Characterization of Murine Peritoneal Macrophage Receptors for Fibrinogen Degradation Products

By Shrin Rajagopalan and Salvatore V. Pizzo

The binding of human fibrinogen degradation fragments D1, E, X, and Y, as well as fibrin fragment D, dimer, to mouse peritoneal macrophages was examined. A Scatchard plot of fragment D1 binding was biphasic, suggesting two classes of receptors. Fragments D1, D, dimer, X, and Y in low concentrations bound to macrophages with high affinity ($K_d = 23$ to $73 \times 10^{-11}$ mol/L). Fragment E bound specifically but at a much lower level than the other fragments. Fragment D1 was able to compete for the binding of radiolabeled fragments X and Y but not radiolabeled fragment E. These studies indicate that fragments D and E are recognized by separate receptor systems but that all of the fibrinogen degradation products that contain the D domain are recognized by the same receptor system.

In the present study, we further characterize the macrophage receptor for fragment D. We also examine the binding behavior of fragment D1, dimer and investigate for the possible presence of receptors that are capable of binding fragments E, X, and Y. By use of competition studies, it is determined whether these fragments are all recognized by the D receptor previously observed by in vivo methods.

MATERIALS AND METHODS

Fragment D1 was prepared from human fibrinogen (Kabi Co, Stockholm) as previously described. Fragment E was obtained as a high-salt eluent from the same DEAE-Sephasel ion-exchange column used to obtain fragment D1. Although ion-exchange chromatography has been the method of choice for purification of fragment E, we observed a higher molecular weight (mol wt) contaminant by analytic gel filtration high-performance liquid chromatography (HPLC; Fig 1) on an LKB Ultropac TSK G3000 SW gel filtration column. Removal of this contaminant required gel filtration on a Sephadex G-100 column, followed by HPLC ion-exchange chromatography on an LKB Ultropac TSK 545 DEAE column. Fragments X and Y were prepared as previously described, except that Sephacryl S-300 was used instead of Sephadex G-200 for the gel filtration step. Plasminogen was obtained from human plasma by affinity chromatography on lysine-Sephasel, as described by Deutsch and Mertz and modified by Brockway and Castellino to include gradient elution. Plasminogen was activated with urokinase (Calbiochem, San Diego, Calif).

Protein concentrations in purified preparations of fibrinogen and fibrinogen fragments were determined from the following molar extinction coefficients and mol wt: fibrinogen, A600/1 cm, 280 nm = 15.1, M, = 320,000; fragment X, A550/1 cm, 280 nm = 14.2, M, = 240,000; fragment Y, A550/1 cm, 280 nm = 17.6, M, = 155,000; fragment D1, A550/1 cm, 280 nm = 20.8, M, = 100,000; fragment E, A550/1 cm, 280 nm = 10.2, M, = 50,000. For plasminogen, A550/1 cm, 280 nm = 16.8, M, = 92,000.

Proteins were radioiodinated by the solid-state lactoperoxidase method. Radioactivity was measured with an AW14-120 gamma counter (Scientific Products, McGraw Park, Ill).

The binding of fibrinogen fragments to murine peritoneal macrophages was examined. Plated macrophages were allowed to equilibrate for one hour at 4 °C and were then washed four times with 1 mL of cold binding medium (10% Earle’s balanced salt solution, 50 mmol/L HEPES, 150 mmol/L NaCl, 1% albumin, pH 7.4). After an additional 30 minutes’ incubation, the binding medium was replaced with fresh binding medium containing radiolabeled ligand at varying concentrations. After 50 hours (the time required for binding equilibrium; Fig 2) the ligand solution was removed; the cells were washed with cold buffer (50 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4), and allowed to dry. Fibrinogen fragments remain intact after 50 hours under these conditions as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and auto-

From the Departments of Pathology and Biochemistry, Duke University Medical Center, Durham, NC.

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Address reprint requests to Dr S.V. Pizzo, Box 3712, Department of Pathology, Duke University Medical Center, Durham, NC 27710.

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MACROPHAGE D DOMAIN RECEPTORS

Fig 1. High-performance liquid chromatography of fragment E preparation. Fragment E was obtained by DEAE ion-exchange chromatography and analyzed on a gel filtration column.

radiography of the culture media (data not shown). The cells were then solubilized with 0.3 mL of 0.1% NaOH overnight. The amount of bound ligand was determined by measuring the radioactivity of the solubilized cell solution in a gamma counter. The protein content of each sample was then determined by the Lowry protein assay, as modified by Peterson. This value was converted to numbers of cells by dividing by the average mass of a thioglycollate-stimulated macrophage (80 ng per cell). The amount of bound ligand in each well was expressed as a function of cell number in that well (fmol bound per cell). Well-to-well variations in cell number were compensated for by multiplying this value by the average cell number per well, resulting in an estimate of the total bound ligand in each well.

In one set of experiments, cells were incubated with radiolabeled ligand either alone or in the presence of a 100-fold molar excess of unlabeled ligand to determine nonspecific binding. The amount of radiolabeled ligand bound in the presence of unlabeled ligand (nonspecific binding) was subtracted from the amount bound in the absence of unlabeled ligand (total binding) to determine specific binding. The values for specifically bound ligand, along with values for free ligand at different concentrations, were used to perform Scatchard analyses and obtain estimates of the K_d values for the binding of fibrinogen fragments to macrophage receptors. Because total binding was always less than 1%, the free ligand concentration in each test solution could be estimated from the total amount of ligand originally added to that solution. In another set of experiments, unlabeled fragment D_1 was used to compete with radiolabeled fragments E, X, and Y for binding to macrophages, in order to determine whether these fragments are recognized by the same receptor as fragment D_1.

RESULTS

The binding of fragments D_1 and D_1 dimer to macrophages is shown in Figs 3 and 4 as femtomoles of fragment bound per microgram of cell protein v the logarithm of the free fragment concentration. In neither case does the binding curve assume a sigmoidal shape, indicating that no saturation of a finite number of binding sites has been attained. Therefore, it is not possible to predict the number of binding sites on macrophages for these fragments. However, because we have demonstrated specific binding, it is valid to derive dissociation constants for such a system. The binding data for fragment D_1 are also shown in the form of a Scatchard plot (Fig 5). The Scatchard plot is biphasic, suggesting either the presence of multiple classes of receptors or cooperativity of binding. As an approximation, we assumed the existence of two classes of receptor, a high-affinity receptor for low concentrations of fragment D_1 and a lower-affinity receptor for the higher concentration ranges. The “high-affinity” portion of the Scatchard curve for fragment D_1 binding was more carefully examined by using additional data points for low concentrations of radiolabeled protein (Fig 6). The data fit well to a straight line with a correlation coefficient of -0.99. The corresponding dissociation constant (K_d) was very low, 4.25 x 10^{-11} mol/L. The possibility that binding to the plastic well accounted for the apparent high-affinity binding was eliminated by performing the study in the absence of cells. In this case, binding of radiolabeled fragments occurred at extremely low levels and was not specific (data not shown).

The binding of fragments E, X, and Y to macrophages in the high-affinity concentration range (1 nmol/L to 100 nmol/L) was also examined and K_d values for fragments D_1,


dimer, X, and Y are shown in Table 1. Fragment Y has a slightly higher binding constant and fragments X and D, dimer have slightly lower constants than fragment D. Fragment E binding (Fig 7) occurred at low levels, less than 10% of those of the other fragments. Moreover, specific binding constituted less than 20% of total binding as opposed to 70% to 80% for the other fragments (data not shown). The higher affinity of binding of fragments X and D, dimer as compared with fragment D, could be explained if these fragments share the same receptor, since fragments X and D, dimer contain two D domains and could therefore be expected to bind more tightly to the cell surface. Again, these $K_d$ values should be regarded only as approximations, due to complexities arising from ligand multivalency and the possible presence of multiple classes of binding sites.

Previous studies have demonstrated that human fibrinogen fragments X and Y, but not E, compete with fragment D, for clearance from the circulation of mice, suggesting that the fragment D receptor also mediates the clearance of fragments X and Y. We used the in vitro macrophage binding system to examine directly the competition of fragment D for the binding of radiolabeled fragments E, X, and Y (Fig 8). Fragment D competes for the binding of both X and Y in a dose-dependent fashion analogously to the in vivo clearance data. However, fragment D does not compete for the binding of radiolabeled fragment E. These data indicate that all of the fibrinogen degradation products containing the D domain are recognized by the same receptor, and the that D domain contains the recognition site for this receptor system.

![Fig 5. Scatchard analysis of binding of 125I-fragment D, to mouse peritoneal macrophages. Data are obtained from Fig 2.](image)

![Fig 6. Scatchard analysis of binding of 125I-fragment D, to mouse peritoneal macrophages at low ligand concentrations.](image)

Table 1. Dissociation Constants for Binding of Fibrinogen Degradation Products to Macrophages

<table>
<thead>
<tr>
<th>Fragment</th>
<th>$K_d$ (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>$4.25 \times 10^{-11}$</td>
</tr>
<tr>
<td>X</td>
<td>$2.30 \times 10^{-11}$</td>
</tr>
<tr>
<td>Y</td>
<td>$7.30 \times 10^{-11}$</td>
</tr>
<tr>
<td>D, dimer</td>
<td>$2.40 \times 10^{-11}$</td>
</tr>
</tbody>
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DISCUSSION

Two of the major functions for biological receptor systems are the removal of specific components from the circulation and participation in the regulation of metabolic pathways. Recent studies in different systems have implicated receptors for plasmin-derived fibrinogen fragments in both roles. The clearance of human fibrinogen fragment D from the circulation of mice is mediated by a specific receptor system in the liver. Furthermore, the clearances of fragments X and Y, but not E, are mediated by the same receptor, suggesting that some determinant in the D domain serves as the receptor recognition site.

Fibrinogen fragments are also involved in the regulation of fibrinogen synthesis by rat hepatocytes. Franks et al showed that fragment D, but not fragment E, stimulated fibrinogen synthesis when perfused into rats. However, Richie et al and Sanders and Fuller demonstrated the stimulatory effect with both fragments D and E in their in vitro system. In addition, the latter group showed that the stimulation occurs by way of an indirect feedback pathway, in which fragments D and E elicit the release of hepatocyte-stimulating factor from human blood monocytes and rat Kupffer cells. Hepatocyte-stimulating factor in turn stimulates hepatocytes to synthesize fibrinogen. Mouse peritoneal macrophages appear to participate in a similar regulatory pathway, and supernatants from fragment D-exposed macrophages stimulated the synthesis of fibrinogen, $\alpha$-antitrypsin, and antithrombin III by cultured rat hepatocytes.

The apparent conflict between these studies is further complicated by in vivo observations in the murine system, which suggest that fragment E is not recognized by the D receptor. In order to clarify these issues, we applied Scat-
chard analyses and competition studies to the in vitro binding of fragments D1, D2, dimer, E, X, and Y to cultured mouse peritoneal macrophages. A Scatchard analysis of fragment D1 binding resulted in a biphasic curve, consistent with, although not strictly indicative of, two classes of receptors. Assuming two receptor classes, the binding at low concentration ranges occurred with an exceptionally low dissociation constant (Kd), 42.5 x 10^-11 mol/L. The binding of fragments D1, dimer, X, and Y, all of which contain the D domain, also occur with high affinity. Fragment D1 also was able to compete for the binding of radiolabeled fragments X and Y, indicating that fragments containing the D domain are recognized by the same receptor. However, fragment D did not compete for the binding of fragment E, indicating that separate receptor systems recognize these fragments. The binding of fragment E to macrophages conforms to the requirements of specific binding. However, so little ligand is bound relative to fragment D binding that uptake of fragments X and Y must occur almost exclusively by the fragment D domain receptor.

During the course of in vivo catabolism of fragments X, Y, D, and E, it is likely that specific uptake mechanisms account for the disappearance of all but fragment E. Clearance of fragment E most likely results from passive glomerular filtration. Consistent with this hypothesis, fragment E is the only one of these fragments that has a mol wt, M, ~ 50,000, which is smaller than albumin. Albumin, M, of ~67,000, is generally thought to represent the renal clearance threshold for a protein.

In light of these observations, it seems unlikely that fragment E would regulate fibrinogen synthesis. Previous studies that showed that fragment E elicits release of hepatocyte-stimulating factor may have used a preparation of fragment E that was contaminated with fragment D. Of interest in this regard is our observation that our fragment E preparation, also obtained by the traditional method of ion-exchange chromatography, displayed a higher mol wt contaminant by HPLC.

Recently, an adenosine diphosphate-dependent platelet receptor system for fibrinogen and von Willebrand factor has been described.34-38 This receptor, consisting of glycoproteins GP IIb and GP IIIa, specifically recognizes a continuous sequence of 12 amino acids at the carboxyl terminal end of the γ-chain of fibrinogen, in the D domain of the molecule. The isolated glycoprotein complex binds fragment D but not fragment E. Presumably, the GP IIb–GP IIIa complex on the surface of activated platelets interacts with fibrinogen and von Willebrand factor, forming "bridge" structures with the damaged vessel wall and with other platelets.

The macrophage receptor for fragment D1, described in this study is similar to the platelet GP IIb–GP IIIa receptor, in that it recognizes fragment D but not fragment E. However, unlike the platelet receptor, the macrophage receptor does not recognize the intact fibrinogen molecule.7 The macrophage receptor does recognize fragment X, which differs from fibrinogen in that the carboxyl terminus of the α2-chain has been removed. It appears that the receptor recognition site in the D domain for the macrophage receptor is "shielded" by the α2-chain of intact fibrinogen, while the recognition site for the platelet receptor is exposed in the intact molecule, despite its occurrence in the same domain.

The differences between these two receptor systems are readily understood if one considers their physiologic roles. The GP IIb–GP IIIa receptor on activated platelets uses circulating fibrinogen molecules to facilitate one of the early steps in hemostasis, the formation of the platelet plug. The macrophage receptor, on the other hand, detects fibrinogen degradation products after fibrinolysis has occurred. By way of the release of hepatocyte-stimulating factor from these macrophages, the synthesis of fibrinogen is increased and circulating levels are returned to normal.

REFERENCES


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S Rajagopalan and SV Pizzo