Degradation of Fibronectin and Vitronectin in Chronic Wound Fluid: Analysis by Cell Blotting, Immunoblotting, and Cell Adhesion Assays

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We used a combination of cell blotting, immunoblotting, and cell adhesion assays to analyze fibronectin and vitronectin in wound fluid from acute and chronic wounds. Acute wound fluid (e.g., suction blister fluid, mastectomy fluid) contained intact fibronectin and vitronectin as major cell adhesion proteins. In marked contrast, chronic wound fluid samples from three of 11 patients with venous stasis ulcers showed complete degradation of vitronectin and degradation of fibronectin into small molecular mass polypeptides less than 125 kDa. Three of these polypeptides — 54, 93, and 125 kDa — were biologically active in promoting cell attachment and were recognized by monoclonal antibodies that bind fibronectin near the arg-gly-asp (RGD) domain. In wound fluid samples from the other eight of 11 patients, only slight degradation of vitronectin and fibronectin occurred, which resulted in a mixture of mostly intact molecules along with large fragments. Intact fibronectin in chronic wound fluid samples contained the ED-A domain, which showed that fibronectin synthesis occurred locally in the wound bed. Wound fluid containing extensively degraded vitronectin and fibronectin reversibly inhibited cell adhesion, and excess fetal bovine serum, but not purified fibronectin, neutralized the inhibitory effect. We suggest that protease activity in some chronic wounds may cause degradation of adhesion proteins and prevent cell adhesion necessary for normal wound closure. J Invest Dermatol 98:410–416, 1992

We have been studying the role of fibronectin in wound repair. Fibronectin, an adhesion protein found in blood and many tissues [1], is deposited at the wound interface after injury [2,3]. Both plasma and cellular fibronectin occur in the wound bed [4,5]. Elevated levels of fibronectin occur in granulation tissue [2,6] and persist in a variety of inflammatory states (cf. [7]). Of particular relevance to cutaneous healing, fibronectin promotes keratinocyte migration [8,9], and keratinocytes within wounds transiently activate fibronectin receptor function [10]. In clinical studies, topical application of fibronectin has been found to increase epithelization of chronic corneal ulcers [11,12]; it may also improve epithelization of some venous stasis ulcers [13].

The presence of fibronectin fragments in bronchoalveolar lavage fluid [14] and in tear fluid isolated from chronic corneal ulcers [15] showed that fibronectin degradation can occur in the wound bed under some circumstances. Recently, we reported that partial degradation of fibronectin occurred in wound fluid samples from several patients with venous stasis and diabetic ulcers [16]. In the present research, we present detailed characterization of fibronectin degradation in chronic wound fluid using cell blotting to identify biologically active fragments [17] in combination with immunoblotting by polyclonal and monoclonal antibodies. In addition, we analyzed a second adhesion protein — vitronectin. Vitronectin is another major adhesion protein in blood [18] that increases in some tissues during inflammation [19]. Finally, we tested the effects of chronic wound fluid on cell adhesion. Details of these studies are reported herein.

MATERIALS AND METHODS

Materials

Baby hamster kidney (BHK) cells adapted for growth in suspension culture were cultured as described previously [20]. Human plasma FN was obtained from the New York Blood Center, New York, NY. Human vitronectin was prepared from outdated human blood by heparin affinity chromatography [21]. Polyclonal antifibronectin antibodies were prepared in our laboratory. Polyclonal antivitronectin antibodies were a gift from Dr. Deanne Mosher, Department of Medicine, University of Wisconsin. Mouse monoclonal antibody against the cellular fibronectin-specific ED-A domain was a gift from Dr. Luciano Zardi, National Institute for Cancer Research, Genova, Italy. Mouse monoclonal antibody against the fibronectin cell-binding domain was purchased from Telios Pharmaceuticals, Inc. (San Diego, CA). The synthetic peptides, gly-arg-gly-glu-ser-pro (GRGESP) and gly-arg-gly-asp-ser-
pro (GRGDSP), were purchased from Peninsula Laboratories (Belmont, CA).

**Wound Fluid Samples**  Informed consent was obtained for all procedures. Patients undergoing surgical mastectomy (5 persons) or with chronic leg ulcers (11 persons) were recruited from inpatient and outpatient populations of a large, metropolitan hospital. Suction blister fluid was obtained from normal volunteers (3 persons). Chronic leg ulcers were present for more than 1 year or healing less than 1 mm per week. Fluid was collected from beneath a transparent polyurethane occlusive dressing (Tegaderm, 3M, St. Paul, MN) placed over the wounds for 4 to 12 h and collected with a sterile tuberculin syringe and 20 gauge needle. Leg ulcers showed no signs of clinical infection at the time wound fluid was obtained. Mastectomy fluid was collected from fluid collection devices (HemoVac and ConstaVac) in sterile test tubes beginning 24 h after surgery. Suction blister fluid was collected from blisters made by a Dermovac (Medico Medical, Espoo, Finland) placed on the forearms of normal volunteers for 2–4 h under a heat lamp as described by the manufacturer. After collection, all fluids were centrifuged (Microfuge II, Beckman) for 4 min at 11,600 × g, and the supernatants were frozen at −70°C until use.

**Collection of Blood and Preparation of Plasma and Serum**  Blood samples were obtained from the antecubital vein of patients with chronic wounds and volunteers with suction blisters using a 21-gauge needle and ethylenediaminetetraacetic acid (EDTA) as anticoagulant. To obtain plasma, cells were removed from the samples by centrifugation (1800 × g for 15 min at 22°C followed by 22,000 × g for 30 min at 4°C). The supernatants were filter sterilized using a 0.2 µm filter, and stored at −70°C until use. To obtain serum, CaCl₂ was added to samples at a final concentration of 14 mM. After 2 h at 37°C, samples were clarified by centrifugation at 22,000 × g for 30 min at 37°C. Supernatants were dialyzed overnight at 4°C against Dulbecco’s phosphate-buffered saline (DPBS) (150 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 1 mM KH₂PO₄, 6 mM Na₂HPO₄, pH 7.2), reconstituted at 22,000 × g for 30 min at 4°C, filter sterilized, and stored as above.

**SDS-PAGE and Immunoblotting**  Samples for electrophoresis were dissolved in reducing sample buffer (62.5 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.01% bromophenol blue, 5% mercaptoethanol, pH 6.8) unless indicated otherwise and subjected to electrophoresis in a 4% to 16% acrylamide gradient slab gel containing a gradient of 3 M to 8 M urea [22]. Purified fibronectin was 0.2 µg/lane for immunoblotting and 0.15 µg/lane for cell blotting (see below). Purified vitronectin was 1 µg/lane for immunoblotting and 0.5 µg/lane for cell blotting. Wound fluid samples, plasma, and serum were 150 µg/lane for immunoblotting and 200 µg/lane for cell blotting. Protein concentrations were determined by the Lowry method [23].

For immunoblotting, proteins were transferred to nitrocellulose paper (Schleicher & Schuell) by electrophoresis at 4°C for 1.5 h at 150 mA followed by 1.5 h at 300 mA, similarly as described previously [16]. The transferred proteins were incubated with polyclonal or monoclonal antibodies for 2 h at 22°C followed by incubation with alkaline phosphatase conjugated goat–anti-rabbit IgG or anti-mouse IgG (Bio-Rad, Richmond, CA) for 1 h at 22°C. Visualization was accomplished using alkaline phosphatase conjugate substrate kit according to the manufacturer’s instructions.
solubilized with 2% sodium dodecyl sulphate. Samples of non-attached and attached cells were mixed with 10 ml of Budget SolvE (RPI Corp.), radioactivity was measured in a Beckman scintillation spectrophotometer, and the percentage of attached cells calculated.

RESULTS

Adhesion Protein Profiles, Fibronectin, and Vitronectin in Suction Blister Fluid, Plasma, and Serum In our experiments, we compared acute and chronic wound fluid. Controls were plasma and serum obtained at the same time that wound fluid was collected. We used cell blotting to analyze the profile of adhesion proteins in the samples and immunoblotting to analyze fibronectin and vitronectin. Under the assay conditions used, cell blotting was as sensitive as immunoblotting.

Figure 1 shows that the adhesion protein profile of suction blister fluid was very similar to serum and plasma. Major adhesion proteins migrating at 250 and 230 kDa corresponded to fibronectin and a 230-kDa fibronectin fragment. Major low molecular weight components migrating around 60 kDa coincided with vitronectin. Although vitronectin appeared as an approximate 60-kDa doublet by cell blotting or immunoblotting, this doublet probably corresponds to the 75- and 65-kDa vitronectins [24], which apparently were compressed beneath the large albumin band. Vitronectin purified from outdated human blood showed higher molecular weight components including a third polypeptide, probably the 57-kDa thrombin fragment [25]. Sometimes, but not always, cell blotting and antivitronectin antibodies also indicated the presence of a 40-kDa component.

There were several other adhesion-promoting polypeptides in the samples that were detected by cell blotting. High molecular mass molecules (~300 kDa) were consistently observed. The appearance of 138- and 180-kDa molecules, on the other hand, was somewhat variable. To determine if these other adhesion-promoting peptides promoted adhesion through an RGD cell-binding domain [26], we tested the effects of RGD-containing peptides on their activity. Figure 2 shows that the addition of GRGDSP to the incubations inhibited cell attachment to all the components, whereas the control peptide GRGESP had no effect. In the case of the major fibronectin band, attachment was not completely inhibited.

Therefore, under these conditions, BHK cells may also be able to bind to RGD-independent attachment-promoting domains of fibronectin [27,28].

The group of adhesion proteins with a molecular mass of approximately 300 kDa was more apparent in blister fluid and serum than in plasma, suggesting that these components developed as a consequence of blood clotting. Also, if we overdeveloped the immunoblots, then the higher molecular mass bands could be stained with antifibronectin antibodies (data not shown). Therefore, the approximately 300 kDa polypeptides may be covalently crosslinked fibronectin complexes.

Adhesion Protein Profiles, Fibronectin, and Vitronectin in Chronic Wound Fluid Figure 3 compares the adhesion protein profiles, fibronectin, and vitronectin in wound fluid from chronic stasis ulcers (WFA, WFB) with acute wound fluid: suction blister fluid (B) and mastectomy fluid (M). Samples from three of 11 patients with stasis ulcers showed a common pattern (WFA), namely, complete degradation of vitronectin and degradation of fibronectin into numerous fragments ranging from 30-125 kDa. Some of the fibronectin fragments detected by immunoblotting — 54 kDa, 93 kDa, and 125 kDa — were active in the cell blotting assay. Samples from the other 8 of 11 patients with stasis ulcers also showed a common pattern (WFB). The adhesion protein profile was broader than observed with suction blister fluid or mastectomy fluid. Also, immunoblotting showed intact fibronectin as well as large fibronectin fragments and a reduction in vitronectin.

Figure 4 provides additional information about fibronectin and fibronectin fragments in WFA and WFB chronic wound fluids. In lane 1, samples were immunoblotted with monoclonal antibodies that bind near fibronectin's RGD cell attachment domain; samples
in lane 2 were immunoblotted with polyclonal antifibronectin antibodies as in Fig 3. In WFA chronic wound fluid, only the three fragments that had cell attachment activity, 125 kDa, 93 kDa, and 54 kDa, were detected by the antiscell binding domain antibodies. Cell adhesion to these fragments was inhibited by 0.5 mg/ml GRGDSP (data not shown).

In lane 3 of Fig 4, samples were immunoblotted with monoclonal antibodies against the alternatively spliced ED-A domain of fibronectin, which is more prominent in cellular fibronectin than in plasma fibronectin [29]. In the samples from plasma, little staining of fibronectin by anti-ED-A was evident. But in WFB chronic wound fluid, intact fibronectin subunits and larger (but not smaller) fibronectin fragments showed staining by anti-ED-A, indicating that chronic wound fluid contains substantial cellular fibronectin as well as plasma fibronectin. Probably, the ED-A domain was absent from the small fragments remaining after extensive degradation of fibronectin in WFB or WFA chronic wound fluid. Also, we cannot rule out the possibility that the level of cellular fibronectin in WFA chronic wound fluid is decreased.

Biologic Activity of Adhesion Proteins in Chronic Wound Fluid Compared to Serum Degradation of adhesion proteins in the wound bed could be an important factor in the inability of chronic wounds to close. It was of interest, therefore, to test the effect of wound fluid on cell adhesion. Figure 5 shows representative results from an assay to measure adhesion-promoting activity of chronic wound fluid compared to serum. BHK cell adhesion to gelatin requires adhesion proteins such as fibronectin or vitronectin so there was little attachment to gelatin in the absence of wound fluid or serum. As the concentration of serum or WFB chronic wound fluid increased, the extent of cell adhesion increased. The total protein concentration in serum and wound fluids was similar. Therefore, it appeared that adhesion proteins in WFB chronic wound fluid had biologic activity equivalent to adhesion proteins in serum. On the other hand, WFA chronic wound fluid, in which vitronectin was completely degraded and fibronectin was broken down into fragments, contained little cell adhesion-promoting activity. The adhesion-promoting polypeptides in WFA wound fluid detected by cell blotting would have promoted cell attachment to gelatin only if they also contained the gelatin binding domain.

Figure 6 shows a parallel experiment that was designed to detect adhesion-inhibiting activity. Here, substrata previously coated with gelatin followed by 2.0 mg/ml serum were incubated with chronic wound fluid or additional serum. In this case, WFA chronic wound fluid inhibited cell adhesion. Therefore, WFA chronic wound fluid not only lacked adhesion proteins necessary for cells to attach to gelatin, but also contained factors that could prevent adhesion from occurring.

Finally, WFA chronic wound fluid reversed the adhesion of already attached and spread cells. Figure 7A shows the appearance of BHK cells after 2 h on fibronectin-coated substrata. When cells were incubated with WFA chronic wound fluid (0.5 mg/ml), the cells rounded up and began to detach over the next 90 min (Fig 7B). Wound fluid was not toxic, however, and replacing wound fluid with fresh serum-containing medium resulted in rapid respreading of the cells (Fig 7C).

Recently, we found that WFA chronic wound fluid contains high levels of metalloproteases [30], which could account for the inhibitory effects of WFA chronic wound fluid on cell adhesion. Consistent with this idea, the addition of 10% fetal bovine serum, which contains a broad spectrum of protease inhibitors, neutralized the adhesion-inhibiting activity of type A chronic wound fluid (Fig 7E), whereas 100 µg/ml fibronectin had no effect (Fig 7D). Also, the addition of 10% fetal bovine serum to WFA chronic wound fluid neutralized the ability of WFA chronic wound fluid to inhibit cell adhesion to serum-coated gelatin (data not shown). These re-
Figure 4. Analysis of fibronectin and fibronectin fragments in wound fluid. Samples of plasma and chronic wound fluid were immunoblotted as follows: lane 1, monoclonal antibodies that bind near fibronectin’s RGD cell attachment domain; lane 2, polyclonal antifibronectin antibodies; lane 3, monoclonal antibodies against the alternatively spliced ED-A domain of fibronectin. In WFA chronic wound fluid, the three fragments with cell attachment activity, 125 kDa, 93 kDa, and 54 kDa, were detected by the antifibronectin antibodies. Fibronectin in plasma and fibronectin degradation fragments did not contain the ED-A domain, which was found in intact fibronectin in WFB chronic wound fluid.

Figure 5. Effect of chronic wound fluid on BHK cell attachment to gelatin. Culture dishes were coated with gelatin, incubated with serum or chronic wound fluid at the concentrations indicated, and then incubated with radiolabeled BHK cells. Cell attachment to gelatin occurred with the addition of serum or WFB chronic wound fluid. WFA chronic wound fluid, however, showed little cell adhesion promoting activity.

Figure 6. Effect of chronic wound fluid on BHK cell attachment to serum-coated gelatin. Culture dishes were coated with gelatin, then with serum (2 mg/ml), and then incubated with radiolabeled BHK cells in medium containing serum or chronic wound fluid at the concentrations indicated. Addition of WFA chronic wound fluid resulted in a dose-dependent inhibition of attachment.

RESULTS

Initial results show that WFA chronic wound fluid interferes with cell adhesion because of wound fluid protease(s).

DISCUSSION

To learn more about the role of adhesion proteins in wound healing, we studied the appearance of these proteins in acute and chronic wounds. In these studies, we used cell blotting to identify adhesion protein profiles and immunoblotting with polyclonal and monoclonal antibodies to identify fibronectin, vitronectin, and their degradation products. The profiles of adhesion proteins in acute wound fluid were similar to those observed in serum and plasma. Polypeptides migrating at 250 and 230 kDa corresponded to fibronectin and the 230-kDa fibronectin fragment. Polypeptides migrating around 60 kDa corresponded to vitronectin. Vitronectin appeared as a doublet around 60 kDa, which is lower than the expected 65- and 75-kDa polypeptides [24], but the vitronectin bands appeared to be compressed beneath the large albumin band. The method for cell blotting that we used in these studies was very sensitive. Nevertheless, other adhesion proteins might have been present that were not detected by BHK cells.

In addition to the major fibronectin and vitronectin bands, there were several other adhesion-promoting polypeptides in acute wound fluid that could be detected by cell blotting. The high molecular mass molecules of approximately 300 kDa were more apparent in blister fluid and serum than in plasma, and could be stained with antifibronectin antibodies if we overdeveloped the immunoblots. Therefore, these polypeptides may be covalently crosslinked fibronectin complexes that developed during blood clotting. The appearance of 138- and 180-kDa molecules was somewhat variable and their identity is unknown at present. The 40-kDa polypeptide that stained with antifibronectin antibodies is probably a vitronectin fragment [31]. Biologic activity of all of the adhesion-promoting polypeptides detected by cell blotting appeared to depend in part on the RGD recognition mechanism [26] based on inhibition studies with GRGDSP. The inability of GRGDSP to inhibit completely cell attachment to fibronectin indicated that cells might also bind to RGD-independent cell adhesion domains [27,28] under these assay conditions.

Chronic wound fluid differed markedly from acute wound fluid. Samples from three of 11 patients with venous stasis ulcers (WFA chronic wound fluid) showed complete degradation of fibronectin into small molecular mass polypeptides. Three of these polypeptides, 54, 93, and 125 kDa, had cell-attachment activity and were recognized by monoclonal antibodies that bind fibronectin near the RGD domain. GRGDSP, but not GRGEVP, inhibited the biologic
activity of these fragments. Wound fluid samples from the other eight of 11 patients with venous stasis ulcers (WFB chronic wound fluid) showed partial degradation resulting in a mixture of intact subunits and fragments. Surprisingly, vitronectin in these wounds was degraded completely; no fragments were detected in wound fluid even though the 40-kDa fragment occurred sometimes in serum and plasma.

Intact fibronectin and large fibronectin fragments in WFB chronic wound fluid gave a strong signal when stained with monoclonal antibodies directed against the ED-A domain of cellular fibronectin [29]. This shows that at least part of the fibronectin in wound fluid is derived locally from cells in the wound bed. Previous immunofluorescence [4] and in situ hybridization studies [5] showed that cellular fibronectin was present in experimental wounds. Apparently, the cells in chronic wounds retain the ability to synthesize and secrete fibronectin, and probably other extracellular matrix proteins. In WFA as well as WFB chronic wound fluids, the ED-A domain probably was not present in the fragments remaining after more extensive degradation of fibronectin. Also, it is possible that the level of cellular fibronectin in WFA chronic wound fluid is decreased.

Adhesion assays with chronic wound fluid demonstrated that WFB chronic wound fluid samples containing partially intact fibronectin and vitronectin promoted cell attachment to gelatin as effectively as serum. WFA chronic wound fluid, on the other hand, was unable to promote cell attachment to gelatin, inhibited cell attachment to serum-coated gelatin, and caused rounding of cells previously spread on fibronectin. These effects were reversible, indicating that WFA chronic wound fluid was not toxic to the cells. Inhibition could be neutralized by excess fetal bovine serum, which contains a broad spectrum of protease inhibitors. The identity of the protease responsible for fibronectin degradation in wound fluid currently is unknown. On the other hand, inhibition could not be overcome by excess fibronectin, which shows that the defect in wound fluid was not simply an absence of adhesion proteins containing the necessary domains to mediate binding of BHK cells to gelatin. The results are consistent with the idea that wound fluid proteases are the factors responsible for inhibition of cell adhesion. These proteases may act on both the adhesion proteins and their cell surface receptors.

Little is known about proteases in wound fluid. Collagenase and elastase have been detected in blister fluid from bullous skin diseases [32], and corneal ulcers contain increased levels of plasminogen activator [33]. Recently, we found that metalloproteases were overexpressed in WFA chronic wound fluid [30]. Once fibronectin fragments occur in the wound bed, the fragments could promote continued high protease levels by stimulating neutrophil degranulation [34] and fibroblast secretion of metalloproteases [35]. Also, some fibronectin fragments exhibit endogenous protease activity toward gelatin and laminin [36]. Elevation of proteases in wound fluid would interfere with normal healing by degrading adhesion proteins and other factors necessary for repair, many of which have been found in wound fluid, e.g., transforming growth factor (TGF)-β [37], EGF [38], platelet-derived growth factor (PDGF)-related peptides [39], and several cytokines [40].

Although chronic skin ulcers continue to present a serious clinical problem [41,42], the underlying cause(s) responsible for poor wound closure remains unknown. Our studies suggest that in at least a subset of venous stasis ulcers, elevated protease activity in the wound bed may account for impaired keratinocyte migration and failed wound closure. Despite the marked differences between the cell adhesion protein profiles of WFA and WFB chronic wound fluids, there were no obvious clinical differences in the stasis ulcers from which these samples were obtained (e.g., size, duration) or demographic features of the patients (e.g., age, race, sex). More work is necessary to determine whether there are biochemically distinct subclasses of venous stasis ulcers or whether these ulcers go through periods of low and high proteolytic activity. In either case, the expression of high proteolytic activity in the wound bed has
significant implications for therapy, because procedures such as skin grafting or the application of growth factors might be affected adversely by elevated proteases (cf. [43]).

We are indebted to Charles Baxter, M.D. for his advice and encouragement; Larry Kim, M.D. and Elise Tidwell, R.N., for helping collect wound fluid; and William Snell, Ph.D., Louis Picker, M.D., and Paul Bergstresser, M.D., for their help in preparing the manuscript.

REFERENCES