Blood-Borne Fragments of Fibronectin After Thermal Injury

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Fibronectin is an adhesive protein that can promote phagocytosis and endothelial cell adhesion. Plasma fibronectin declines following burn in animals and patients, potentially due to its complexing with circulating collagenous debris as well as its rapid binding to sites of tissue injury. Such depletion of fibronectin initiates an opsonic deficiency of the plasma. In view of the sensitivity of fibronectin to proteolytic enzymes, an additional factor that could contribute to the decrease of plasma opsonic activity after burn is the proteolytic fragmentation of fibronectin in the blood. In the current study, we determined if fibronectin fragments appear in the blood of anesthetized rats after a sublethal full-thickness skin burn of 15% to 16% of body surface. Plasma fibronectin concentration was quantified by enzyme-linked immunosorbent assay and the presence of fibronectin fragments in plasma was determined by immunoblot analysis. All blood was collected in an antiprotease mixture to yield final plasma concentrations of 0.15% EDTA, 3 mmol/L phenylmethylsulfonyl fluoride, and 3 mmol/L iodoacetate to prevent degradation of fibronectin after sampling. Plasma fibronectin decreased 60% to 70% within 30 minutes post-burn, and this low level lasted for at least 4 hours. Within 30 minutes post-burn, two prominent fragments of fibronectin with a molecular weight of 110 ± 2.2 Kd and 122 ± 3.3 Kd, respectively, were also detected in the plasma. Peak concentration of these fragments was detected at 60 minutes post-burn, but their level declined by 4 hours. By 4 hours, both bands appeared to resolve into doublets. To rule out the possibility that the fragments of fibronectin detected in the plasma were actually generated by coagulation enzymes activated at the site of peripheral blood sampling, rapid direct inferior vena cava sampling was performed, which also yielded the presence of the fragments. Thus, fibronectin fragments exist in the plasma following thermal injury. Because fragments of fibronectin can compete with the intact fibronectin molecule with respect to its ability to stimulate macrophage phagocytosis, such fragments may contribute to altered systemic phagocytic host defense following thermal injury. Furthermore, because fibronectin peptides can compete with matrix fibronectin and impair adhesion of cultured endothelial cells, such circulating fragments may also influence the integrity of the vascular barrier.

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increase in lung vascular permeability with postoperative sepsis,19 apparently 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,20 whose plasma concentrations increase following burn,13,21 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 either 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.22 Blood samples were collected either serially from the tail tip and 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.23 Antibody specificity was verified by precipitant arc electrophoretic analysis. Each antibody produced a single 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.22 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 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; a-chymotrypsinogen, 25.7 Kd; b-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 milk protein, 2% rat serum, and 0.15% ethylenediamine tetraacetate (EDTA), 3 mmol/L PMSF, and 3 mmoL iodoacetate. Fibronectin was isolated from the plasma by gelatin-Sepharose affinity chromatography as previously described.22 Fibronectin was eluted from the column with 4 mol/L urea and dialyzed in 0.2 mol/L phosphate buffer, pH 7.4, with 0.9% saline before storing at −80°C.

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 ±
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.

**Fig 2.** Nitrocellulose immunoblots of SDS-PAGE gels showing intact and fragmented rat plasma fibronectin in samples collected serially at various times (0 to 240 minutes) after sham or burn trauma. Samples were collected into an antiproteolytic solution to yield final plasma concentrations of 0.15% EDTA, 3.0 mmol/L PMSF, and 3.0 mmol/L iodoacetate to inhibit coagulation and general proteolysis. The immunoblots in three separate rats are presented to demonstrate the consistency of the result. After burn (right side), fibronectin fragments with molecular weights of 110 ± 2.2 and 122 ± 3.3 Kd (calculations were based on band migration distances in the immunoblots) were detected in the plasma of each rat within 30 minutes. Molecular weights (Kd) of standards are noted.

**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. 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. Situations in which this clearance is impaired have been associated with decreased resistance to sepsis and trauma. 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 al 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. Ozkan et al 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. Easter et al 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. 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 particle 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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