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Research Article

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Mechanism of Acute Depletion of Plasma Fibronectin Following Thermal Injury in Rats

Appearance of a Gelatinlike Ligand in Plasma

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Abstract. Plasma fibronectin was depleted within 15 min following sublethal burn, followed by partial recovery at 8 h and complete restoration by 24 h in anesthetized rats. Radiolabeled ^{75}Se-plasma fibronectin, injected intravenously before burn, was rapidly sequestered in burn skin as well as the liver. Fibronectin levels at 2 h postburn as detected by immunoassay vs. ^{75}Se-plasma fibronectin indicated that more fibronectin was in the plasma than detected by electroimmunoassay. Crossed immunoelectrophoretic analysis of fibronectin in early postburn plasma demonstrated a reduced electrophoretic mobility of the fibronectin antigen. Addition of heparin or fibrin, both of which have affinity for fibronectin, to normal plasma was unable to reproduce this altered fibronectin electrophoretic pattern. In contrast, addition of gelatin or native collagen to normal plasma reproduced the abnormal electrophoretic pattern of fibronectin seen in burn plasma. Extracts of burned skin, but not extracts of normal skin, when added to normal plasma, elicited a similar altered electrophoretic pattern for fibronectin. By gel filtration, fibronectin in burn plasma had an apparent molecular weight $\sim 40\%$ greater than that observed in normal plasma. These data suggest the release into the blood of a gelatinlike ligand from burned skin, which complexes with plasma fibronectin. Thus, fibronectin deficiency acutely postburn appears mediated by (*a*) its accumulation at the site of burn injury; (*b*) its removal from the circulation by the liver; and (*c*) its presence in the plasma in a form that is less detectable by immunoassay.

Introduction

Plasma fibronectin is a 440-kD glycoprotein with an affinity for collagen, fibrin, Clq, actin, heparin, as well as sites of tissue injury (1–4). It becomes incorporated into the fibrin clot during coagulation; it is sequestered extravascularly at sites of tissue injury; and is believed to facilitate wound healing by promoting cell-cell interaction and cell attachment to a substratum (3, 5–8). Plasma fibronectin can be incorporated into basement membranes and the extravascular matrices of many cells and may serve as a reservoir for the fibronectin found in tissues (6, 9, 10). Plasma fibronectin is identical to cold-insoluble globulin or opsonic α-2-surface binding (SB) glycoprotein, which is believed to augment macrophage clearance of nonbacterial particulates (1, 3). Burn-induced alterations in circulating plasma fibronectin may alter opsonic activity and phagocytic removal of products of tissue injury.

Early following burn, immunoreactive plasma fibronectin concentration is reduced in association with decreased reticuloendothelial system (RES)[1] phagocytic activity and impaired plasma opsonic activity (11–14). Documentation of plasma fibronectin deficiency following thermal injury has primarily relied upon immunologic techniques. Recently, it has been shown that the in vitro addition of gelatin (denatured collagen) to

1. *Abbreviations used in this paper:* %ID/g, %ID/ml, %ID/TO, percentage of injected dose per gram, milliliter, or total organ, respectively; RES, reticuloendothelial system.

normal plasma reduces the apparent plasma fibronectin concentration as measured by electroimmunoassay (15). Similarly, the in vitro or in vivo addition of gelatin or actin to normal plasma depresses the in vitro opsonic activity (15, 16). Thus, it has been suggested that the presence of collagen or collagenous debris in the blood early following injury may contribute to immunoreactive fibronectin and bioassayable opsonic deficiencies. Although numerous studies have documented opsonic deficiency and diminished plasma fibronectin early after burn, the etiology of this acute fibronectin deficiency has not been delineated.

The present study was designed to investigate the mechanisms of plasma fibronectin deficiency following acute, sublethal burn injury in anesthetized rats. Accordingly, dual isotopic experiments with ^{75}Se-plasma fibronectin and ^{125}I-albumin were used. Emphasis was placed on plasma fibronectin localization at the site of thermal injury; accumulation of plasma fibronectin in the liver and spleen, which are rich in RES cells; and the nature of the plasma fibronectin molecule that continues to circulate shortly after burn trauma.

Methods

Sublethal burn model

Healthy male Sprague-Dawley rats weighing 250–300 g were used in all studies. Under ether anesthesia, they received a sublethal 20% body surface area burn by 3-s exposure of the shaved dorsal skin to 90°C water. Control nonburned rats were anesthetized and exposed to 20°C water for 3 s. The rats rapidly recovered and were allowed food and water ad lib. throughout the experimental period. Histologic examination revealed a second degree, partial thickness burn with no apparent involvement of the underlying striated muscle.

Immunoelectrophoretic assays

Plasma was obtained from blood anticoagulated with 7.5% EDTA and supplemented with 3 mM of the antiprotease benzamidine to prevent proteolysis of plasma constituents. All assays were corrected for dilution by the anticoagulant. Antiserum specific for rat albumin was purchased commercially (Cappel Laboratories, Cochranville, PA), while monospecific antiserum to rat plasma fibronectin was developed in rabbits as previously described (17). Purified rat albumin and rat plasma fibronectin served as standards for the electroimmunoassay techniques as described by Laurell (18) and previously used by Blumenstock et al. (17) for quantification of plasma fibronectin in rats. Electroimmunoassay and crossed immunoelectrophoresis (19) were conducted in 1% ME agarose (Seakem, FMC Corp., Rockland, ME) using a 0.1 M Tris buffer (pH 8.5) containing 0.50 mM Ca lactate and 0.02% Na azide.

Isotopically labeled plasma fibronectin and albumin

Plasma fibronectin was metabolically labeled with the amino acid analogue [^{75}Se]selenomethionine (Sethotope, E. R. Squibb & Sons, Princeton, NJ). Normal rats, which served as plasma donors, were exsanguinated under anesthesia 4 h after intravenous injection of 500 μCi [^{75}Se]selenomethionine. The ^{75}Se-labeled fibronectin from this plasma was isolated by gelatin-Sepharose affinity chromatography (2, 20). After elution of the fibronectin with urea (4 M), the isolated protein was

dialyzed against 0.2 M sodium phosphate buffer (pH 7.4) with 0.02% 2-mercaptoethanol at 4°C for 24 h, the dialysate was changed twice. Dialysis of the purified plasma fibronectin was done in 50,000-mol wt dialysis tubing (Spectra/Por 6, Spectrum Medical, Los Angeles, CA). The purified ^{75}Se-labeled plasma fibronectin was stored at 4°C in the dialysis buffer and used within 24 h for in vivo disappearance kinetics studies. Gradient polyacrylamide gel electrophoresis (Gradipor 2.5–27% survey gel, Isolab Inc., Akron, OH) verified the purity of the isolated ^{75}Se-plasma fibronectin. Retention of its opsonic activity was documented by in vitro opsonic bioassay (21). Iodinated human serum albumin (^{125}I-albumin) was purchased commercially (^{125}I-HSA, Mallinckrodt Inc., St. Louis, MO) and its purity confirmed by gradient polyacrylamide gel electrophoresis.

Isotopic localization and plasma disappearance studies

To evaluate the basis of the plasma fibronectin deficiency after burn, the rats were first injected with both ^{75}Se-fibronectin and ^{125}I-albumin and its clearance and tissue distribution pattern studied at 2 h postburn. The 1.2-ml volume injection contained 0.5 μCi of ^{125}I-albumin (0.5 mg albumin) and 20 nCi of ^{75}Se-plasma fibronectin (0.5 mg fibronectin). 6 min later (0.1 h) a 1.0 ml venous blood sample was obtained from a femoral vein and the small skin incision was closed with a single skin clip. The rats ($n = 10$) were then divided into two equal groups and exposed to either burn or sham burn under ether anesthesia. At 2 h, they were anesthetized again with ether and a second blood sample obtained from the inferior vena cava via a laparotomy. At this time, they were also exsanguinated and reperfused (20-gauge catheter in the inferior vena cava) with saline until the arterial hematocrit was <2%. This whole body perfusion was done to remove both the labeled fibronectin and labeled albumin from the vascular compartment so that the 2-h tissue measurements of radioactivity would represent the extravascular tissue content of these labeled proteins. The following organs and/or aliquots of tissues were removed: liver, spleen, lungs, kidneys, small intestines, colon, brain, abdominal aorta, vena cava, back skin, back muscle, abdominal skin, and abdominal muscle. Back skin and muscle corresponded to the site of injury. In addition, the tail, which was rich in collagen, was studied. In contrast to organs that were directly weighed in their entirety, the total wet weights of skin and muscle were estimated as 20 and 48% of the animal's body weight, respectively.

Substances tested for interaction with plasma fibronectin

Heated plasma. Normal plasma was heated in a 55°C water bath for 0.5 min and then cooled in 20°C water bath. This temperature and time was selected to correspond with previous determinations of muscle temperature at the site of a similar thermal injury in rats (22). Duplicate aliquots of normal plasma maintained at 20°C served as controls. The plasma was subsequently used in the electroimmunoassay mixing experiments at a final concentration of 10% and in the crossed immunoelectrophoresis experiments at a 50% concentration.

Skin extracts. Aliquots of skin were obtained from sham and burned rats 1 h after burn. Skin samples (0.25 g) were cut into six smaller pieces and incubated with 0.5 ml 0.9% NaCl in a shaking water bath at 37°C for 1 h. After incubation, the samples were centrifuged at 10,000 g for 15 min and the supernatant extracts were analyzed without dilution for fibronectin concentration. The extracts were mixed with equal volumes of 40% normal plasma for the electroimmunoassay mixing experiments (final plasma concentration = 10%; see Table IV), and with equal volumes of 100% normal plasma for the crossed immunoelectrophoresis studies.

Fibrin monomer. Rat fibrin monomer was prepared in 0.05 M

Tris–HCl buffer (pH 7.4) containing 3 M urea by the method of Kaplan and Snedeker (23). The rat fibrin monomer solution was added to diluted normal rat plasma so that the final plasma concentration was again 10% for the electroimmunoassay mixing experiment. This resulted in a fibrin monomer concentration of 39 µg/ml and a urea concentration of 0.15 M. For crossed immunoelectrophoresis experiments, 50% rat plasma contained fibrin monomer at 37 µg/ml and urea at 0.15 M.

Heparin. Heparin USP (porcine intestines, Elkins-Sinn, Inc., Cherry Hill, NJ) was combined with diluted rat plasma to yield a final plasma concentration of 10% containing 10 U/ml of heparin for electroimmunoassay mixing experiments. In the crossed immunoelectrophoresis experiments, 50% rat plasma supplemented with heparin at a concentration of 100 U/ml was used.

Gelatin. Gelatin (USP type A), which is a partial acid hydrolysate of bovine skin, white connective tissue, and bone (pI = 8–9) was commercially purchased (ICN Pharmaceuticals Inc., Covina, CA). It was dissolved in 0.9% NaCl and added to 10% normal plasma for electroimmunoassay mixing experiments at a final concentration of 20 µg/ml. It was added to 50% rat plasma and used in the crossed immunoelectrophoresis experiments at a final concentration of 100 µg/ml.

Collagen. Rat tendon type I collagen (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) was prepared at 2 mg/ml in 0.5 M acetic acid and 0.9% NaCl and allowed to dissolve until there was no visible particulate collagen. The mixture was diluted with 0.9% NaCl and added to normal plasma for the Laurell electroimmunoassay mixing experiments at a final concentration of 20 µg/ml in 10% rat plasma and 5 mM acetic acid. It was used for crossed immunoelectrophoresis at a final concentration of 100 µg/ml in 50% rat plasma and 25 mM acetic acid.

Gel filtration

Gel filtration determination of the approximate molecular weight of fibronectin in sham and burn plasma was performed on Sephacryl S-400 using a 1.5 × 100-cm column at a flow rate of 4 ml/h. The buffer consisted of 0.1 M Tris buffer (pH 8.5) containing 5 mM EDTA and 0.15 M NaCl. A 1.5-ml plasma sample was applied to the column and fractions obtained at 30-min intervals. Molecular weight standards consisted of high-molecular-weight gel filtration markers (Pharmacia Fine Chemicals, Piscataway, NJ) in addition to rat albumin. Each fraction obtained from the column was monitored for protein content by measuring the absorbance at 280 nm. Immunoreactive plasma fibronectin and isotopic ^{75}Se-plasma fibronectin were also assayed in each fraction. Additionally, to test directly if denatured collagen (gelatin) would alter the gel filtration profile of fibronectin, a 1.5-ml aliquot of normal rat plasma supplemented with gelatin at a concentration of 200 µg/ml was also applied to the column and analyzed as above.

Gelatin infusion

The effects of intravenous injection of gelatin on circulating isotopic and immunoassayable fibronectin was undertaken in chronically catheterized rats (n = 3) pulse-labeled with ^{75}Se-fibronectin. 6 h after an intravenous bolus injection of 1.2 ml containing 20 nCi ^{75}Se-plasma fibronectin the rats were injected with 0.5 ml/100 g body wt of a 0.3% gelatin in 5% dextrose solution (pH 7.0). Arterial blood samples were obtained before gelatin infusion and 2 h after gelatin infusion.

Isotopic techniques

Samples were assayed for ^{75}Se and ^{125}I activity utilizing a gamma counter with correction for isotope decay (model 4218, Nuclear-Chicago Corp.,

Des Plaines, IL). In experiments utilizing both ^{75}Se and ^{125}I, the overlap of these two energy peaks was corrected.

Recovery analysis for nonadditivity

Studies were also performed to test if the addition of various test solutions to normal fibronectin of known concentration would result in the anticipated recovery of the added antigen as determined by electroimmunoassay. The inability to recover the added antigen by immunoassay was used to demonstrate nonadditivity. The statistical test for nonadditivity (interaction) was based on a modification of the standard two-way analysis of variance (24) as outlined in the Appendix.

Results

Presented in Fig. 1 is the temporal alteration of plasma fibronectin concentration as measured by electroimmunoassay over 24 h following sublethal burn injury. Minimal alteration in plasma fibronectin was observed in control rats. Burned rats demonstrated a marked diminution of immunoreactive plasma fibronectin from 731±49 µg/ml (mean±SE) to 112±9 µg/ml at 1.5 h postburn, and then increased to 959±48 µg/ml at 24 h. The hematocrit alterations after burn are also presented and depict early hemoconcentration.
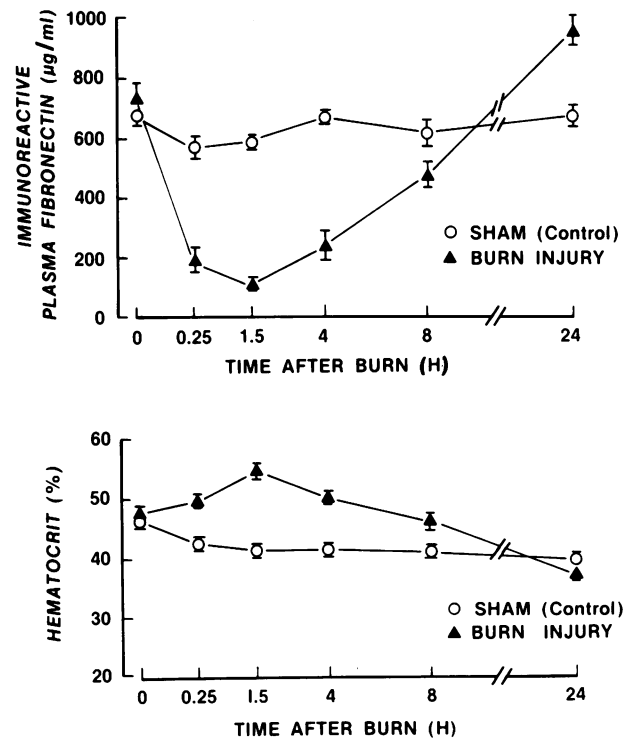

Figure 1. The effect of burn injury on plasma fibronectin concentration. Two groups of five rats each received burn or sham burn. Plasma was obtained from tail vein bleeding immediately before injury and serially thereafter. The development of hemoconcentration following burn is reflected in the hematocrit alterations.

To clarify the mechanism of acute plasma fibronectin loss, labeled plasma fibronectin and labeled albumin were injected together into normal rats, and plasma and tissue samples were obtained for analysis at 2 h after burn. Tables I and II contain the tissue localization data for ^{75}Se-plasma fibronectin and ^{125}I-albumin, expressed as a percentage of injected dose per gram (%ID/g) and as a percentage of injected dose per total organ (%ID/TO). In the sham group, the loss of ^{75}Se-fibronectin and ^{125}I-albumin from plasma at 2 h postburn was secondary to a redistribution from plasma into extravascular tissue sites located primarily in the liver and other tissues (Table II). After burn injury there was an increased loss of both ^{75}Se-fibronectin and

Table I. Plasma Clearance and Tissue Localization of Labeled Plasma Fibronectin and Albumin on a Per Gram Basis After Burn

Tissue sample	Animal group	^{75}Se-Fibronectin time		^{125}I-Albumin time	
		0.1 h	2.0 h	0.1 h	2.0 h
Plasma	Burn	8.850±0.250	4.590±0.160*	9.310±0.490	5.090±0.320*
	Sham	9.140±0.130	6.980±0.430	9.180±0.280	6.480±0.320
Liver	Burn	—	1.760±0.110*	—	0.039±0.004
	Sham	—	0.960±0.070	—	0.087±0.019
Spleen	Burn	—	0.733±0.072	—	0.095±0.022
	Sham	—	0.931±0.113	—	0.179±0.018
Lung	Burn	—	0.123±0.003	—	0.176±0.024
	Sham	—	0.206±0.049	—	0.338±0.062
Kidney	Burn	—	0.312±0.024	—	0.127±0.033
	Sham	—	0.465±0.059	—	0.277±0.074
Small intestine	Burn	—	0.124±0.011	—	0.027±0.002
	Sham	—	0.238±0.045	—	0.060±0.013
Colon	Burn	—	0.089±0.003*	—	0.040±0.003*
	Sham	—	0.130±0.011	—	0.082±0.006
Brain	Burn	—	0.021±0.002	—	0.008±0.003
	Sham	—	0.020±0.006	—	0.003±0.001
Aorta and vena cava	Burn	—	0.314±0.043	—	0.046±0.009
	Sham	—	1.830±0.490	—	0.281±0.082
Tail	Burn	—	0.059±0.006	—	0.094±0.006
	Sham	—	0.087±0.011	—	0.117±0.013
Back skin‡	Burn	—	0.672±0.118*	—	1.290±0.130*
	Sham	—	0.057±0.011	—	0.069±0.011
Back muscle	Burn	—	0.025±0.009	—	0.027±0.091
	Sham	—	0.060±0.007	—	0.037±0.004
Abdominal skin	Burn	—	0.062±0.016	—	0.063±0.029
	Sham	—	0.095±0.032	—	0.104±0.017
Abdominal muscle	Burn	—	0.038±0.004	—	0.024±0.004*
	Sham	—	0.086±0.026	—	0.039±0.004

Tissue localization is expressed as %ID/g. Plasma is on a %ID/ml basis. At 0.1 h, before burn, the concentration of both labeled proteins in plasma was similar for each group. Data are expressed as mean±SE with five rats per group.
* $P < 0.05$, significantly different from sham.
‡ Region of burn injury to the skin.

Table II. Total Isotopic Localization of Labeled Plasma Fibronectin and Albumin After Burn

Tissue	Animal group	^{75}Se-Fibronectin time		^{125}I-Albumin time	
		0.1 h	2.0 h	0.1 h	2.0 h
Plasma	Burn	94.60±1.00	49.00±0.70*	99.50±3.80	54.40±2.90*
	Sham	96.10±1.00	73.40±4.50	96.50±2.60	68.20±3.40
Liver	Burn	—	21.80±0.60*	—	0.48±0.05
	Sham	—	11.20±0.80	—	1.02±0.25
Back skin	Burn	—	8.16±1.36*	—	15.70±1.40*
	Sham	—	0.68±0.12	—	0.83±0.12
Other tissues‡	Burn	—	12.90±1.70*	—	8.52±1.60*
(combined)	Sham	—	21.80±2.50	—	14.40±1.80
Total isotope	Burn	94.60±1.00	90.90±2.70	99.50±3.80	79.10±3.60
recovered	Sham	96.10±1.00	107.10±5.10	96.50±2.60	83.70±3.50

Isotopic localization is expressed on a %ID/TO basis. Data are expressed as mean±SE with five rats in each group.
* $P < 0.05$, significantly different from sham.
‡ This represents the sum of all measured tissues except plasma, liver, and back skin. Total isotope recovered was calculated by summing all measured isotopic localization data and reflects the completeness with which the labels were accounted for.

^{125}I-albumin from plasma within the 2-h period. As seen from Table II, the isotopic plasma albumin content of burned rats decreased to 80% of that observed in sham rats at 2 h and the isotopic plasma fibronectin content of burned rats decreased to 67% of that observed in sham rats.

After burn, liver uptake of ^{75}Se-plasma fibronectin increased by 95% in spite of the marked decline of plasma ^{75}Se-fibronectin, implying a selective uptake of the ^{75}Se-fibronectin by the liver after burn. Specificity was suggested since the hepatic localization of ^{125}I-albumin decreased after burn injury. Compared with controls, the burned skin exhibited greatly increased extravascular localization of both ^{125}I-albumin (19-fold) and ^{75}Se-plasma fibronectin (12-fold). Other measured tissues, as a whole, demonstrated less extravascular localization of both albumin and plasma fibronectin after burn compared with controls (Table II), perhaps reflecting the decline in circulating label that would alter the gradient for flux into tissues.

The relative 2-h distribution of ^{125}I-albumin and ^{75}Se-fibronectin in control and after burn is portrayed in Fig. 2. Intense localization in injured skin of the labeled albumin and fibronectin was observed. All other tissues show reduced localization of both the labeled proteins except for the liver, which displayed a marked increase in its content of labeled fibronectin after burn.

Fig. 3 contrasts isotopic and immunoassayable decreases for plasma fibronectin and albumin as influenced by sublethal burn.

The isotopic loss of either protein should equal or exceed immunoassayable loss since resupply of the plasma level via synthesis and extravascular equilibration initially contains no labeled proteins. This was observed for albumin but was not true for plasma fibronectin. For example, after burn isotopic ^{75}Se-plasma fibronectin decreased an additional 32% below that observed with the normal loss in controls. However, a 78% decline in plasma fibronectin concentration was observed by immunoassay. This results in an unaccounted for 46% drop in immunoassayable fibronectin concentration. In contrast, ^{125}I-albumin decreased by an additional 19% in burned rats at 2 h by isotopic assay and 12% by immunoassay.

One possible explanation for this observation was that the immunoassay was providing an erroneous estimation of the fibronectin concentration in burn plasma. A series of mixing experiments was conducted to test this hypothesis. Table III shows the results of a mixing experiment conducted with sham and burn plasma. When normal fibronectin (obtained by a dilution of normal plasma) was mixed with sham plasma, the effects were almost perfectly additive, i.e., the fibronectin concentration of the mixture was as expected from the sum of the concentrations of the constituent solutions. However, when burn plasma was mixed with normal fibronectin some interaction developed that blunted the expected increase in immunoassayable plasma fibronectin. This suggested that the electroimmunoassay was underestimating the true concentration of plasma
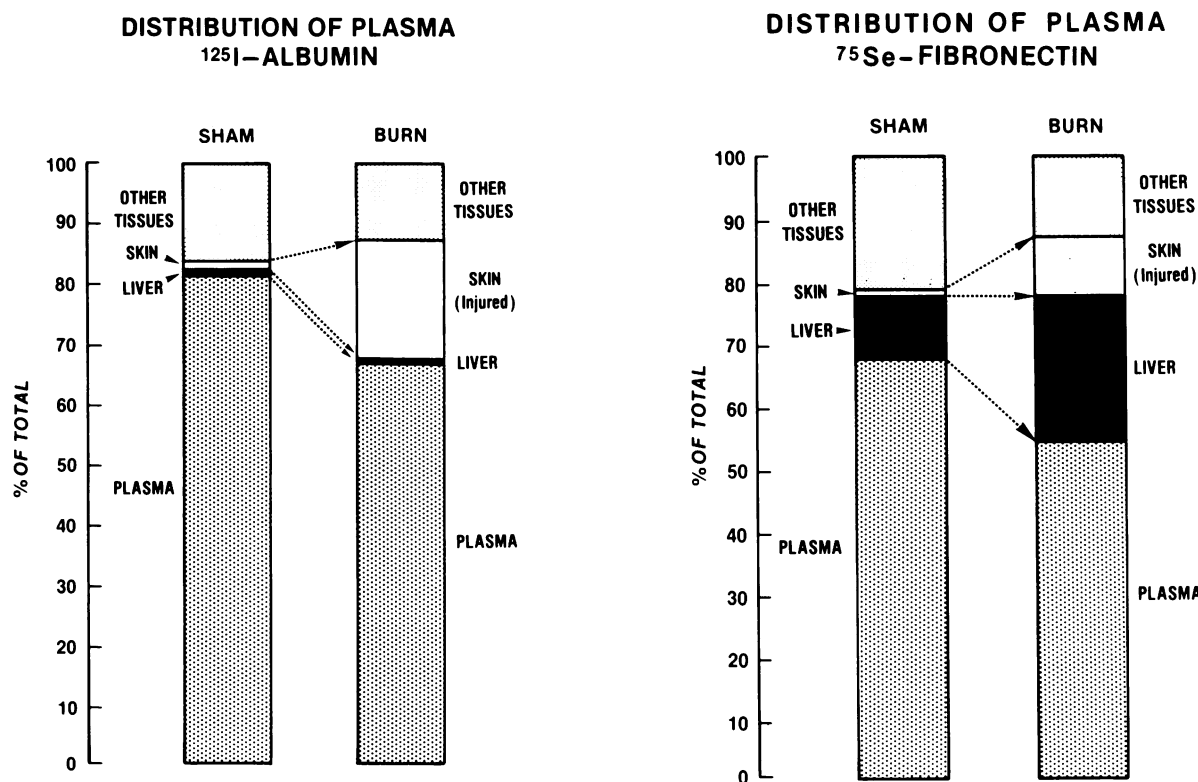
DISTRIBUTION OF PLASMA
125I−ALBUMIN

DISTRIBUTION OF PLASMA
75Se−FIBRONECTIN

Figure 2. Relative distribution of isotopically labeled albumin and plasma fibronectin 2 h after burn. As a result of burn, ^{125}I-albumin localization in injured skin increases 19-fold while localization in plasma, liver, and other tissue sites is diminished. In contrast, ^{75}Se-fibronectin localization increases in injured skin (12-fold) as well as liver (twofold), while localization is reduced in plasma and other tissue sites. Therefore, the loss of labeled fibronectin from plasma is accompanied by an increased localization in injured skin and an increased hepatic localization (which appears specific to fibronectin).

fibronectin in burn plasma. Since this nonadditivity occurred when burn plasma was added to normal plasma, the burn plasma contained some factor that interacted with and/or altered the fibronectin in normal plasma.

To test if burn directly produces some altered plasma constituent that interacts with plasma fibronectin, plasma samples were heated to 55°C to mimic the temperature measured subcutaneously after a similar burn injury in the rat (22). Immunoreactive fibronectin concentration in plasma was unchanged by heating to 55°C (586±37 [SE] μg/ml normal vs. 582±25 μg/ml heated normal; $P = 0.91$, t test). In addition, as seen in Table III, heated plasma behaved additively and did not alter the detection of fibronectin by electroimmunoassay.

Since the burn did not appear to be acting directly on plasma, it must be acting indirectly through the thermally injured skin. This hypothesis was tested using extracts of normal and burned skin. As shown in Table IV, extracts from burned skin, in contrast with extracts from normal skin, altered detection of fibronectin by electroimmunoassay. This suggests that the substance(s) in plasma following burn, which interacts with fibronectin, may

originate in the burn skin and subsequently appear in the circulation.

We investigated several substances known to interact with fibronectin in an effort to reproduce the blunted, nonadditive response seen in the electroimmunoassay. As seen in Table V, fibrin monomer as well as heparin did not alter the detection of fibronectin by electroimmunoassay. In contrast, gelatin or collagen reproduced the type of blunted immunoreactive response seen with burn plasma.

The crossed immunoelectrophoretic patterns for purified plasma fibronectin as well as the fibronectin in sham plasma or burn plasma are shown in Fig. 4. The antigenic fibronectin in burn plasma displays less anodic electrophoretic mobility and less immunoreactive fibronectin content compared with fibronectin in sham plasma. Heating normal plasma to 55°C did not reproduce the altered crossed immunoelectrophoretic pattern. Extracts of normal rat skin did not alter the crossed immunoelectrophoretic pattern of normal plasma but extracts of burn skin, when added to normal plasma, were able to reproduce the altered crossed immunoelectrophoretic pattern of
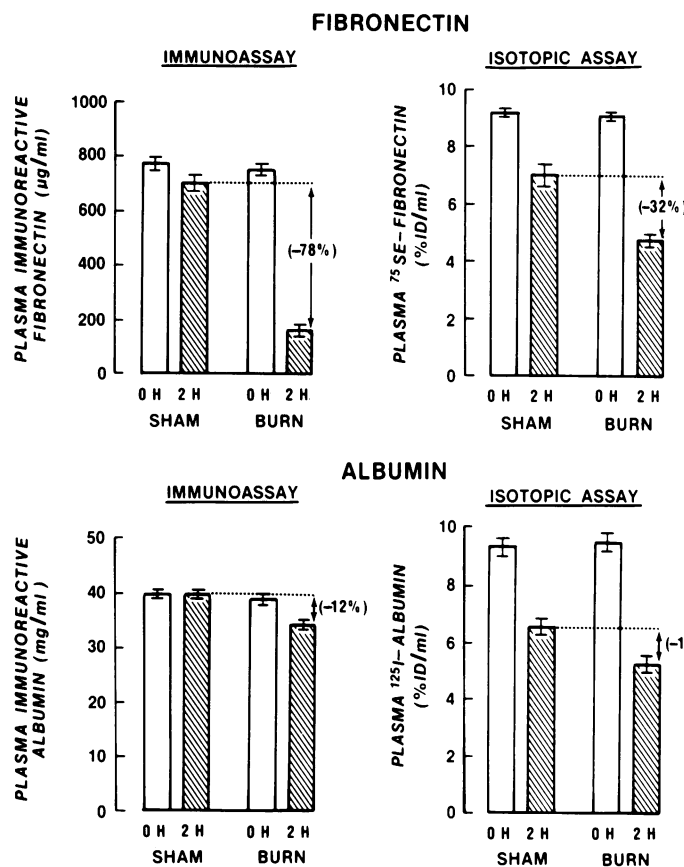
Figure 3. Comparison of immunoassay and isotopic loss of plasma fibronectin and albumin in acute burn. Plasma was obtained from burn and sham-burn rats immediately before and 2 h after burn. A comparison of the extent of plasma fibronectin depletion reveals a much greater immunoassayable drop than isotopic drop. For albumin, isotopic plasma loss slightly exceeds immunodetectable plasma loss resulting from burn, as anticipated from an absence of label in newly synthesized and extravascular albumin.

fibronectin in normal plasma. Moreover, as shown in Fig. 5, this altered electrophoretic pattern was reproduced with addition of gelatin or collagen but not with heparin or fibrin. Thus, nonadditivity detected in the electroimmunoassay coincided with diminished electrophoretic mobility of the fibronectin as demonstrated by crossed immunoelectrophoresis.

Gel filtration was performed to elucidate the effective size of fibronectin circulating in burn plasma with emphasis on the possibility that it may be complexed to another species or present in labeled but nonimmunoreactive form. The results using plasma obtained from sham (nonburned) rats at 2 h after injection of ^{75}Se-plasma fibronectin are shown in Fig. 6. The elution profiles of isotopic and antigenic fibronectin are essentially identical in the plasma of these normal rats. However, as seen in Fig. 7, plasma from rats 2 h after tracer injection and subsequent burn injury revealed that both isotopic and antigenic fibronectin were displaced toward a higher molecular size, exhibiting a broad peak centered at >700 kD. Again, there was no discordance of antigenic and isotopically labeled plasma fibronectin—confirming that the exogenously administered isotopic fibronectin is behaving similarly to the endogenous plasma fibronectin. Thus, there is no evidence to support nonimmunoreactive but labeled fibronectin fragments as the explanation

for the observed discrepancy between isotopic and immunoreactive deficiencies after burn (Fig. 3). Fig. 8 shows the results achieved when normal plasma was supplemented with gelatin. Again, antigenic fibronectin eluted in a broad peak with a molecular mass centered at >700 kD. Thus, gelatin added in vitro to normal plasma produces a higher apparent molecular mass for plasma fibronectin, comparable to that seen in burn plasma, suggesting that a gelatinlike ligand may be released after burn, which complexes with fibronectin in the plasma.

To investigate the in vitro effects of circulating gelatin, rats were injected intravenously with gelatin. Immunoassayable plasma fibronectin levels decreased 81% (from 912±60 [SE] μg/ml to 169±27 μg/ml) at 2 h after gelatin infusion. However, the total isotopic fibronectin content in plasma only declined by ~50% (58.6±1.4 [SE] %ID to 28.4±1.0 %ID) within 2 h after gelatin infusion, similar to that observed after burn. The hepatic localization of circulating ^{75}Se-fibronectin after gelatin infusion increased acutely 2.5-fold from 8.9 %ID to 31.3 %ID/TO at 60 min. Therefore, circulating gelatin interacts with plasma fibronectin to initiate the rapid uptake of fibronectin in the liver, perhaps due to an opsonic role for plasma fibronectin in augmenting Kupffer cell uptake of blood-borne collagenous material (1, 3, 15, 17).

Table III. Recovery of Fibronectin by Electroimmunoassay after Adding Sham, Burn, or Heated Plasma to Fibronectin

Plasma	Assay mixture	Measured fibronectin concentration. Mean±SD	Nonadditivity P value
		µg/ml	
Sham plasma	Diluent plus fibronectin	291±33	
(n = 6)	Sham plasma plus diluent	721±56	
	Sham plasma plus fibronectin	980±48	0.35
		(1012)*	
Burn plasma	Diluent plus fibronectin	291±33	
(n = 6)	Burn plasma plus diluent	149±26	
	Burn plasma plus fibronectin	290±19	
		(440)*	<0.01
Heated plasma	Diluent plus fibronectin	287±37	
(n = 5)	Heated plasma plus diluent	582±56	
	Heated plasma plus fibronectin	885±72	
		(869)*	0.72

Each assay mixture was composed of two solutions of equal volumes. The fibronectin added to test for additivity was obtained by diluting normal plasma. Diluent consisted of 0.9% saline without fibronectin. Under these circumstances, the fibronectin concentration of the combined test plasma (sham; burn; heated) and fibronectin should equal the sum of the test plasma and fibronectin individually.
* Anticipated calculated concentrations appear directly beneath measured concentrations for comparison.

Table IV. Recovery of Fibronectin by Electroimmunoassay after Adding Normal and Burn Skin Extracts in Normal Plasma to Fibronectin

Skin site	Assay mixture	Measured fibronectin concentration. Mean±SD	Nonadditivity P value
		µg/ml	
Normal skin extract	Diluent plus fibronectin	287±37	
(n = 5)	Extract/normal plasma plus diluent	567±49	
	Extract/normal plasma plus fibronectin	899±60	0.27
		(854)*	
Burned skin extract	Diluent plus fibronectin	287±37	
(n = 5)	Extract/normal plasma plus diluent	248±33	
	Extract/normal plasma plus fibronectin	373±31	<0.01
		(535)*	

Extracts from either normal skin or burned skin were mixed equally with normal plasma and then used in the assay mixture. Extracts from burned skin alone displayed a detectable immunoreactive fibronectin concentration (4.4 µg/ml±0.5 [SE]; $P < 0.01$, unpaired-sample t test), whereas the normal skin extracts did not. Fibronectin used to test additivity was obtained from diluted normal plasma.
* Anticipated calculated concentrations appear directly beneath measured concentrations for comparison.

Table V. Recovery of Fibronectin by Electroimmunoassay after Adding, Fibrin, Heparin, Gelatin, or Collagen in Normal Plasma to Fibronectin

Test substance	Assay mixture	Measured fibronectin concentration. Mean±SD	Nonadditivity P value
		µg/ml	
Fibrin	Diluent plus fibronectin	286±41	
(n = 5)	Fibrin/normal plasma plus diluent	491±27	
	Fibrin/normal plasma plus fibronectin	780±40 (777)*	0.93
Heparin	Diluent plus fibronectin	286±41	
(n = 5)	Heparin/normal plasma plus diluent	615±39	
	Heparin/normal plasma plus fibronectin	954±58 (901)*	0.17
Gelatin	Diluent plus fibronectin	286±41	
(n = 5)	Gelatin/normal plasma plus diluent	168±13	
	Gelatin/normal plasma plus fibronectin	296±50 (454)*	<0.01
Collagen	Diluent plus fibronectin	286±41	
(n = 5)	Collagen/normal plasma plus diluent	153±10	
	Collagen/normal plasma plus fibronectin	274±29 (439)*	<0.01

Fibrin monomer alone produced no detectable rocket and did not change the measured fibronectin concentration in normal plasma (503±27 [SE] µg/ml normal plasma vs. 491±12 µg/ml normal plasma plus fibrin, $P = 0.51$). Heparin alone produced no detectable rocket and produced an increase in immunoassayable fibronectin concentration (503±27 [SE] µg/ml normal plasma vs. 615±18 µg/ml normal plasma plus heparin, $P < 0.01$). Gelatin alone produced no detectable rocket and when added to normal plasma resulted in a significant fall in apparent fibronectin concentration (503±27 [SE] µg/ml normal plasma vs. 168±6 µg/ml normal plasma plus gelatin, $P < 0.01$). Collagen alone produced no detectable rocket and when mixed with normal plasma resulted in a fall in apparent fibronectin concentration (503±27 [SE] µg/ml normal plasma vs. 153±7 µg/ml normal plasma plus collagen, $P < 0.01$). Anticipated calculated concentrations (*) appear directly beneath measured concentrations for comparison.

Discussion

Immunologically measured fibronectin levels appear to be acutely decreased by intravenous infusion of foreign particulate matter, stored blood with microaggregates, and various gelatin-coated test particles cleared by the RES (1, 25). In addition, starvation will rapidly deplete plasma fibronectin (26), consistent with the concept of a rapid turnover of this protein (1, 9). Severe trauma and burn as well as sepsis postinjury is often associated with plasma fibronectin deficiency (1, 27, 28). Fibronectin deficiency in plasma is also observed with severe disseminated intravascular coagulation (3). The deficiency of fibronectin in plasma after injury is believed to be due to a consumptive depletion as a result of its opsonization of blood-borne microag-

gregates and collagenous and cytoskeletal "tissue debris." This investigation attempted to put this hypothesis on a more firm experimental basis as well as to delineate the mechanism of plasma fibronectin deficiency after burn.

Several previous studies have documented alterations of plasma fibronectin concentration following burn. Turinsky et al. (29) reported that plasma fibronectin concentrations as measured by electroimmunoassay in rats were elevated from 1 to 7 d after burn. Lanser et al. (13) measured plasma fibronectin levels by electroimmunoassay and plasma opsonic activity by liver slice assay in adult burn patients. They found fibronectin levels and opsonic activity generally paralleled each other with an acute depletion after burn followed by restoration by 1–3 d. A secondary deficiency was observed in those burn patients who
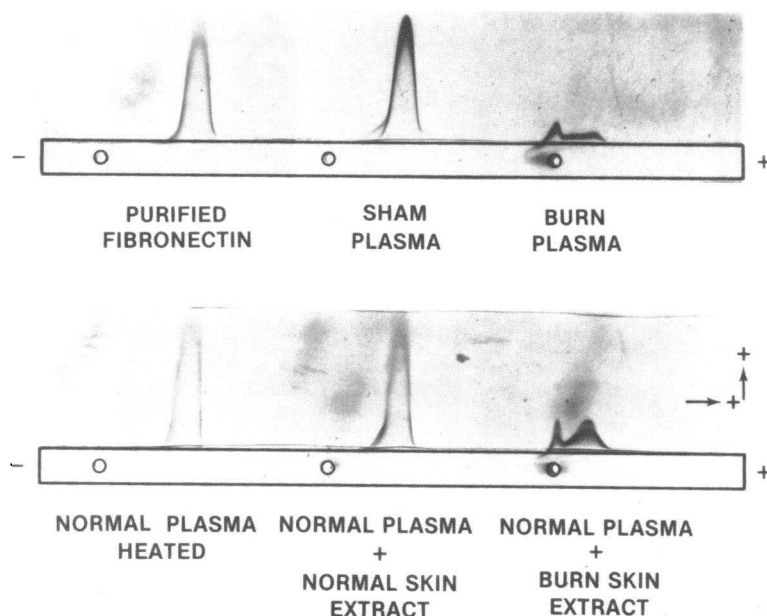
PURIFIED FIBRONECTIN   SHAM PLASMA   BURN PLASMA

NORMAL PLASMA HEATED   NORMAL PLASMA + NORMAL SKIN EXTRACT   NORMAL PLASMA + BURN SKIN EXTRACT

Figure 4. Crossed immunoelectrophoresis for fibronectin in sham plasma, burn plasma, heated plasma, and plasma mixed with skin extracts. Purified plasma fibronectin migrated as far as fibronectin in plasma and produced a very similar crossed pattern. The fibronectin in burn plasma produced a distinctive altered pattern indicating less electrophoretic mobility and suggesting less antigenic fibronectin content. Heating normal plasma did not affect its crossed pattern. Saline extracts from skin burned 1 h earlier, when added to normal plasma, reproduced the crossed pattern of burned plasma, unlike extracts of normal skin, which did not affect the electrophoretic pattern of fibronectin in normal plasma.
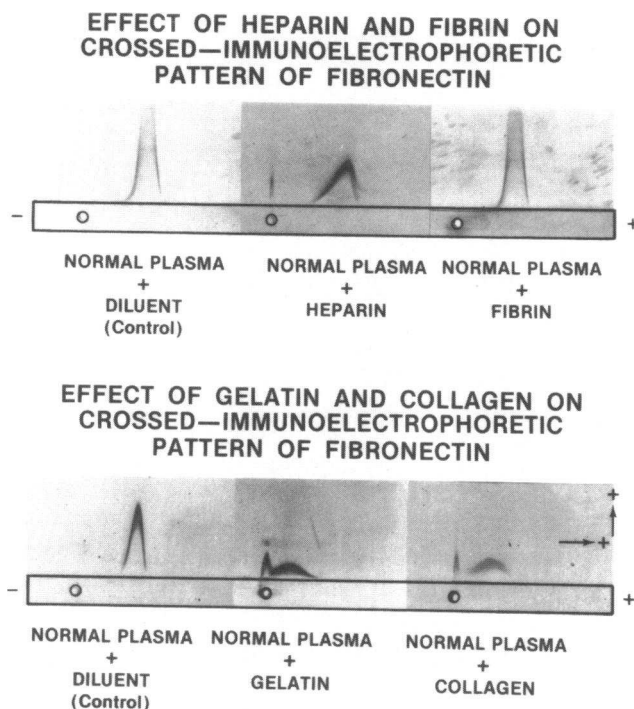
EFFECT OF HEPARIN AND FIBRIN ON CROSSED—IMMUNOELECTROPHORETIC PATTERN OF FIBRONECTIN

NORMAL PLASMA + DILUENT (Control)   NORMAL PLASMA + HEPARIN   NORMAL PLASMA + FIBRIN

EFFECT OF GELATIN AND COLLAGEN ON CROSSED—IMMUNOELECTROPHORETIC PATTERN OF FIBRONECTIN

NORMAL PLASMA + DILUENT (Control)   NORMAL PLASMA + GELATIN   NORMAL PLASMA + COLLAGEN

Figure 5. Effects of adding heparin, fibrin, gelatin, and collagen on the crossed immunoelectrophoretic pattern of fibronectin in normal plasma. Heparin and fibrin had no effect but gelatin and collagen reproduced the distinctive crossed pattern for plasma fibronectin from burn plasma.

later became septic. Grossman et al. (11) utilized a burn model in sheep and documented decreased plasma fibronectin levels from 4 to 96 h after burn using the Laurell electroimmunoassay. A recent study has shown that this early deficit in immuno-reactive fibronectin and opsonic activity was partially but not completely restored by administration of purified plasma fibronectin (12).

In contrast to the numerous studies documenting plasma fibronectin deficiency following burn injury, less emphasis has been placed on the mechanism inducing the fibronectin deficiency. The current study suggests that three factors contribute to the early deficiency of plasma fibronectin following burn. First, there appears to be a rapid accumulation of plasma fibronectin in the liver following burn injury. Although the precise intrahepatic localization of the label is unknown, plasma fibronectin is known to be removed from the vascular compartment in association with RES clearance of blood-borne test particles (1, 17, 30). It is possible that this rapid uptake of plasma fibronectin by the liver represents the consumption of plasma fibronectin in association with the RES removal of opsonized blood-borne material generated after thermal injury. The decrease in ^{125}I-albumin localization in the liver following burn suggests that fibronectin is selectively accumulated there.

Plasma fibronectin sequestration in areas of tissue injury, as shown by the current study, may represent a second mechanism. Such sequestration is also observed with plasma albumin and reflects not only the affinity that fibronectin may have for sites of tissue injury but also increased vascular permeability at the site of thermal injury to the skin. Plasma fibronectin that enters the skin under normal circumstances is retained as pre-
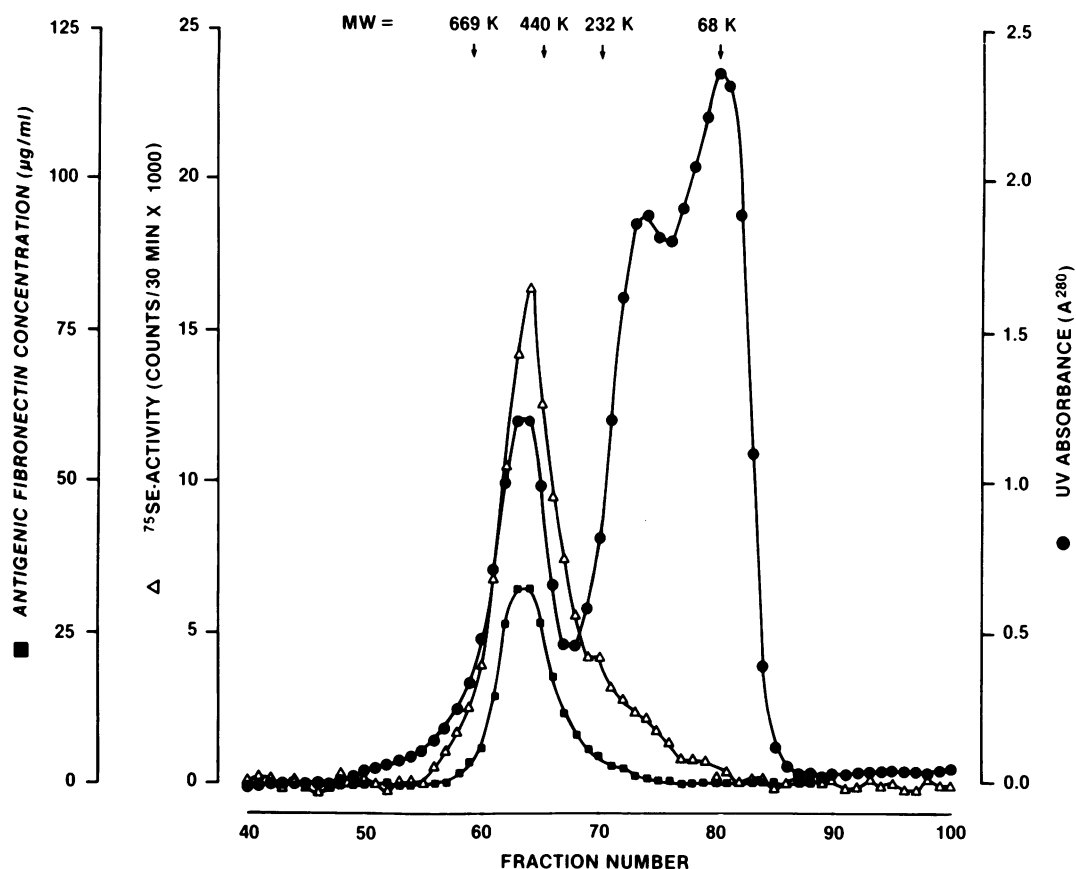
Figure 6. Effect of sham burn on the gel filtration pattern of fibronectin in plasma. Fractions collected from plasma 2 h after sham burn produced three elution profiles: UV absorbance (●); ^{75}Se-labeled plasma fibronectin (△); and antigenic fibronectin (■). Isotopic and antigenic fibronectin profiles are similar and indicate an approximate molecular mass of 500 kD.

viously shown (9), whereas plasma albumin that enters the skin returns to the plasma compartment. The accumulation of both albumin and fibronectin at the site of thermal injury in skin was clearly distinct from the localization pattern observed in noninjured skin.

Several studies have shown increased antigenic fibronectin at areas of inflammation and wound repair (6–8, 30, 31). Kaplan et al. (30) and Clark et al. (8) have used radiolabeled plasma fibronectin and radiolabeled albumin to confirm increased extravascular localization of both proteins following surgery and hypersensitivity reactions, respectively. Although fibronectin sequestration at sites of tissue injury may serve a physiologic role in wound healing by promoting chemotaxis of fibroblasts and monocytes, this sequestration may also contribute to a deficiency of fibronectin in the plasma that may limit systemic reticulo-endothelial phagocytic host defense mechanisms.

Oh et al. (10), in a series of in vivo studies in mice, documented the ability of human plasma fibronectin to become incorporated into tissue matrices in a distribution pattern com-

parable to that manifested by endogenous tissue fibronectin. Hayman and Ruoslahti (32) also documented incorporation of exogenously added plasma fibronectin into the extracellular matrix of kidney cells in culture. Deno et al. (9) have shown sequestration of radiolabeled plasma fibronectin in various tissues with retention of the fibronectin by such tissues, consistent with its potential role as an adhesive matrix protein. Although the exact mechanism initiating such incorporation remains to be delineated, studies by Mosher and McKeown-Longo (33) suggest a role for Factor XIII$_a$ in the cross-linking of fibronectin and/ or its conversion into an insoluble form in the extracellular connective tissue matrix. Thus, plasma fibronectin may serve as a reservoir for fibronectin found in tissues and sustained plasma fibronectin deficiency following trauma or burn injury, may limit this normal pathway for incorporation.

The third mechanism contributing to the apparent fibronectin deficiency was the existence in plasma early following burn of a gelatinlike ligand that has a high affinity for fibronectin and alters its electrophoretic pattern. Although burn plasma is
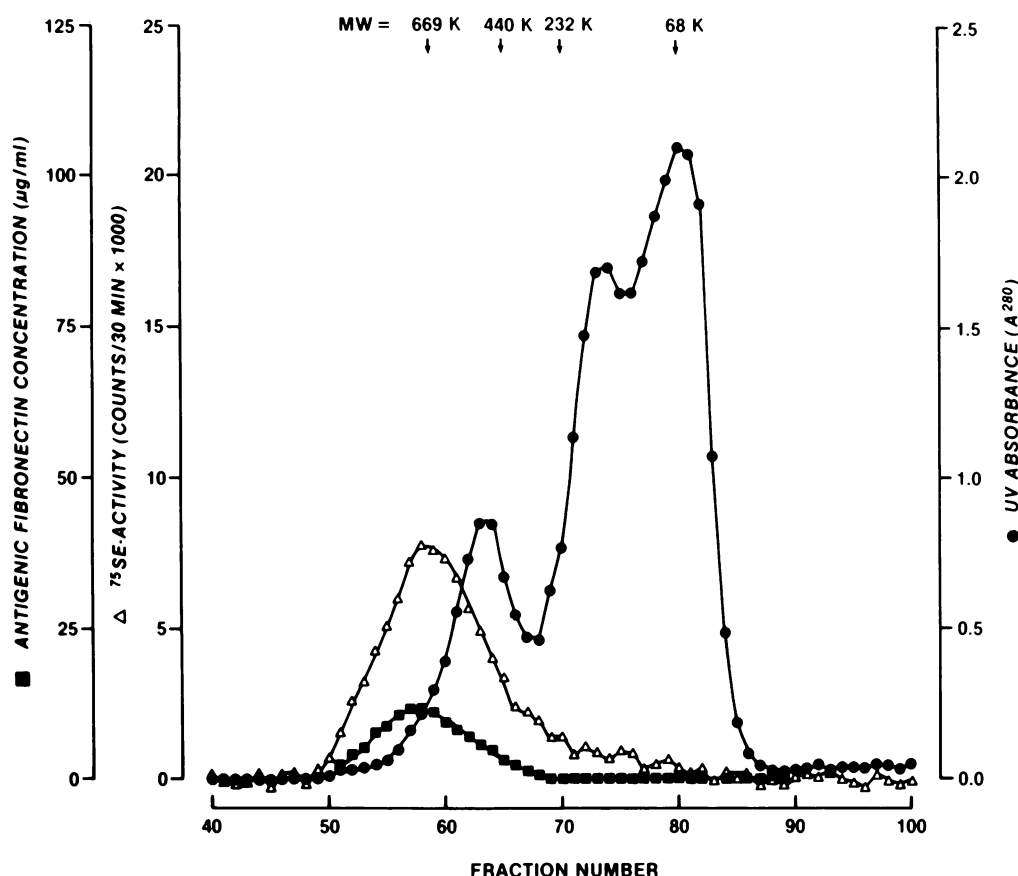
Figure 7. Effect of burn on the gel filtration of fibronectin in plasma. 2 h after burn, the elution profiles of ^{75}Se-labeled fibronectin (△) and antigenic fibronectin (■) have been displaced toward higher

molecular mass, peaking at >700 kD. Closed circles (●) denote UV absorbance.

really deficient in fibronectin, it was actually present in plasma at a higher concentration than detected by electroimmunoassay. This was because the ability to quantitate the molecule was limited since the molecule appears to be present in a complexed form. Comparative studies utilizing fibrin and heparin, demonstrated that an affinity for fibronectin by itself was not sufficient to alter the immunoelectrophoretic migration of fibronectin. However, native collagen and denatured collagen (gelatin) when added to normal plasma, distorted the crossed immunoelectrophoretic profile of fibronectin, comparable to that observed with fibronectin in burn plasma. Since skin has a high content of collagen, it is possible that collagenlike fragments and/or denatured collagen may be released into the blood from sites of thermal damage. A comparative analysis of extracts of normal and burned skin demonstrated the existence in burn skin of factor(s) that might enter the plasma and complex with fibronectin. How long this factor remains in the circulation following burn injury is not known. However, the fact that restoration of fibronectin levels occurred within ~8 h postthermal injury suggests that it is ultimately cleared from the circulation, perhaps by the Kupffer cells of the liver.

Dobke et al. (34) have demonstrated that burn injury produces a plasma factor appearing early after burn, which can inhibit the ability of serum from rabbits injected with collagen to agglutinate collagen-coated erythrocytes. They were able to extract this inhibitor of hemagglutination from burned rat skin but not normal skin. Thus, this inhibitor of agglutination, which was speculated to be collagen, may be similar to the gelatinlike ligand detected in burn plasma in the present study. They interpreted this response as an interaction with anticollagen antibodies. However, these anticollagen antibodies, which are naturally occurring, may actually be plasma fibronectin (34–37).

Recently, several reports documenting altered forms of plasma fibronectin in various pathological states have appeared. Lohmann and Pott (38) using isoelectric focusing, found an increase in the isoelectric point of plasma fibronectin in patients during septic shock. The reduced anodic electrophoretic mobility of plasma fibronectin acutely following burn is consistent with
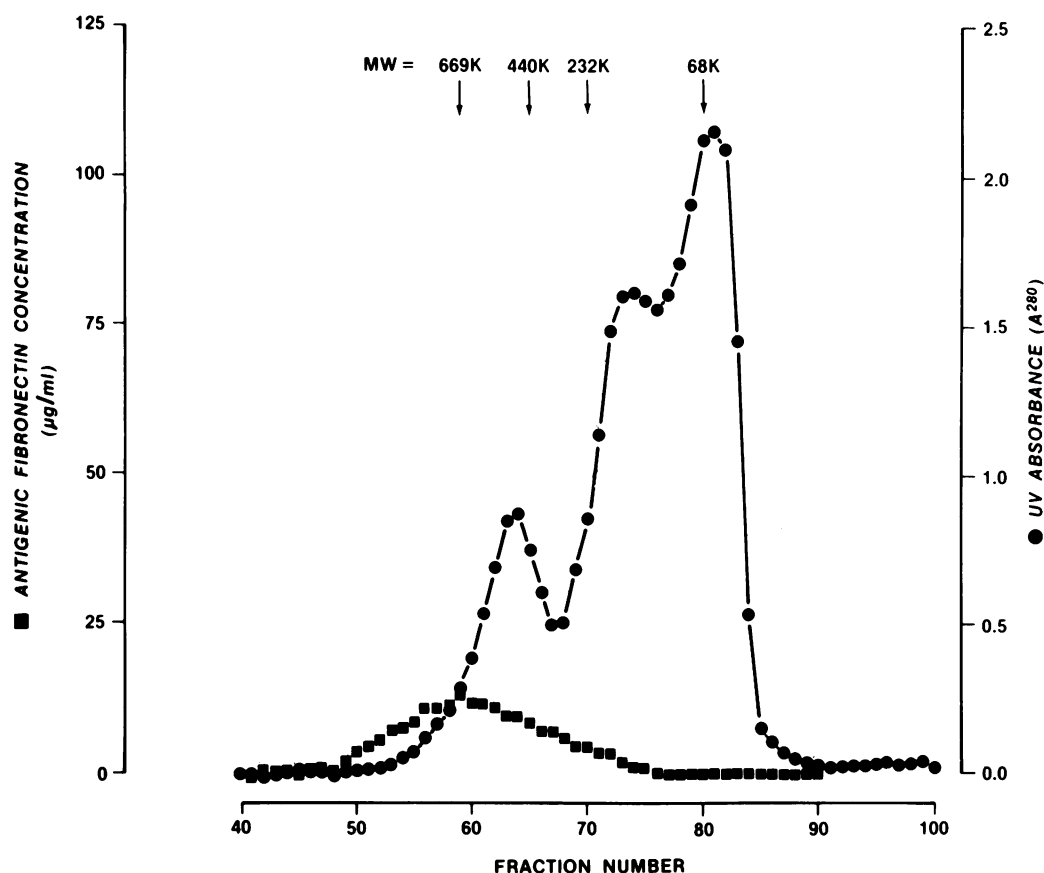
Figure 8. Effect of gelatin on the gel filtration pattern of fibronectin in normal plasma. Normal rat plasma and gelatin resulted in an antigenic elution profile for fibronectin (■) that closely resembles that of burn plasma. The peak of antigen detection also occurred at >700 kD. Closed circles (●) denote UV absorbance.

an increased effective size and/or an increase in its isoelectric point. In support of this concept, Ingham et al. (39) recently published a study showing that the addition of gelatin to purified plasma fibronectin results in the appearance on exclusion chromatography of a fibronectin-gelatin complex with an increased molecular size. This complex also exhibited reduced anodic electrophoretic mobility on immunoelectrophoresis. A distortion of the crossed immunoelectrophoretic pattern for fibronectin was also found by Klingemann et al. (40) in the citrated plasma but not serum of patients with acute leukemia. A portion of the antigenic fibronectin possessed a reduced electrophoretic mobility. They concluded that the altered fibronectin reflected a Factor XIII$_a$ cross-linked complex of fibronectin and fibrinogen. Our results appear to differ in that the altered crossed immunoelectrophoretic pattern for fibronectin was present in both plasma and serum of burned rats (unpublished data) and that burn plasma, when added to normal plasma, in the absence of free calcium, altered the detection of normal fibronectin by electroimmunoassay (Table III).

The potential existence of such a factor in burn patients needs to be evaluated, as it may influence phagocytic host defense mechanisms and the extent of tissue incorporation. Recent clinical studies documenting reversal of plasma fibronectin deficiency in trauma and burn patients by intravenous infusion of fresh fibronectin-rich plasma cryoprecipitate (27, 28) emphasize the potential clinical significance of the current observations, especially in view of interpretation of the significance of plasma fibronectin levels as determined by immunoassay before and after replacement therapy.

Thus, acute plasma fibronectin deficiency early after burn is followed by rapid restoration. Multiple factors contribute to this acute deficiency. These include: uptake of plasma fibronectin by the liver; sequestration at sites of tissue injury; and complexing of fibronectin in the blood to a gelatinlike ligand that can blunt its detectability by immunoassay. Such observations provide experimental support for the concept of the existence of collagenouslike debris in the circulation following trauma and burn, which may interact with plasma fibronectin.

Appendix

The model for a 2^2 design (2 by 2 data table) with n observations in each cell may be written:

$$y_{ijk} = \bar{\mu}.. + (\bar{\mu}_i. - \bar{\mu}..) + (\bar{\mu}._j - \bar{\mu}..) + (-1)^{i+j} \cdot \gamma + \epsilon_{ijk}$$

$$i = 1, 2 \quad j = 1, 2 \quad k = 1, \ldots, n, \tag{1}$$

where y_{ijk} denotes the kth observation in the cell of the ith row and j^{th} column of the table; μ_{ij} is the mean or expectation value in the cell of the ith row, and j^{th} column; the subscript · notation means that sums are formed over the index that has been replaced by a dot; the overbar notation denotes the average; γ represents the interaction term describing nonadditivity; and ϵ_{ijk} are independent and normally distributed errors with mean = 0 and common variance σ^2. The null hypothesis, H_0, in the test for interaction or nonadditivity is:

$$H_0: \gamma = \mu_{12} + \mu_{21} - \mu_{11} - \mu_{22} = 0. \tag{2a}$$

Considerable computer software exists for the above balanced case. A modification was developed to allow the mean and variance of one cell to be zero. For convenience this cell is chosen to be $i = 1$, $j = 1$ and so its mean, μ_{11}, equals zero. In this modified design, the null hypothesis that there is no interaction (i.e., the model is additive) is:

$$H_0: \gamma = \mu_{12} + \mu_{21} - 0 - \mu_{22} = 0. \tag{2b}$$

To test H_0, one forms two estimates of variance. One of which, $\hat{\sigma}_1^2$, is always an unbiased estimate of the variance, whether H_0 is true or not, and appears below with $3n - 3$ degrees of freedom.

$$\hat{\sigma}_1^2 = \frac{\sum_{i=1}^{2}\sum_{j=1}^{2}\sum_{k=1}^{2} (y_{ijk} - \bar{y}_{ij}.)^2}{3n - 3}. \tag{3a}$$

The other estimate, $\hat{\sigma}_2^2$, is unbiased only if $\gamma = 0$ and appears below with 1 degree of freedom.

$$\hat{\sigma}_2^2 = (n/3) \cdot (\bar{y}_{12}. + \bar{y}_{21}. - \bar{y}_{22}.)^2. \tag{3b}$$

Proceeding as in the usual analysis of variance, the ratio $\hat{\sigma}_2^2/\hat{\sigma}_1^2$ is formed to compare the two estimates of variance which, if H_0 is true, has an F distribution with $(1, 3n - 3)$ degrees of freedom.

$$F_{1,3n-3} = \frac{\hat{\sigma}_2^2}{\hat{\sigma}_1^2} = \frac{(n-1) \cdot n \cdot (\bar{y}_{12}. + \bar{y}_{21}. - \bar{y}_{22}.)^2}{\sum_{i=1}^{2}\sum_{j=1}^{2}\sum_{k=1}^{2} (y_{ijk} - \bar{y}_{ij}.)^2}. \tag{4}$$

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