

INVESTIGATIVE REPORT

Degradation of Antiproteinases, Complement and Fibronectin in Chronic Leg Ulcers

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It has been proposed that excessive and uncontrolled proteolytic activity is an important pathogenetic factor for chronic wounds. Identification of molecules that either control or reflect proteolysis in wounds may prove to be useful in determining wound healing activity. In this study wound fluid was sampled under a polyurethane dressing or on hydrophilic glass filters. Multiple chronic wound fluid components were identified; viz. the previously described α_2 -macroglobulin, α_1 -antitrypsin and fibronectin, as well as “novel” wound fluid molecules such as complement factor C3, inter- α -inhibitor, kininogen, IgG, IgA, C-reactive protein, tetranectin, orosomuroid and ceruloplasmin. There appeared to be a highly variable degradation of α_1 -antitrypsin in the wounds; furthermore, the activation of C3 appeared to correlate with the appearance of fibronectin breakdown products. In wound fluid, inter- α -inhibitor was degraded. The influence of the sampling procedures was studied. It was shown that contact phase activation must be taken into account in the study of molecules (such as kininogens) activated by hydrophilic charged surfaces. Key words: proteolysis; antiproteinases; complement; fibronectin.

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Wound healing is a complex process requiring the concerted and sequential activation of different growth factors and proteinases. Recent studies indicate the presence of excessive proteolytic activity in and near chronic wound areas (1, 2). Several enzymes and inhibitors have been identified in chronic ulcers, such as plasmin (3), collagenase 1 and 3 (2), stromelysin-1 and 2, matrilysin, urokinase plasminogen activator (4), elastase (5), gelatinase (6) and the inhibitors TIMP-1 (4), α_1 -antitrypsin and α_2 -macroglobulin (5). Lack of control of proteases, such as neutrophil elastase, leads to excessive degradation of matrix and cell adhesion molecules such as fibronectin and tenascin-C (5, 7), possibly resulting in delayed wound healing (5). Furthermore, chronic wound fluid has been reported to inhibit cell adhesion (8) and cell growth (9) and, hence, it is likely that all these factors together contribute to the chronicity of venous wounds. Hypothetically, biochemical analysis of selected wound fluid components could aid in determining the grade of proteolysis in individual wounds. As a first step towards this goal, this study describes the identification of a “set” of plasma-derived proteins in chronic wound fluid. Selective processing of some

of these was identified, and the influence of sampling methods determined.

MATERIAL AND METHODS

Patients

Wound fluid from 12 patients with venous ulcers (duration >4 months) was used for the experiments. The venous insufficiency was routinely determined either by a handheld doppler (5 MHz probe; examination of reflux in the popliteal vein, great saphenous vein and small saphenous vein) or by colour duplex examination. Patients with diabetes or signs of general infection (malaise, fever) or local infection (cellulitis, erysipelas) were excluded. The research project was approved by the local ethics committee. Informed consent was obtained from the patients.

Materials

Tegaderm dressing was from 3M Health Care, Germany. Fibronectin, α_2 -macroglobulin, α_1 -antitrypsin, orosomuroid, inter- α -inhibitor, ceruloplasmin, prothrombin, IgG, IgA, CRP and tetranectin polyclonal antibodies were from Dakopatts (Denmark). Factor B and C3 antibodies were from Serotec (England). Antibodies against kininogens was a gift from Dr Heiko Herwald, Lund. Other materials were from sources previously listed (10, 11).

Wound fluid and plasma

Wound fluid was collected either by sampling on filters as described previously (11) or under a Tegaderm dressing (5). In a group of 12 patients (patient 1 was studied during 3 visits) glass filters (Whatman) were applied to the center of the wounds and these were saturated with wound fluid for a period of 2 h, gently peeled off the ulcer area and stored at -20°C until further use. The filters were then extracted with ice-cold phosphate buffered saline and supplemented with the proteinase inhibitors N-ethylmaleimide (NEM), di-isopropyl phosphorofluoridate (DFP) and ethylenediamine tetra-acetic acid (EDTA) (11). In one patient (no. 12) a Tegaderm dressing was applied (before filter sampling) on the wound and approximately 600 μl wound fluid was collected underneath the film after 2 h, centrifuged at 10000 rpm in an Eppendorf centrifuge, aliquoted (50 μl portions) and stored at -20°C until further use. Protein content was measured by the method of Bradford. A volume corresponding to 10 μg of protein was solubilized in SDS-containing buffer (20–40 μl) before analysis by SDS-PAGE.

SDS-polyacrylamide gel electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis was performed on 3–12% polyacrylamide gradient gels (T/C = 30/0.8), with a 3% stacking gel, using the buffer system of Laemmli (12) as previously described (10). Immunoblotting was performed after electrophoresis as previously described (11). The following dilutions for the different antibodies were used; rabbit polyclonal antibodies against α_1 -antitrypsin 1:10000; α_2 -macroglobulin, inter- α -inhibitor, C3, Factor B, fibronectin, pro-

thrombin, IgG, IgA, ceruloplasmin 1:1000; tetranectin antibodies 1:500; orosomucoid antibodies 1:5000; polyclonals (goat) against kininogens 1:6000. Dilution for all antirabbit polyclonals (HRP) was 1:1000, antigoaat polyclonals were diluted 1:3000. The membranes were developed using the ECL system (Boehringer, Germany) (11).

Control experiment

50 μ l serum or EDTA-plasma (patient no. 12) was incubated with 1/10 of a glass filter for 2 h at 37°C. Extraction was performed as described above with 1/10 of extraction buffer supplemented with the 3 proteinase inhibitors. 10 μ g of total protein was analyzed by immunoblotting.

RESULTS

Antiproteases in plasma and wound fluid

Wound fluid has been reported to contain α_2 -macroglobulin (molecular weight 180 kDa, reduced) and variable amounts of cleavage products have been detected (5). In this study, α_2 -macroglobulin was identified in wound fluid (polyurethane dressing) and the corresponding plasma (Fig. 1a, α_2 -M). Both the intact protein and degraded 80–90 kDa fragments were detected in the wound fluid sample.

α_1 -antitrypsin (54 kDa) undergoes a selective cleavage in wound fluid, which results in the formation of smaller fragments of about 45 kDa. In addition, variable amounts of complexed forms with molecular weights of about 66 kDa have been detected (5). In the present material, all these forms were detected in the wound fluid samples. The wound fluid of patient no. 12 appeared to contain mostly intact antiproteinase (Fig. 1a, α_1 -AT). The activation of α_1 -antitrypsin seemed to vary between patients; some, such as patient no. 1 (third sampling), 7 and 12 contained mostly intact antiproteinase (Fig. 2a). Others, most notably patient no. 6, contained more of the \approx 50 kDa products.

Inter- α -inhibitor in plasma has been reported to contain immunoreactive bands with molecular weights of about 230–240 kDa, 140–150 kDa and 70–80 kDa, corresponding to H1+H2+bikunin, H3+bikunin and H2 or H1, respectively (13). All these forms were detected in the plasma from

patient no. 12 (Fig. 1a, I- α -I), and also (not shown) in the plasmas from patients 1–11. In wound fluid from patient no. 12, (Fig. 1a, I- α -I) it was noted that the high molecular weight bands (above 100 kDa) yielded weaker signals after immunoblotting when compared with the same bands in the corresponding plasma sample. The dominant band in wound fluid corresponded to free heavy chains (H2 or H1). The intensity of this band was higher in wound fluid than in the plasma sample, and since the same amount of total proteins were analyzed in both cases, there appeared to be a higher absolute amount of heavy chains in this wound fluid sample than in plasma. Similar results were obtained with filter-derived wound fluid (results not shown, Table I).

Plasma as well as wound fluid from patient no. 12 contained H-kininogen (molecular weight 120 kDa) and L-kininogen (molecular weight 68 kDa). In the wound fluid sample from this patient, a slight activation was noted (Fig. 1a, KIN).

Antichymotrypsin (molecular weight 69 kDa) was identified in plasma as well as in wound fluid in patient no. 12 (polyurethane dressing, Fig. 1a, ACT), and similar results were achieved with the filter-derived wound fluid from patients no. 1–12 (not shown).

Fibronectin, complement factors and antibodies in wound fluid

Fibronectin, a disulphide-bonded dimer (monomeric forms \approx 200 kDa; see plasma, Fig. 1b, FN) (24) was identified. Wound fluid contained fragments of this molecule with molecular weights of about 36, 64, 100 kDa and of higher molecular weights (Fig. 1b, FN and Fig. 2c). The intensities of the different peptide fragments and the degree of degradation seemed to vary between the patients. In some wound fluids (Fig. 2c: 1b, 2, 9 and 11 and 12) the 100-kDa bands, as well as a weaker 36-kDa bands, were detected. Other patients contained more of the high molecular weight fragments. It appeared that samples containing predominantly intact fibronectin displayed little degradation of complement C3 (e.g. patients no. 5 and 10).

Complement factor C3, a disulfide-bonded molecule consisting of an α -chain (116 kDa) and a β -chain (70 kDa) was

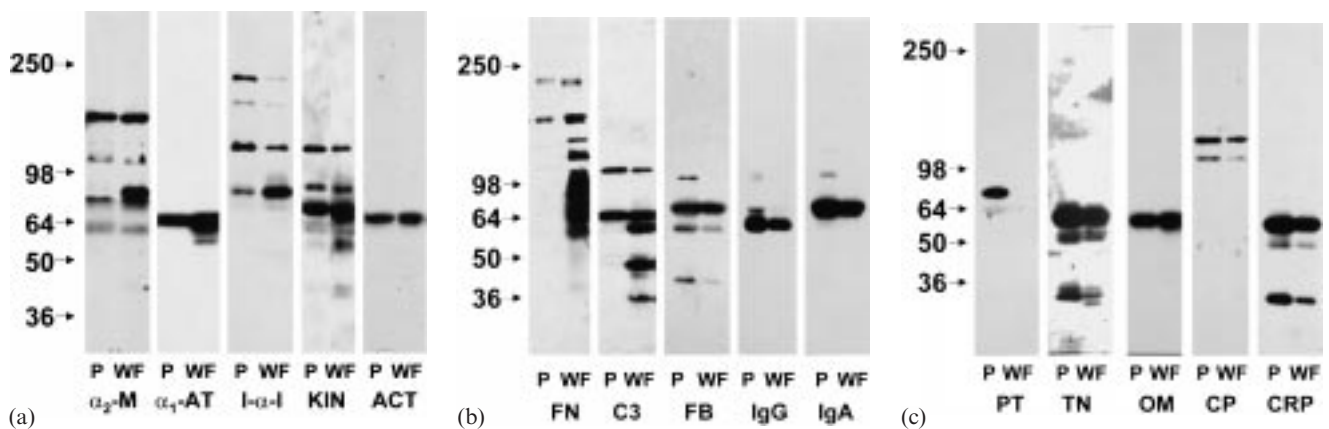


Fig. 1. a–c. Proteins in plasma and wound fluid directly collected under Tegaderm. Plasma (P) and wound fluid (WF) (patient no. 12) was electrophoresed (3–12% SDS-PAGE), transferred to nitrocellulose and immunoblotted with antibodies against (Fig. 1a) α_2 -macroglobulin; α_2 -M, α_1 -antitrypsin; α_1 -AT, inter- α -inhibitor; I- α -I, kininogen; KIN, antichymotrypsin; ACT, (Fig. 1b) fibronectin; FN, complement factor C3; C3, factor B; FB, IgG, IgA, (Fig. 1c) prothrombin; PT, tetranectin; TN, orosomucoid; OM, ceruloplasmin; CP, C-reactive protein; CRP. 10 μ g of protein was applied in each lane. Molecular weight standards (in kDa) are indicated to the left.

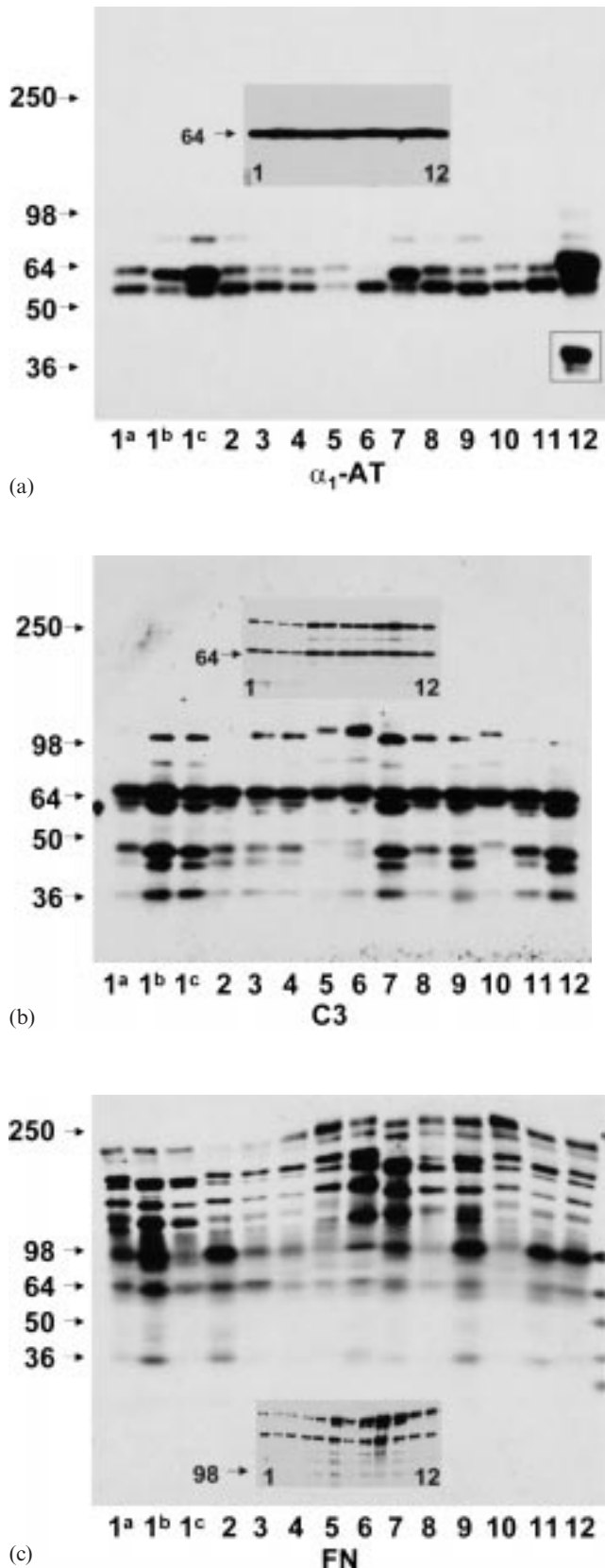


Fig. 2. a–c. α_1 -antitrypsin, C3 and fibronectin in plasma and filter-derived wound fluids. Plasma (inset) and wound fluid from 12 patients (1–12) was studied. Wound fluid from patient 1 was collected during 3 visits. The samples (10 μ g protein) were electrophoresed on 3–12% SDS-PAGE, transferred to nitrocellulose and

identified in all the plasma samples (Fig. 1b, C3 and inset in Fig. 2b) (14). In wound fluid sampled under the polyurethane dressing, there was evidence for extensive complement degradation. The α -chain signal in the blots was, when compared with plasma, weaker in this patient's wound fluid, which also contained additional fragments of apparent molecular weights 60, 40–50 and 30–35 (Fig. 1b, C3). The polyclonal antiserum used detects various parts of C3; in addition to the intact polypeptide chains of C3, C3b as well as C3a, C3b, iC3b, C3c and C3d (product information, Serotec, AHC007) are detected. It was beyond the scope of this work to identify the exact nature of each fragment in the immunoblots, although the apparent molecular weights of the fragments were of the same sizes as two α -chain derived fragments of iC3b (68 kDa and 43 kDa) and chains of C3c (36–43 kDa) and C3d (30–40 kDa), respectively (15). Analysis of wound fluid from patients no. 1–12 (filter method, Fig. 2b) showed that the C3 activation seemed to vary. Although the majority of patients contained C3 degradation products, some, such as no. 5, 6, and 10, contained very little of the scission fragments.

Factor B (proform of molecular weight 93 kDa) was identified only in plasma (patient no. 12, Fig. 1b, FB). In wound fluid from the same patient (Fig. 1b, lane 3B), the polyclonal antiserum did not detect any intact factor B. The antiserum cross-reacted with other plasma proteins (comigrating with albumin and immunoglobulins).

In patient no. 12, a polyclonal antiserum against the Fc part of IgG detected a 60-kDa signal in plasma as well as in wound fluid (sampled under polyurethane dressing) of this patient (Fig. 1b, IgG). Antibodies against IgA detected a slightly larger product in plasma and in wound (70 kDa, Fig. 1b, IgA). No degradation of IgA or IgG was noted in the 12 patients studied (results not shown, Table I).

Prothrombin, tetranectin, orosomuroid, ceruloplasmin and C-reactive protein

In patient no. 12, prothrombin (69 kDa, Fig. 1c, PT), tetranectin (20 kDa, reduced, Fig. 1c, TN), orosomuroid (55 kDa, Fig. 1c, OM), ceruloplasmin (130 kDa, Fig. 1c, CP) and C-reactive protein were identified (Fig. 1c, CRP). In wound fluid of this patient, ceruloplasmin, CRP, orosomuroid and tetranectin were identified (Fig. 1c). No signal for thrombin or related peptides was detected in the wound fluid sample from patient no. 12. Tetranectin in wound fluid appeared to be slightly degraded and contained a smaller fragment in addition to the 20-kDa form (wound fluid in Fig. 1c, TN). The film was overexposed to visualize the rather weak tetranectin bands, and hence, unspecific bands were detected; the 64 and 55 kDa bands comigrated with albumin and IgG, respectively.

Determination of interference by the sampling method

Human serum and plasma (patient no. 12) was incubated with or without glass filter material for 2 h at 37°C. Plasma

immunoblotted with antibodies against α_1 -antitrypsin (a), C3 (b) or fibronectin (c). Inset to the lower right in (a) shows a less exposed α_1 -antitrypsin band. Molecular weight standards (in kDa) are indicated to the left.

Table I. A summary of the changes found in wound fluid molecules using the 2 sampling techniques (N.D.; not determined)

Molecule	Sampling under Tegaderm dressing (patient 12)	Sampling in filters (patients 1–12)
α_1 -antitrypsin	degraded partially	degraded partially
α_2 -macroglobulin	degraded partially	degraded partially ^a
inter- α -inhibitor	heavy chains dominating	Heavy chains dominating
H, L-kininogen	degraded slightly	degraded completely ^a
antichymotrypsin	intact	intact
fibronectin	degraded partially	degraded partially
C3	α -chain degraded partially	α -chain degraded partially
factor B	not detected	N.D.
IgG	intact	intact
IgA	intact	intact
prothrombin	not detected	N.D.
orosomucoid	intact	intact
ceruloplasmin	intact	intact
tetranectin	intact+small shift in size	N.D.
c-reactive protein	intact	N.D.

^aIncreased degradation was seen in filter-sampled material.

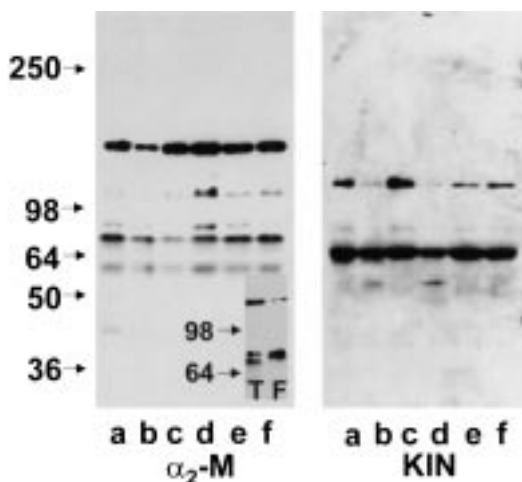


Fig. 3. Effects of filter matrix on plasma proteins. Plasma (a and b) and serum (c and d) from patient no. 12 was incubated with (b and d) or without (a and c) glass filter material for 2 h at 37°C to simulate sampling conditions. Material (10 µg protein) was electrophoresed (3–12% SDS-PAGE) and transferred onto nitrocellulose. (e) and (f) show plasma and serum analyzed directly (without incubation). The material was immunoblotted with antibodies against α_2 -macroglobulin (α_2 -M) or kininogen (KIN). Inset shows α_2 -macroglobulin in wound fluid from patient no. 12 sampled under Tegaderm (T) or on filters (F).

contained small amounts of activated α_2 -macroglobulin after incubation with filters (compare Fig. 3c and d). In serum a slight degradation was noted (Fig. 3a). The H-chain of kininogen was completely degraded after filter incubation (Fig. 3 b and d). The polyurethane dressing material did not exhibit these effects (results not shown). Inter- α -inhibitor, C3, α_1 -antitrypsin and fibronectin were unaffected (results not shown). Analogously, increased degradation was noted for α_2 -macroglobulin (inset Fig. 3, α_2 -M) whereas H-kininogen was completely degraded (not shown) when filter sampled wound fluid was compared with material directly aspirated under the Tegaderm dressing (Table I).

DISCUSSION

Several lines of evidence show that increased proteolysis plays a role in the pathogenesis of chronic venous wounds. Proteases might be expected to affect various molecules in the wound environment, such as matrix proteins, antiproteases, kininogens and complement factors. Previous studies have shown that the degradation of α_2 -macroglobulin and α_1 -antitrypsin, wound fluid antiproteases also identified in this study (Table I), as well as the matrix component fibronectin, depends on the levels of neutrophil elastase in wound fluid (5, 16).

In this study, novel findings showed that C3, the dominant and most abundant factor in the complement cascade, was partially degraded in chronic wounds. The products were not directly identified as individual species, but should correspond to various forms of C3 degradation products. These findings relate to those obtained by immunohistochemical methods, showing leukocytoclastic vasculitis in venous wounds, implicating the presence of complement activation in these ulcers (17). In the patients reported here, it appeared that wound fluid from those patients exhibiting a high degree of fibronectin fragmentation, contained more C3 degradation products and vice versa. This may be interpreted in several ways. It is possible that proteases capable of cleaving fibronectin (such as elastase) also degrade C3. The effects of elastase on C3 have been studied in some extent *in vitro* and *in vivo*; elastase cleavage sites have been identified (18) and it has been shown that C3 is cleaved by neutrophil proteases, such as elastase in pleural empyemas (19). Interestingly, bacteria, such as *Pseudomonas aeruginosa* secrete proteases that degrade C3 (20). This could lead to low complement-mediated opsonic activity by direct inactivation of C3 (19, 20). C3 can also be activated by bacterial surfaces directly; the alternate route (14). Which of the activations that occur *in vivo* has to be investigated.

Another novel finding was the identification of inter- α -inhibitor in wound fluid. Although plasma contained, as expected, all forms of this inhibitor family, it was noted that wound fluid contained mostly the low molecular weight forms. Interestingly, similar changes have been noted in serum

from patients with acute infections (13); bikunin-containing forms (bikunin+H1+H2 and bikunin+H2) were down-regulated at the mRNA level in the liver whereas the single H3 chain was up-regulated. However, since plasma contained all 3 forms (bikunin containing complexes seemed to dominate), altered synthesis patterns in the liver could not explain the changes in wound fluid reported here. More probably, the present findings reflect the antiproteolytic nature of inter- α -inhibitor; the molecule is an inhibitor of trypsin, chymotrypsin, cathepsin G and neutrophil elastase (21), the last enzyme being a dominant protease in wound fluid (5, 16). Indeed, recent observations indicate that neutrophil proteases are able to degrade inter- α -inhibitor (22, 23). A further possible processing mechanism could be related to possible hyaluronidase and chondroitinase activities in the wound environment, perhaps produced by different bacteria (24, 25).

Kininogen represent a potentially interesting, not previously described, antiproteinase in the chronic wound environment. These molecules may undergo degradation, releasing specific proinflammatory peptides, kinins, which mediate vasodilatation, pain and edema (26). When wound fluid sampled under the polyurethane dressing was analyzed, an additional component of about 55–60 kDa was detected in the patient's wound fluid, perhaps identical to one of the products of H-kininogen (denoted light chain; 58 kDa). The complete degradation of H-kininogen, together with a increased activation of α_2 -macroglobulin, indicated that the glass filters, in contrast to the polyurethane dressing, activated the contact phase system (27). Thus, in studies using different hydrophilic wound fluid sampling matrices, including various absorbent wound dressings, the extent of contact activation should be determined.

Finally, the possibility that other plasma proteins were affected by wound proteolysis was also investigated (Table I). Surprisingly, several high molecular weight proteins (such as orosomucoid, ceruloplasmin, IgG and IgA) were identified unaffected in wound fluid or, such as tetranectin, slightly degraded (Table I). Others, such as thrombin were not detected in wound fluid. These findings suggest that protein processing in chronic wounds is specific, affecting a subset of proteins.

Concluding remarks

This descriptive study, identifying molecules such as C3, kininogens, fibronectin, inter- α -inhibitor and other antiproteinases in chronic wound secretions, is a prerequisite for experiments aimed at relating the expression and processing of these molecules to clinical parameters such as inflammation, bacterial load and wound healing activity. Ongoing development of matrices with non-activating properties should facilitate the collection of molecules such as kininogens as well as other components involved in the contact phase system.

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