown in Table 3. Plasmas from patients with diseases commonly associated with the presence of circulating fibrinogen/fibrin cleavage fragments, and in which noncoagulable cleavage fragments were demonstrable by radial immunodiffusion assay (9, 14), were all positive for fg-Dneo by radioimmunoassay. The competitive inhibition slopes observed upon assay of plasmas from two subjects with disseminated

Table 3. Plasmin cleavage derivatives of fibrinogen and fibrin in human plasma

<table>
<thead>
<tr>
<th>Number</th>
<th>Plasma samples</th>
<th>Slope</th>
<th>Total (nM)</th>
<th>Fg-derivative (nM)</th>
<th>Fb-derivative (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X107</td>
<td>Acute meningoencephalitis</td>
<td>0.338</td>
<td>510</td>
<td>0 (0%)</td>
<td>570 (100%)</td>
</tr>
<tr>
<td>X104</td>
<td>Sepsis with consumption coagulopathy</td>
<td>0.308</td>
<td>1,850</td>
<td>416 (22.5%)</td>
<td>1,434 (77.5%)</td>
</tr>
<tr>
<td>X102</td>
<td>Metastatic adenocarcinoma, prostate</td>
<td>0.342</td>
<td>1,950</td>
<td>362 (0%)</td>
<td>1,588 (100%)</td>
</tr>
<tr>
<td>X105</td>
<td>Metastatic melanoma</td>
<td>0.310</td>
<td>2,940</td>
<td>647 (22.0%)</td>
<td>2,293 (78.0%)</td>
</tr>
<tr>
<td>X106</td>
<td>Peritonitis (E. coli, Proteus); renal tubular necrosis; adenocarcinoma, prostate</td>
<td>0.281</td>
<td>2,360</td>
<td>991 (42.0%)</td>
<td>1,369 (58.0%)</td>
</tr>
<tr>
<td>X103</td>
<td>Abruptio placenta, fetal defibrinogenation</td>
<td>0.203</td>
<td>36,800</td>
<td>36,800 (100%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

* See Appendix for calculations.

![Fig. 2. Expression of fg-Dneo by plasmin cleavage fragments of fibrinogen or fibrin produced in vitro. Fg-Dneo expression by normal plasma (O—O), by plasma incubated directly with plasmin (fibrinolytic, △—△), and by plasma coagulated with thrombin (20 units/ml) at 37° for 30 min and then incubated with plasmin (fibrinolytic, ●—●) was assessed. 4 μg of plasmin was added to 1 ml of plasma, followed by incubation for 3 hr at 37°. The reaction was terminated by the addition of 0.1 volume of 1.0 M ε-aminocaproic acid.](image-url)
in intravascular coagulation (X107—acute meningococcemia; X102—metastatic adenocarcinoma of the prostate) were indistinguishable from that of standard fb-D, suggesting the presence of fibrin derivatives. By contrast, one plasma (X103—abruptio placentae and fatal hemorrhage) exhibited a competitive inhibition slope identical with that of fg-D, indicative of the presence of fibrinogen derivatives. Other plasma samples (X104, X105, and X106) exhibited intermediate competitive inhibition slopes, suggesting the presence of a mixture of plasmin cleavage derivatives of both fibrinogen and fibrin.

**DISCUSSION**

Modification of molecules—whether associated with function, denaturation, or cleavage—may induce complex modulation of antigenic expression. Physiological cleavage of fibrinogen is associated not only with progressive loss of native antigenic expression, but also with emergence of specific neoantigenic expressions (9). Conformational events induced in the D region by plasmin cleavage result in the exposure of specific aminoacid sequences, fg-Dneo, that are sterically concealed in the intact native fibrinogen molecule (8). This exposure of fg-Dneo determinants occurs initially in the generation of the X-fragment, and the sites remain uniformly expressed by each plasmin cleavage fragment containing a D region. Fibrinogen, fibrin, and their X-fragments each contain two D regions and, thus, two potential fg-Dneo expressions, yet expression of only 1 fg-Dneo site by the X-fragments indicates exposure of only one of the two latent sites. The Y-fragments, the D:E complexes, and the D-fragments of fibrinogen and fibrin each contain one D region, and demonstrate expression of 1 fg-Dneo site. Thus, plasmin cleavage of both fibrinogen and fibrin leading to the X-fragment must be asymmetric, exposing only one fg-Dneo site; subsequent cleavage of the X-fragments must again be asymmetric, fully exposing the second latent fg-Dneo site. The asymmetry of plasmin cleavage of fibrinogen has also been suggested by biochemical studies (15).

This study demonstrates that, although a specific neoantigenic site may be quantitatively expressed by cleavage derivatives of two different forms of a molecule, the affinity of a specific antiserum may vary for one form as contrasted to the other form. Thus, the fg-Dneo site appears to be differentially expressed by the derivatives of fibrin and fibrinogen. This differential expression cannot be attributed to partial expression of a composite of fg-Dneo determinants by one family of fragments, since derivatives of both fibrinogen and fibrin are capable of complete neutralization of anti-fg-Dneo antibody. Furthermore, these differences presumably cannot be due to major differences in secondary structure resulting from differential plasmin cleavage of fibrin or fibrinogen, since the derivative fragments appear to be similar in size and amino-acid composition (4, 5). Thus, the specific differential expression of fg-Dneo must reflect conformational or very minor structural differences in the neoantigenic site per se or in the immediate vicinity of this site. These conformational or minor structural differences in the fg-Dneo locus are influenced by the conversion of fibrinogen to fibrin. Although the fibrin transition does not directly expose the fg-Dneo site, it does apparently control the subsequent exposure and immunochemical characteristics of this region. This finding suggests that cleavage by thrombin of fibrinopeptides from the amino-terminal aspect of fibrinogen is associated with conformational changes in more distant regions of the molecule, changes that may influence biological expressions of the physiological catabolic fragments.

Plasma from subjects with various diseases associated with pathologic cleavage in vivo contained the cleavage-associated neoantigen, fg-Dneo. Competitive inhibition profiles characteristic of fibrin derivatives were observed in the plasma from some individuals, whereas only fibrinogen cleavage fragments were recognized in the plasma of another subject. The fibrin derivative-containing plasmas appear to reflect fibrinolysis after in vivo coagulation, whereas the fibrinogen derivative containing plasma appears to reflect primary fibrinogenolysis. Plasma from other subjects exhibited competitive inhibition slopes in fg-Dneo assays that were intermediate to the fb-D and fg-D standards. We demonstrated that such slopes may arise from mixtures of fibrin derivatives and fibrinogen derivatives. Other enzymes may cleave fibrinogen in vitro, and possibly in vivo, but assay of the fg-Dneo expression generated by other enzymes—such as leukocyte proteases—have yielded slopes distinctly different from plasmin cleavage (8, 9) that cannot directly account for the results observed in this study.

It may be significant that anti-fg-Dneo antiserum derived from the early phase of the immune response and used in this study was capable of discriminating between the cleavage fragments of fibrinogen and fibrin. During the later phases of this same immune response, no such discrimination was observed (9). It is well known that the binding affinity of antibody is low early in the immune response (16). These preliminary observations suggest that the capacity to recognize conformational differences may be dependent upon early, low-affinity antibody rather than a difference in antigenic specificity.

The present study not only introduces evidence for discrete molecular differences between the products of fibrinogenolysis and fibrinolysis, but also provides a highly sensitive and precise approach to the recognition of the products of these processes in vivo and in vitro.

**APPENDIX**

The molar concentration of fibrin and fibrinogen cleavage fragments in an unknown mixture may be calculated from the
competitive inhibition profile and by application of the following equations. The fraction of cleavage fragments of fibrin-derivation in an unknown, Fbₙ, is determined by comparison of the slope of the unknown, slopeₙ, with those of the fb-D and fg-D standards.

\[
Fbₙ = \frac{slopeₙ - slopeₙ-D}{slopeₙ-D - slopefg-D}
\]  

[1]

The fraction of cleavage fragments derived from fibrinogen, Fgₓ, is given by:

\[
Fgₓ = (1 - Fbₙ)
\]  

[2]

The molar concentration of fragments in the unknown is then determined from the concentration required for 50% competitive inhibition. Since the observed molar concentration of the unknown will be influenced by the relative proportion of fibrin and fibrinogen fragments, a factor, C.F., must be derived that corrects for the quantitative difference between fg-D and fb-D standards. If C.F. is the correction factor, \([M_{obs}]\) is the observed molar concentration of the unknown calculated at 50% competitive inhibition, \([fg-D_{std}]\) is the molar concentration of fg-D for 50% competitive inhibition, and \([fb-D_{std}]\) is the concentration of the fb-D standard, then:

\[
C.F. = Fbₙ([fb-D_{std}] - [fg-D_{std}]) + [fg-D_{std}]
\]  

[3]

The correction factor may be used to calculate the total concentration of cleavage fragments present in the unknown, \([Mₓ]\)

\[
[Mₓ] = \frac{C.F.}{[M_{obs}]}\]

[4]

The molar concentration of fibrin derivatives, \([Fbₓ]\), and fibrinogen derivatives \([Fgₓ]\), is then given by:

\[
[Fbₓ] = [Mₓ]·Fbₓ
\]  

[5]

\[
[Fgₓ] = [Mₓ]·Fgₓ
\]  

[6]

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