Blood-Borne Fragments of Fibronectin After Thermal Injury

By Peter La Celle, Frank A. Blumenstock, and Thomas M. Saba

Fibronectin is an adhesive glycoprotein (GP) found in a soluble form in plasma and lymph as well as in a relatively insoluble form in basement membranes and connective tissue matrices. It is a dimeric, large molecular weight GP consisting of unique domains with high affinity for actin, collagen, fibrin, and cells. The polypeptide chains between these globular domains are highly sensitive to proteolytic attack, which can generate defined fragments of fibronectin as characterized by their amino acid sequence as well as binding affinities. Fibronectin’s opsonic activity enables it to stimulate macrophage phagocytosis of nonbacterial particulate debris. Fibronectin’s adhesive activity influences endothelial cell-cell interaction and cell adhesion to a collagenous substratum. Fragments of fibronectin as well as a unique peptide unit can compete with the intact molecule for selected biologic functions.

Fragments of fibronectin as well as fibrin degradation products (FDP) are beginning to emerge as potentially important modulators of the cellular response to inflammation and injury. Ehrlich et al. reported that plasmin-generated fragments inhibit macrophage uptake of gelatinized particles under in vitro and in vivo conditions. Accordingly, depletion of intact fibronectin coupled with the presence of fibronectin fragments may collectively inhibit liver reticuloendothelial phagocytic clearance of fibronectin-opsonized blood-borne debris occurring as a result of intravascular coagulation and/or tissue injury.

Additionally, Clark et al. have reported the existence of a 120-Kd fragment of fibronectin that is chemotactic for blood monocytes. This chemotactic activity only appears expressed upon fragmentation of the fibronectin molecule. Its prompt expression following fibronectin fragmentation suggests that proteolysis of the fibronectin may amplify tissue injury by recruiting inflammatory cells. Ozkan et al. have identified a peptide suppressor of neutrophil chemotaxis in the blood of trauma and burn patients that appears to be a fragment of fibronectin. Lymphocyte-immunosuppressive effects of the peptide-containing serum may also be due to products of fibrinolysis that are known to be present in plasma after trauma or burn. Such observations suggest that fibronectin fragments released at a site of tissue injury, due to the enhanced proteolytic environment, may influence leukocyte and monocyte infiltration into wounds.

Fibronectin fragments may also influence endothelial cell adhesion and vascular permeability. For example, fibronectin peptides have been documented to dissociate cultured endothelial cells from their substratum and discrete fibronectin fragments appear in the plasma and lung lymph in sheep following thrombin-induced lung vascular injury. FDP may also alter the integrity of the lung vascular barrier, and the endothelial detachment observed with exposure of cultured endothelial cells to fibrinogen fragment D may be mediated, in part, by redistribution of the actin cytoskeleton in substrate attached cells. Conversely, elevation of plasma fibronectin can limit the

From the Department of Physiology and Cell Biology, Albany Medical College, Albany, NY.


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Address reprint requests to Thomas M. Saba, PhD, Professor and Chairman, Department of Physiology & Cell Biology (A-134), Albany Medical College, 47 New Scotland Ave, Albany, NY 12208.

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increase in lung vascular permeability with postoperative sepsis, approximately due to the incorporation of the plasma fibronectin into the adhesive tissue fibronectin pool in the lung vascular barrier. Because fibronectin is especially sensitive to proteolytic enzymes whose plasma concentrations increase following burn, we determined if fibronectin fragments could be detected in the plasma following sublethal burn trauma.

MATERIALS AND METHODS

Sublethal burn. Male Sprague Dawley rats weighing 250 to 300 g were provided food and water ad libitum. These rats were ether-anesthetized and subjected to either a 90°C (experimental) or a 25°C (sham) 3-second burn over 15% of the body surface area, as previously described. Blood samples were collected either serially from the tail tip before and at various times after burn or under ether anesthesia from the inferior vena cava at 60 minutes post-burn. All blood was collected into an antiprotease cocktail yielding final plasma concentrations of 0.15% ethylenediamine tetraacetate (EDTA), 3 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 3 mmol/L iodoacetate to prevent coagulation and proteolysis. Immediately after centrifugation, plasma was combined either with sodium dodecyl sulfate (SDS) sample buffer in preparation for immunoblot analysis or diluted for subsequent enzyme-linked immunosorbent assay (ELISA) of fibronectin concentration.

Antibodies to rat fibronectin. Affinity-purified goat antibody to rat fibronectin was provided by Dr Vera Stecher, formerly of Sterling Winthrop Research Institute (Rensselaer, NY). Affinity-purified rabbit antibody to rat fibronectin was prepared as previously described. Antibody specificity was verified by precipitant arc when diffused against rat plasma at an electrophoretic migration distance identical to that of purified rat plasma fibronectin.

Western blot analysis of plasma fibronectin. Plasma proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted onto nitrocellulose as previously reported. Plasma samples were diluted 1:8 in sample buffer (0.1 mol/L tris, pH 6.8 containing 3% SDS, 16% glycerol) and boiled for 5 minutes. Sample proteins (10 μL per lane) were stacked at 20 mA through 3% acrylamide (tris pH 6.8), and separated at 40 mA in 4% to 15% gradient polyacrylamide slab gels (tris pH 8.8). Molecular weight markers (Bethesda Research Laboratories, Gaithersburg, MD) consisted of myosin (H chain), 200 Kd; phosphorylase B, 97.4 Kd; bovine serum albumin (BSA), 68 Kd; ovalbumin, 43 Kd; α-chymotrypsinogen, 25.7 Kd; β-lactoglobulin, 18.4 Kd; and lysozyme, 14.3 Kd. Proteins were transferred electrophoretically onto nitrocellulose (0.02 mol/L tris with 0.15 mol/L glycine in 20% methanol, pH 8.3) at 100 V for 2 hours. The remaining protein binding sites on the paper were blocked by exposure to 5% milk protein (Carnation, Los Angeles, CA) and 2% goat serum in 0.05 mol/L tris with 0.2 mol/L NaCl (TBS), pH 7.4, for 2 hours at room temperature. Affinity-purified rabbit antirat fibronectin antibodies were added directly to the blocking solution and oscillated overnight at room temperature. After a 10-minute TBS wash, blots were incubated in TBS with 5% milk protein, 2% rat serum, and hors eradish peroxidase-conjugated goat antibody to rabbit IgG (Organon Teknika, West Chester, PA) for 2 hours at room temperature. Blots were again washed in TBS for 10 minutes, and stained for 30 minutes at room temperature using 3.4 mmol/L 4-chloro-1-naphthol in TBS with 17% methanol. The approximate molecular weights of the fragments were estimated from measurements of sample band positions in the three immunoblots as compared with plots of standard band position versus molecular weight for each blot.

ELISA for rat fibronectin. Fibronectin concentrations of plasma samples were measured using a double-antibody ELISA as described. In this process, 96-well ELISA plates (Corning, Corning, NY) were coated with goat antirat fibronectin primary antibody at pH 9.6. Three plate washes were performed between incubations. Nonspecific binding was blocked with 3% BSA in the alkaline buffer. After incubation with samples and purified fibronectin standards diluted in PBS-Tween (0.01 mol/L phosphate buffer, pH 7.4 with 0.5% BSA, and 0.15% Tween-20 [Sigma, St Louis, MO]), rabbit antibody to rat fibronectin in PBS-Tween was adhered to bound antigen, and horseradish peroxidase-conjugated goat antibody to rabbit IgG was used with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) for detection. The ELISA assay was sensitive to a fibronectin concentration as low as 2 ng/mL.

RESULTS

Rat plasma fibronectin concentrations measured by ELISA at various times after burn are shown in Fig 1. In the burn group, the fibronectin concentration was significantly decreased (P < .01) to 34% ± 4.7% of zero time levels (mean ± SEM), with partial restoration to 41% ± 6.8% of control by 4 hours. Accordingly, as shown in Fig 1, the plasma fibronectin concentration decreased from 645 ± 91 μg/mL at zero time to 200 ± 13 μg/mL within 30 minutes. When the serial plasma samples were evaluated by immunoblot analysis, we observed the appearance of two prominent fibronectin fragment bands with molecular weights of 110 ± 40 Kd.
2.2 Kd and 122 ± 3.3 Kd by 30 minutes after burn in each animal, reaching an apparent concentration maximum by 1 hour. In each case, the quantity of these fragments diminished by 4 hours after burn. No such fragments of fibronectin existed in any of the plasma samples harvested from control animals, which were anesthetized and bled in an analogous manner. These fragments were distinct as compared with the intact dimer (440 Kd) and the monomer (220 Kd) as shown in Fig 2.

To verify that fragmentation of fibronectin was not the result of surface activation of proteases at the tail-tip sampling site, plasma was also collected by exsanguination via the inferior vena cava (in a separate study) at 1 hour post-burn, using the same antiprotease cocktail. As seen in Fig 3, the fragments found in the vena cava sample (far right lane) are identical to those detected in the serial tail vein samples (seven left hand lanes), confirming the existence of these fragments in the circulation post-burn.

**DISCUSSION**

The plasma glycoprotein fibronectin consists of two nearly identical 220-Kd subunits joined near the carboxy-termini by a pair of disulfide bonds. These polypeptide chains are characterized by discrete globular domains comprised of three types of repeating homology units, designated types I, II, and III. Flexible regions connecting the domains, which allow the otherwise linear chains to kink and bend, are also very sensitive to proteolytic cleavage. Treatment of purified fibronectin with enzymes such as leukocyte elastase, cathepsin G, cathepsin D, chymotrypsin, trypsin, plasmin, thrombin, or with combinations of enzymes in succession, can be used to prepare protease-resistant fragments of the parent molecule. Domain structure is highly retained following proteolysis, although specific functions of the intact molecule can be altered. Interest in fibronectin fragments extends beyond their usefulness in elucidating structure-function relationships of the parent molecule. Experimental evidence suggests their importance in host defense by at least three mechanisms. First, fragments may impede the ability of intact fibronectin dimer to opsonize debris produced by tissue injury, which appears in the circulation after burn injury.22 Using exogenous gelatin or gelatin-coated particles as a model debris, fibronectin has been shown to bind the collagenous ligands leading to clearance of the complexes in the liver.5,22 Situations in which this clearance is impaired have been associated with decreased resistance to sepsis and trauma.24-26 Because plasmin-derived degradation products of fibronectin can impede the reticuloendothelial clearance of particles, it is plausible to suggest that fibronectin fragments may limit macrophage host defense after injury.

Second, fragments of fibronectin may also influence recruitment of phagocytic cells into wounds. Clark et al10 have determined that a 120-Kd fragment is chemotactic for monocytes in vitro, but not for neutrophils. Neither the intact protein nor an overlapping 150-Kd fragment showed monocyte chemotactic activity. When the 120-Kd fragment was introduced into the rabbit lung bronchoscopically, there was an increased migration of intravenously injected labeled monocytes into the alveoli as detected by bronchoalveolar lavage.27 Ozkan et al11,12 have isolated a peptide suppressor (SAP) of lymphocyte blastogenesis and neutrophil chemotaxis from the blood of immunosuppressed trauma and burn patients. A monoclonal antibody to this peptide that blocks its suppressor activity cross-reacts with both intact fibronectin as well as a low molecular weight fibronectin fragment.28 Easter et al29 found that an elastase-derived small molecular weight fibronectin fragment also inhibits neutrophil chemotaxis, and that the inhibition is blocked by the SAP antibody. Fibronectin fragments released at sites of injury may modulate leukocyte and monocyte infiltration of wounds.30 Accordingly, characterization of the potential chemotactic activity of the fibronectin fragments detected early after burn in the present study may provide valuable information on the biologic effects of these fragments. While such fragments may have adverse effects, their generation may contribute in a beneficial manner to the regulation of the host response to tissue
injury by modulating the recruitment of monocytes/macrophages to sites of tissue injury to assist in local wound defense and debridement by phagocytic cells.

The molecular weight we observed for the larger fibronectin species corresponds well with that fragment observed by Clark et al to be chemotactic for monocytes. Smaller molecular weight fragments were not observed in our system, perhaps due to their rapid clearance. In support of this possibility is the observation that fibronectin fragments with a molecular weight less than 100 Kd have been observed in rat urine. Alternatively, low molecular weight fragments may be produced at concentrations below the detection limits of our Western blot analysis. Small molecular weight fragments can also stain poorly, reducing the likelihood of detection.

Third, fibronectin appears to influence lung vascular integrity by limiting the increase in lung transvascular protein clearance seen in sheep with experimental postoperative bacteremia. Given the well-established cell binding capabilities of fibronectin, it is conceivable that fibronectin mediates endothelium-basement membrane contacts important in maintaining barrier function, and that fibronectin fragments undermine barrier integrity by competing with basement membrane fibronectin for binding sites on endothelial cells. This concept is supported by the findings of Hayman et al, which demonstrate that the addition of fibronectin peptides corresponding to the cell binding region of the molecule to cultured endothelial cells will actually limit their adhesion. In terms of the current study, whether the fibronectin fragments observed contain the gelatin-binding and/or the cell-binding domain remains to be determined.

Increased proteolytic activity associated with inflammation and injury is well documented. Neutrophil activation in response to injection of foreign particles, as well as complement activation or disseminated intravascular coagulation results in degranulation and release of proteases such as leukocyte elastase and cathepsin G, which can degrade fibronectin. Increased elastase levels have been measured in the plasma after burn injury, and activated leukocytes can trigger the release of fibronectin degradation products from endothelial monolayers. Indeed, the rapid appearance of soluble collagenous material within skin after burn injury directly suggests an acute increase in protease activity in injured tissues after burn trauma. Thus, a proteolytic environment exists in both injured tissue and blood after burn that can result in fibronectin proteolysis.

Given the documented influences of fibronectin fragments on phagocytic host defense and the potential for their formation by protease activity after injury, the current direct documentation of fibronectin fragments in the plasma following sublethal burn trauma may have important clinical implications. Further studies are required to determine how such fragments may affect phagocytic function as well as vascular integrity after burn injury.

REFERENCES


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P La Celle, FA Blumenstock and TM Saba