Fibronectin Dependent Macrophage Fibrin Binding

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Plasma fibronectin has been shown to increase the binding of fibrin monomer to macrophages in vitro. In the present study we began characterization of the mechanism underlying this fibronectin activity. Fragments of fibronectin containing the amino terminus enhanced macrophage fibrin binding to the same extent as intact fibronectin on an equimolar basis. However, fibronectin fragments containing the gelatin-binding domain or the cell-binding domain, but lacking the amino terminus, had no effect on fibrin binding. Fibronectin enhanced fibrin binding was not affected by the addition of synthetic peptides containing the RGD adhesion sequence. The ability of fibronectin to augment fibrin binding remained after paraformaldehyde fixation of macrophage monolayers. Fixation did not alter the basal levels of fibrin binding by macrophages. Preincubation of macrophages with exogenous fibronectin did not increase the binding of fibrin. Fibronectin enhanced fibrin binding remained unaltered after the removal of endogenous cell surface fibronectin by capping with F(ab’), fragments of antibodies to fibronectin. These results suggest that the amino terminus of fibronectin supports the attachment of fibrin to macrophages by an initial fluid-phase interaction that precedes cellular binding and does not require a cellular response.

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MATERIALS AND METHODS

Cell isolation and culture. Casein-elicited rat peritoneal macrophages were prepared as previously described10 under serum-free conditions. Macrophage monolayers were maintained in culture in Dulbecco’s Modified Eagle Media (DMEM; GIBCO, Grand Island, NY) containing 100 U/mL penicillin/streptomycin (GIBCO). Some macrophage monolayers were fixed before study with buffered 2% paraformaldehyde for 1 hour at 4°C followed by rinsing. Cells for immunofluorescence were cultured on 13-mm glass coverslips in multiwell plates (Costar, Cambridge, MA) under identical conditions.

Removal of cell-surface fibronectin. Cell-surface fibronectin was depleted by modification of a method of Colvin.28 Monolayers of peritoneal macrophages cultured on glass coverslips were incubated with 100 µg of F(ab’), fragment of polyclonal antifibronectin

cytes. Fibronectin binding to VLA-5 (α5β1) and the Ib/IIa-like receptor (αIIbβ3) appears dependent on the arginyl-glycyl-aspartyl (RGD) adhesion sequence17; however, VLA-3 (αβ1) and VLA-4 (αβ2) binding of fibronectin is RGD independent.20,26 Binding to VLA-4 occurs via the variable region of the fibronectin cell-binding domain.20

The interaction of fibronectin with fibrin has been documented. Fibronectin contains three domains with fibrin-binding affinity. Low-affinity fibrin-binding sites are contained within the carboxy-terminal 31-Kd region2 and the type III repeats near the gelatin-binding domain.22 These sites have not been reported to be active under physiologic conditions.21,22 The high-affinity fibrin binding site resides within the amino terminus of the molecule.23 This fibrin binding region also contains the critical glutamyl residue (Gln508), which is the site of plasma transglutaminase (factor XIIIa)-mediated covalent cross-linking of fibronectin to a lysyl residue located in the fibrinogen α-chain.24 The available donor lysyl residues are located within residues 508 to 584 in the 610 amino acid α-chain.25

While several investigators have described a fibronectin-dependent aspect of macrophage fibrin binding,8,11 the underlying mechanism whereby this enhancement occurs remains to be elucidated. This study was designed to determine which regions of the fibronectin molecule are important in macrophage fibrin binding, as well as the relative contribution of endogenous cell-surface fibronectin versus exogenous fluid-phase fibronectin. The requirement for a cellular response in fibronectin-dependent fibrin binding by the macrophage was also evaluated.

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antibody in DMEM, 10 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; Sigma, St Louis, MO), 0.5% bovine serum albumin (BSA; Sigma), pH 7.4 at 37°C in 5% CO2-95% air. To verify removal of cell-surface fibronectin, monolayers at 15-minute intervals were fixed with 2% buffered paraformaldehyde and rinsed in 0.01 mol/L PO4, 0.15 mol/L NaCl, pH 7.4 (PBS). The monolayers were then incubated with rhodamine-conjugated goat anti-rabbit IgG (Cappel, Durham, NC) specific to the F(ab) region of the antibody at a 1:50 dilution in PBS. This concentration of secondary antibody failed to react with the cells in the absence of primary antibody. After 1 hour, the unbound secondary antibody was removed. The monolayers were rinsed and the coverslips inverted on glass slides in 5.0 μL of 1.0 mmol/L 1,4 Diazabicyclo-(2.2.2)Octane (Sigma) and sealed. Cells were viewed on an inverted phase contrast microscope (Olympus, Tokyo, Japan) equipped for immunofluorescence. Fibronectin-deficient monolayers prepared for further study were rinsed after a 120-minute incubation with the antibody fragment. These cells were neither fixed nor exposed to secondary antibody.

**Protein isolation and preparation.** Blood for isolation of plasma proteins was collected from normal human volunteers into syringes containing 19% sodium citrate (2 mL/100 mL) as anticoagulant. The blood was centrifuged at 5,000g for 20 minutes and the plasma collected and applied to a 10-mL lysine-Sepharose 4B (Pharmacia, Piscataway, NJ) column equilibrated in 0.10 mol/L PO4, pH 7.4 at 35 mL/h to remove plasminogen. Vitamin-K dependent proteins were removed from the pass through by the addition of 22.3 mg barium chloride/mL plasma and benzamidine hydrochloride to a final concentration of 3 mmol/L. This mixture was stirred gently for 1 hour at 4°C and then centrifuged at 5,000g for 10 minutes. Excess barium was removed by the addition of 9.8 mg (NH4)2SO4/mL plasma while stirring for 15 minutes at room temperature followed by centrifugation at 5,000g for 10 minutes. The plasma was then applied to a 10 mL gelatin-Sepharose 4B (Pharmacia) column2728 equilibrated in 0.01 mol/L PO4, 1.0 mol/L NaCl, 3 mmol/L benzamidine hydrochloride, pH 7.4 and 0.02% 2-mercaptoethanol, at 25 mL/h. The column was then washed with 3 vol of equilibration buffer and bound protein was eluted with 4.0 mol/L urea PBS, pH 7.4. Eluted protein was dialyzed overnight in PBS containing 0.02% 2-mercaptoethanol and reapplied to the gelatin-Sepharose column under the same conditions. The twice-purified fibronectin was eluted and dialyzed against PBS for use.

The 70-Kd amino-terminal fibronectin fragment29 was prepared from fresh intact fibronectin. Fibronectin at a concentration of 1.0 mg/mL was dialyzed into 50 mmol/L sodium acetate, 2 mmol/L phenyl methyl sulfonyl fluoride (PMSF; Sigma), with 2.0 μg/mL soybean trypsin inhibitor (STI; Sigma), pH 5.5. Cathepsin D (Sigma) at 4.0 U/mL in 0.01 mol/L Tris base, 0.014 mol/L PMSF, 1.0 mg/mL STI, pH 7.4 was added to the fibronectin at a final concentration of 3.0 μg cathepsin/mg fibronectin and incubated at room temperature for 12 hours. The reaction was terminated by the addition of pepstatin A (Sigma) to a concentration of 0.025 μg pepstatin A/μg cathepsin D. The reaction mixture was titrated to pH 7.4 by the addition of 3.0 mol/L Tris base and applied to a 10.0-mL gelatin-Sepharose 4B column equilibrated in PBS at 25 mL/h. After washing with three bed volumes of PBS, the bound 70-Kd fragment was eluted with 4.0 mol/L urea, PBS, pH 7.4 and dialyzed against PBS for use.

The 70-Kd fibronectin fragment was further cleaved into a 29-Kd amino-terminal fragment and a 45-Kd gelatin-binding fragment by limited plasmin digestion.30 Porcine plasmin (Sigma) was added to purified 70-Kd fragment in PBS at a ratio of 1:100, wt:wt and incubated for 3 hours at 37°C. The reaction was terminated by the addition of PMSF to a final concentration of 5 mmol/L. The reaction mixture was separated into the 29-Kd and 45-Kd fragments by gelatin affinity chromatography under conditions similar to 70-Kd isolation, and dialyzed against PBS.

A 120-Kd cell-binding fragment of fibronectin33 was prepared by digestion with chymotrypsin (Type VII; Sigma) at a 30:1, wt:wt ratio in PBS for 60 minutes at 37°C. The reaction was halted by addition of diphenylcarbonyl chloride (Sigma) to a 1.0 mmol/L concentration. The 120-Kd fragment was contained in the pass through from sequential chromatography against gelatin-Sepharose 4B and heparin-Sepharose 4B (Pharmacia) affinity columns.

A 180-Kd fibronectin fragment35 was prepared from intact fibronectin by digestion with porcine plasmin at a 100:1, wt:wt ratio in PBS for 3 hours at 37°C. The reaction was halted by the addition of PMSF to a 5 mmol/L final concentration and the 180-Kd fragment isolated by retention on a gelatin-Sepharose 4B affinity column.

Fibronectin was isolated as described36 from the plasma pass through of the first gelatin-Sepharose chromatography step of the fibronectin isolation. Des-AB fibrin was prepared from fibronectin through limited thrombin cleavage as described.31 (α-Thrombin was the generous gift of Dr John W. Fenton II, Wadsworth Center for Laboratories and Research, Albany, NY.) Iodinated fibrin monomer was prepared from fibronectin radiolabeled with 125I (New England Nuclear, Boston, MA) by the lactoperoxidase procedure as previously described.32 Radiolabeled fibronectin was added to unlabeled fibronectin before thrombin cleavage.

The purity of all protein reagents used in this study was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions.

**Macrophage fibrin uptake assay.** Monolayers of 24-hour adherent peritoneal macrophages in Costar 24-well tissue culture plates were washed three times with Hanks’ Balanced Salt Solution (HBSS; GIBCO) and incubated with 40 μg 125I-fibrin for 1 hour with or without 40 μg intact fibronectin or an equimolar amount of a fibronectin fragment. The assay was performed in 1.0 mL DMEM with 0.5% BSA, 10 mmol/L HEPES, pH 7.4 for 1 hour in a humidified 37°C, 5% CO2-95% air incubator. The monolayers were then rinsed three times with HBSS, solubilized in 1.0 mol/L NaOH, and collected for analysis by gamma scintigraphy.

**Antibody preparation and fragmentation.** Antiserum to fibronectin was prepared as previously described.37 Antibodies to fibronectin were purified on a fibronectin-Sepharose 4B affinity column. These antibodies were then cross-purified on a fibrinogen-Sepharose 4B column to remove cross-reactive antibody. F(ab)2 fragments of antibody were prepared by digestion with immobilized pepsin (Pierce, Rockford, IL) according to the manufacturer’s instructions.

**Protein ligand coupling to Sepharose.** Fibronectin and fibrogen affinity columns were prepared by coupling the ligand to cyanogen bromide activated Sepharose 4B (Pharmacia) according to the manufacturer’s instructions.

**Statistics.** Data are expressed as mean ± SEM. Significance was determined by analysis of variance followed by Duncan’s comparison testing. A confidence level of 95% was used in all analyses.

**RESULTS**

To evaluate the ability of individual fibronectin domains to enhance macrophage fibrin binding, proteolytically derived fragments of fibronectin were coincubated with 40 μg of radiolabeled fibrin monomer on the macrophage monolayer as described in Materials and Methods. Figure 1 shows the macrophage binding of fibrin alone (control) or when coincubated with 40 μg intact plasma fibronectin, 2.8 μg 29-Kd amino-terminal fragment, 4.3 μg 45-Kd gelatin-binding fragment, 6.7 μg 70-Kd amino-terminal fragment,
fibronectin. We evaluated the effect of synthetic RGD-45-Kd fragment also failed to affect fibrin binding. How-occur through integrin receptor interaction with the RGD sequence contained within the cell-binding domain of fibronectin.17,18 We evaluated the effect of synthetic RGD-containing peptides on fibronectin-dependent macrophage fibrin binding. Figure 2 shows one of two identical studies. Monolayers of peritoneal macrophages were incubated with 40 |xg of radiolabeled fibrin monomer with and without 40 |xg intact fibronectin as described in Materials and Methods. Synthetic GRGDSP and GRGESP peptides (Telios, La Jolla, CA), solubilized in DMEM with 10 mmol/L HEPES, pH 7.4, were added to parallel cultures. In this study, fibronectin significantly (P < .005) enhanced fibrin binding approximately threefold, from 111 ± 4 ng/well to 326 ± 11 ng/well. The coincubation with 1.0 mmol/L GRGDSP or GRGESP had no effect on either basal fibrin binding or on the fibronectin enhancement of fibrin binding.

To determine if a cellular response was required for the fibronectin augmentation of fibrin binding by macrophages, we fixed monolayers of macrophages with paraformaldehyde. The fibrin-binding assay was then performed as described in Materials and Methods along with parallel studies on nonfixed macrophages. Figure 3 demonstrates that paraformaldehyde treatment (fixed) had no effect on the basal levels of fibrin binding compared with unfixed macrophages (control). Coincubation (FN co-Fibrin) with 40 |xg fibronectin caused a significant (P < .05) increase in the macrophage binding of fibrin by both normal and paraformaldehyde fixed macrophages. Data are expressed as percent of control binding (fibrin alone, normal cells). Mean control fibrin binding was 113 ± 17 ng/well. Fibrin and fibronectin have been shown to be closely associated on the surface of macrophages.20 Therefore, it was possible that exogenously added fibronectin bound to
the macrophage surface and functioned as a receptor for fibrin. To determine if such a mechanism was responsible for enhanced levels of fibrin binding, macrophage monolayers were preincubated with fibronectin. In Fig 3, normal (control) and paraformaldehyde-treated (fixed) macrophages were rinsed three times with HBSS and pretreated (FN pre-Fibrin) with 40 μg fibronectin at 37°C, 5% CO₂, 95% air in DMEM, 10 mmol/L HEPES, 0.5% BSA, pH 7.4. This pretreatment resulted in the retention of an additional 67.2 ng/well of fibronectin on the surface of fixed monolayers. Fibronectin retention by unfixed macrophages was similar (62 ng/well). After 1 hour, the cells were rinsed three times with HBSS and 40 μg radiolabeled fibrin was added for an additional hour under identical conditions. In both fixed and control cells, fibronectin pretreatment did not increase macrophage fibrin binding (Fig 3).

To determine if fibrin was binding to endogenous surface fibronectin on the macrophage, cell-surface fibronectin was removed by the method of Colvin. The F(ab)₂ fragment of antibody against fibronectin was used to link surface fibronectin into an aggregate that caused capping on the cell surface and internalization by the macrophages. Figure 4 shows phase-contrast (A, C, and E) and corresponding immunofluorescence (B, D, and F) micrographs of macrophages after 1-minute (A and B), 30-minute (C and D), and 120-minute (E and F) incubations with the F(ab)₂ antibody fragment. Throughout the time course, no changes in cell morphology were visible by phase-contrast microscopy. After a 1-minute incubation, fibronectin antigen was shown as diffuse granular fluorescence over the entire cell surface (Fig 4B). At 30 minutes, fibronectin antigen was localized to discrete foci on the cell surface (Fig 4D) with larger areas of intense fluorescence (capping) visible on some cells. Fibronectin antigen was no longer detectable on the cell surfaces after a 120-minute incubation with the antibody fragment (Fig 4F), presumably having been internalized by the macrophages. The greatest proportion of the cell population appeared to be aggregating and capping its fibronectin antigen after a 30-minute incubation with the antibody fragment. Therefore, this time point is shown for illustrative purposes. However, it is important to note that the aggregation and capping of fibronectin antigen appeared to begin within 15 minutes after the addition of antibody fragment. This process continued on some portion of the cell population for 45 to 60 minutes. Additionally, after 45 minutes, there was a time-dependent reduction in the overall amount of immunofluorescence visible on the cells, indicating the continual removal of fibronectin antigen from the cell surfaces. For purposes of this study, a 120-minute antibody incubation was chosen to ensure maximal removal of fibronectin antigen from the macrophage surface.

Using the information obtained from this study (Fig 4), we prepared monolayers deficient in cell-surface associated fibronectin and evaluated their ability to bind fibrin. Results of this study are shown in Fig 5. Fibrin with and without 40 μg fibronectin was added to untreated (control) and surface-fibronectin deficient (J- cell-associated Fn) macrophage monolayers under the conditions described in Materials and Methods. Data are expressed as nanograms of fibrin bound per well. In these studies, fibronectin significantly (P < 0.05) enhanced the binding of fibrin by untreated macrophages (142 ± 37 ng to 336 ± 47 ng). The removal of cell-surface fibronectin had no effect on the binding of fibrin alone by these macrophages (142 ± 37 ng and 136 ± 33 ng). The addition of 40 μg exogenous fibronectin to macrophages lacking endogenous fibronectin significantly increased the binding of fibrin by these cells (136 ± 33 ng to 329 ± 34 ng, P < 0.05).

DISCUSSION

Fibrin binding and subsequent degradation by macrophages has important implications in the resistance to potentially detrimental thrombosis associated with disease states. Electron microscopic studies have shown that circulating fibrin binds to the surface of hepatic Kupffer cells. During liver perfusions, radiolabeled fibrin is removed from circulation by Kupffer cells. When low-grade coagulation is induced experimentally in the vasculature of intact animals, the Kupffer cells accumulate fibrin. Furthermore, if mononuclear phagocyte activity is depressed experimentally, the removal of fibrin from the circulation is delayed. Thus, the role of mononuclear phagocytes in the metabolism of fibrin is critical to the maintenance of vascular homeostasis under thrombotic conditions. Several investigators have shown a role for fibronectin in the macrophage uptake of soluble fibrin. This study examined the mechanism of fibronectin-dependent macrophage fibrin binding.

We evaluated the ability of several defined fibronectin fragments to enhance macrophage fibrin binding to determine which domains were critical to this event. Figure 1 shows that the ability to augment macrophage fibrin binding is contained entirely within the 29-Kd amino-terminal fibrin-binding fragment. While this domain does bind fibrin, it is surprising that it could support binding of fibrin to macrophages, as it lacks the regions commonly associated with cellular binding. The amino terminus of fibronectin does bind to fibroblasts and endothelial cells. This binding has been implicated in the assembly of a fibronectin matrix. Interestingly, the 180-Kd and 120-Kd fibronectin fragments, both of which have affinity for fibrin and cell surfaces, did not enhance fibrin binding. This may be attributed to the nonphysiologic conditions under which the Fib II and Fib III sites reportedly bind fibrin. While we are aware that the concentrations of the 120-Kd and 180-Kd fragments used in this study were below ideal for integrin receptor binding, 10-fold higher concentrations also failed to enhance fibrin binding. Additionally, the amino-terminal fragments augmented binding to similar levels as the intact molecule when used at equimolar concentrations. This indicates that the cell binding domain of fibronectin is not necessary for this augmented interaction between fibrin and macrophages.

While the fragments of the fibronectin molecule containing the RGD sequence did not enhance fibrin uptake, this component could play a role during interactions of the intact molecule with fibrin or macrophages. We evaluated the effects of synthetic peptides containing the RGD
sequence to further understand the contributions of this sequence in the fibronectin-dependent binding of fibrin. The results indicate that this important cell-glycoprotein adhesion sequence does not mediate the fibronectin-independent macrophage binding of fibrin (Fig 2).

We used fibronectin pretreatment of both normal macrophages and paraformaldehyde fixed macrophages to determine if fibronectin binding elicits a macrophage response that is manifested as an enhanced fibrin binding. Intracellular stores of some integrin receptors are contained within specific granules termed adhesosomes. The expression of these receptors can be enhanced with stimulatory agents such as phorbol esters. We wanted to determine if fibronectin binding by macrophages was exerting a stimulatory effect on the cells, causing an upregulation or an increase in the number of cellular receptors for fibrin. We found that paraformaldehyde fixation of macrophages had no effect on the binding of fibrin, or on the increase in binding seen when fibrin is coincubated with fibronectin on the macrophage monolayer (Fig 3). Fixation would prevent
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Our second approach to this problem was via the removal of endogenous surface fibronectin from the macrophages. The removal of surface fibronectin antigen was verified by immunofluorescence (Fig 4). Figure 5 shows that fibrin binding occurred independent of fibronectin on the surface of the macrophage under our assay conditions. Basal levels of fibrin binding were similar in normal and fibronectin-deficient macrophages. Additionally, macrophages depleted of cell-surface fibronectin exhibited an increase in fibrin binding when coincubated with exogenous fibronectin.

A fibrin receptor such as that identified by Shainoff et al may, in part, be responsible for the basal levels of fibrin binding shown in this study in the absence of fibronectin. Shainoff et al have demonstrated that fibrin binding to the murine macrophage cell line J774, in a fibronectin-free system, is mediated by a receptor that interacts with the amino-terminal domains of fibrin. This binding is not inhibited by synthetic RGD peptides, but can be blocked by synthetic sequences corresponding to the amino termini of the α- and β-chains of fibrin. Given the low B_max of this high-affinity fibrin receptor ($B_{\text{max}} \approx 200$ to 800 sites/cell, $K_d = 10^{-12}$ mol/L), it is limited in its ability to scavenge circulating fibrin, but may act in concert with other, less limited fibrin clearance mechanisms such as fibronectin-dependent fibrin binding. Additionally, we have previously shown that a small portion of the basal fibrin binding seen in the absence of fibronectin is not sensitive to removal by trypsinization.$^1$ This was approximately equal to the levels of basal fibrinogen binding by these cells and may represent nonspecific fluid-phase pinocytosis.

In a series of studies, Jilek and Hormann$^8$ and Hormann et al$^7,10$ examined macrophage binding of fibrin. They showed that fibronectin augments this process and their data are supportive of a primary role for the amino-terminal domain in this process. They suggested that fibrin can bind to fibronectin fragments which have been attached to the macrophage surface by transglutaminases. In our assay, fixed macrophages exhibit fibronectin-enhanced fibrin binding in serum-free conditions. As fixation presumably eliminates cell-associated transglutaminase activity, it is unlikely that the fibronectin-augmented fibrin binding we observed is due to cellular transglutaminase-mediated cross-linking to the macrophage surface. This does not preclude a factor XIII-mediated cross-linking. The preparations of fibrin used in this study may contain factor XIII. However, we have shown that fibronectin on the cell surface did not serve as a surface receptor for fibrin and that pretreatment of macrophages with fibronectin does not enhance fibrin binding. Thus, it is unlikely that the enhanced fibrin binding we observe is due to fibronectin cross-linked to the macrophage surface. However, the contributions of fluid-phase transglutaminases are not directly addressed in this report. It is possible that fluid-phase transglutaminases play a role in stabilizing the interaction between fibrin and fibronectin before cellular binding or in stabilizing the complex after cellular binding.

We have partially characterized the mechanism whereby fibronectin enhances the binding of fibrin by macrophages. This effect is not unique to this macrophage type. Fibronectin augmentation of fibrin binding also occurs on phorbol ester-treated, adherent U937 cells and by 3-day cultured, adherent human monocytes (data not shown). Additionally, in limited studies, fibrin monomer prepared by acid solubilization (Tris HCl) is bound by macrophages in a manner similar to the binding of urea-soluble fibrin monomer used in these studies (data not shown). The data in this study suggest a mechanism whereby the amino-terminal domain
of fibronectin interacts with fibrin in the fluid phase, followed by the macrophage binding of this complex. The receptor or receptors involved in this mechanism remain to be defined. The data from our studies using fibronectin fragments and synthetic peptides indicate that previously identified integrin-type receptors are not responsible for this augmentation. The macrophage binding of a fibrin-fibronectin complex may occur via the fibronectin component. Therefore, studies with fibronectin fragments would indicate that this binding is directed to the amino-terminal domain of fibronectin. It is also possible that the macrophage binding of such a complex could occur through the fibrin component and the enhancement seen in the presence of fibronectin due to conformational changes in the fibrin molecule. Binding of amino-terminal fibronectin fragments has been demonstrated on fibroblasts and endothelial cells. High-affinity binding of fibronectin to peritoneal macrophages has been shown and exhibits kinetics similar to the amino-terminal fibronectin binding seen on fibroblasts. In related studies in our laboratory we have identified a macrophage cell-surface protein with binding activity directed to the amino-terminus of fibronectin. The identification of the macrophage receptor or receptors responsible for the binding event described in this report as well as a better understanding of the role of transglutaminases in this process will be important to further our understanding of the macrophage metabolism of fibrin.

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