Acta Derm Venereol 1999; 79: 291–295

Chronic Ulcers: A Method for Sampling and Analysis of Wound Fluid

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There is growing evidence that chronic wounds show specific temporospatial patterns with respect to the expression of various molecules such as proteinases and their inhibitors. A careful analysis of molecules in the exudate of chronic wounds can be expected to provide useful information regarding events in the underlying tissue. In this connection there is a need for a method applicable to large amounts of patient material for collecting and analysing wound fluid from leg ulcers. A method of this sort is described. Wound fluid is accumulated for a defined period of time in Whatman GF/D filters. The filters are then extracted with isotonic buffer. Cells and other debris are centrifuged down and the supernatant is analysed further. The analysis can include electrophoresis on polyacrylamide gels, HPLC ion exchange chromatography and Western blotting techniques. The method will be useful for the sampling and analysis of wound fluid from patients with wounds of different aetiologies. ***Key words: leg ulcer; venous insufficiency; immunoblotting; antichymotrypsin.***

(Accepted January 17, 1999.)

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Several parameters are important in the study of events at the surface of chronic wounds. Wound fluid consists mainly of a mixture of extravasated material and factors synthesized in the vicinity of the wound. In addition, since wounds are colonized by a variety of bacteria, wound fluid represents a heterogeneous mixture of endogeneously synthesized proteins from the underlying granulation tissue, bacterial antigens and plasma proteins. Various methods have been employed to study events at the wound surface. Grinnell & Zhu (1), in their study of fibronectin degradation, used a polyurethane dressing for wound occlusion and aspirated wound fluid from under the dressing with a syringe. In a similar study, aimed at identifying different matrix metalloproteinases, Weckroth et al. (2) collected wound fluid directly from the exudating surface of ulcers using blunt-end glass microcapillaries. Others, such as Chen et al. (3), have used plastic wound chambers or collected wound fluid from under hydrocolloid dressings. Bernfield's group (4) identified syndecan 1 and 4 ectodomains in wound fluid obtained by sterile, closed suction drains placed in the subcutaneous tissue (following mammoplasty). Recently, proteinase 3 was identified in acute wound fluid (burn wounds) collected under a sterile glove (5) and plasminogen degrading activity was identified in wound fluid expressed from an absorptive dressing (6).

In the present study, concerning wound fluid components from chronic leg ulcers, it was considered important: (a) that the method used reflects wound fluid composition during an extended period of time (several hours); (b) that the sampling device interferes only minimally with the wound components; and (c) that the sampling be easy to carry out and possible to perform at a leg ulcer unit such as ours. In general, sampling methods for wound fluid may have disadvantages. Dressings, such as hydrocolloids, may stimulate or accumulate proteinases and growth factors in the environment of the wound (3). Use of wound chambers or glass capillaries, although attractive, is too laborious and is not applicable as a general sampling method in larger patient groups. The latter method only reflects wound fluid composition during a short period of time. In the present study, a method using hydrophilic filters is described. The filters exhibited low interactions with the major wound fluid components. They were saturated with wound fluid for a defined period of time, allowing the wound components to be extracted by isotonic buffer and to be analysed further.

MATERIAL AND METHODS

Patients

The research project was approved by the Ethics Committee of Lund University Hospital. Informed consent was obtained from the patients. Wound fluid from 5 patients with venous ulcers was used for the experiments. The patients' venous insufficiency was determined routinely either by a hand-held Doppler (5 MHz probe; examination of reflux in the politeal vein, great saphenous vein and small saphenous vein) or by colour duplex examination. Each of the patients had a systolic index >0.9. Patients with diabetes or immunological disease were excluded.

Materials

Whatman glass microfibre circles (GF/D) were from Whatman International Ltd (UK). Phosphate-buffered-saline solution contained 0.137 M NaCl/3 mM KCl/8 mM NaH_2PO_4/2 mM KH_2PO_4, pH 7.4; 0.5 M NaCl buffer contained 0.5 M NaCl, 10 mM Tris-HCl, pH 7.4; 2 M NaCl buffer contained 2 M NaCl, 10 mM Tris-HCl, pH 7.4 and 4 M guanidinium hydrochloride (GdnHCl) buffer contained 4 M GdnHCl, 10 mM Tris-HCl, pH 7.4. In some cases the proteinase inhibitors N-ethylmaleimide (NEM), diisopropyl phosphorofluoridate (DFP) and ethylenediamine tetra-acetic acid (EDTA) were added. The final concentration was always 10 mM for NEM and EDTA, and 1 mM for DFP. Dextran T-500 was from Sigma (Sweden). ECL Western blotting kit was from Boehringer Mannheim (Germany). Hybond NC was from Amersham (UK). Anti-chymotrypsin polyclonal antibodies were from Dakopatts (Denmark). Other chemicals were from sources described previously (7).

Fibroblast culture and incorporation of radioactive precursors

Cultures of human embryonic skin fibroblasts were established and grown as monolayers on 75 cm^2 plastic dishes, as described by Malmström et al. (8). Cells were grown to confluence in MEM (Gibco BRL) supplemented with 10% (v/v) donor calf serum, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were incubated with medium supplemented with 2% serum followed by no serum for 2 h each. Incorporation of radioactive precursors was performed in serum and methionine-free medium for

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48 h with ^{35}S-methionine (50 μCi/ml). The medium was decanted and centrifuged. The supernatant was dialysed against PBS-buffer and stored at −20°C.

Extraction of filters

Sterilized (autoclaved) filters (Whatman GF/D; diameter 2.5 cm) were applied to wounds for 4 h. The filter and the wound were occluded by a plastic foil (polyethylene; DowBrands, Germany). Filters were weighed before and after application and after saturation were transferred to 12-ml tubes. Wound fluid was extracted from the filter; 10 ml of ice cold PBS was added and the filters were rocked gently (2 oscillations/s) for 5 min in the cold-room. Macromolecular material (erythrocytes, leukocytes, bacteria and other debris) was spun down (1000 g for 15 min at 4°C). The supernatant was supplemented by the proteinase inhibitors DFP, NEM and EDTA (see above), and 1 ml aliquots were stored at −20°C. The material was used in further experiments. In a separate experiment (control), 1/10 of a filter (approximately 8 mg) was placed in eppendorf tubes and incubated with a mixture of 10 μl EDTA-plasma and 90 μl radiolabelled fibroblast products (in PBS) for 15 min at room temperature or 4 h at 37°C. Subsequent extraction was performed as above, but with 1 ml of each buffer. The extracts (100 μl) were precipitated with 9 volumes of 95% ethanol, air-dried briefly and solubilized in SDS-buffer.

Ion exchange chromatography on MonoQ HR

Ion exchange chromatography was performed on a MonoQ HR 5/5 column connected to an FPLC system (LKB) as previously described (7). Samples (10 μl plasma or 800 μl filter extract) were dialysed against 7 M urea/10 mM Tris-HCl/0.1% Mulgophene (pH 8.0) and applied to the column. A 0-0.8 M NaCl gradient (in the 7 M urea buffer) was then applied (flow rate 0.5 ml, fraction volume 1 ml). Volumes of 200 μl of the fractions (4–24) were precipitated by 5 volumes of 95% ethanol after addition of 10 μg dextran T-500.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on either an 8 or a 3–12% polyacrylamide gradient gel (T/C = 30/0.8) with a 3% stacking gel using the buffer system of Laemmli (9). Samples were dissolved in 25 μl 5% (w/v) SDS/20% (v/v) glycerol/4 mM-EDTA/0.04% bromophenol blue/125 mM-Tris/HCl, pH 6.8. To reduce disulphide bonds, β-mercaptoethanol was added to a final concentration of 10% (v/v). Samples were boiled for 3 min and electrophoresed for approximately 6 h at a constant current of 60 mA. After electrophoresis, gels were stained with 0.25% Coomassie brilliant blue R-250, destained, and dried. In one experiment the gel was analysed for radioactivity (radioimaging system; Bas 2000, Fuij).

Western blotting

Immunoblotting was performed after separation by SDS-PAGE. The gel was equilibrated in 50 mM NaCl/2 mM EDTA/10 mM Tris-HCl pH 7.0/0.10 mM DTT/4.5 M urea for 2 h. Proteins were transferred to nitrocellulose membranes (Hybond NC) by diffusion for at least 48 h in the same buffer as above without urea. Blocking was performed in 10 mM Tris pH 7.4/0.2% Tween 20/0.15 M NaCl/3% delipidated milk for 2 h. Incubation with the first antibody was performed for 1 h in the above blocking buffer. The membrane was washed 5 × 10 min in blocking buffer (without milk) and then incubated with the second antibody in blocking buffer as above. After washing (5 × 10 min) with blocking buffer (without milk) the membranes were developed using the ECL system according to the manufacturer's instructions (Boehringer, Germany).

RESULTS

Determination of interference with proteins

SDS-PAGE analysis of wound fluid directly aspirated from the wound surface of a chronic venous wound showed that the major proteins of the exudate had the same molecular weights as the ones found in plasma (not shown). It is also likely that the wound fluid contains proteins originating from the underlying granulation tissue. And thus, in order to examine possible interference of the filters with a defined "set" of wound fluid components, human plasma was mixed with radiolabelled fibroblast extracellular proteins (representing wound-bed derived proteins). The material was then incubated with the filter for 15 min and extracted by PBS followed by 0.5 M NaCl, 2 M NaCl buffer and 4 M GdnHCl buffer. The material was recovered and analysed by SDS-PAGE. The proteins were stained with Coomassie (Fig. 1, protein stain) and the relative intensity was determined by densitometric scanning of the lanes. No significant difference in the approximately 25 individual protein bands was detected between the control (no filter) and the material released from the filter (first PBS extract). Similar results were obtained when the filters were incubated for 4 h (not shown). No additional material was detected after incubation with NaCl or GdnHCl (Fig. 1, protein stain). Protein concentration was measured by the Bradford method, and recoveries were 96

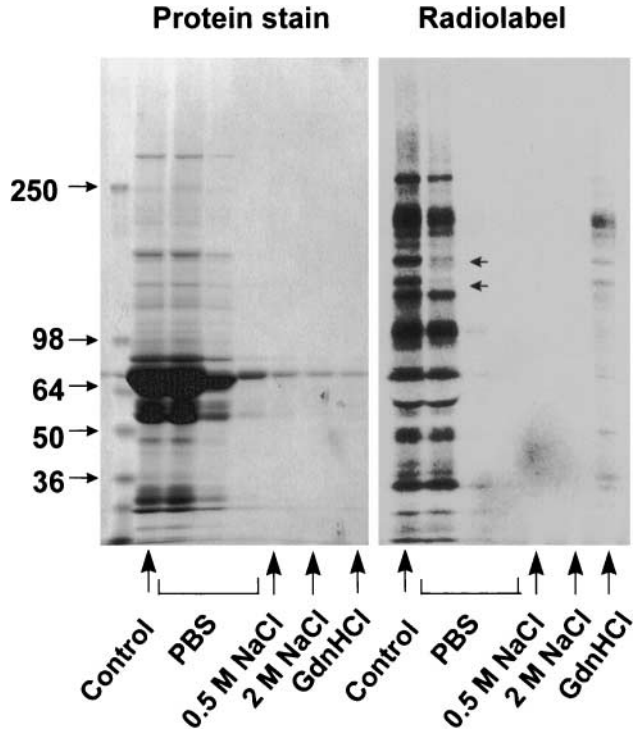


Fig. 1. Analysis of interactions with filter material. Plasma was mixed with radiolabelled secreted fibroblast proteins and added to filters. The filters were extracted 3 times with PBS followed by 0.5 M NaCl, 2 M NaCl and 4 M GdnHCl, respectively. PBS was added to a control sample without filter ("Control"). The material in each fraction was analysed by SDS-PAGE (3–12% gels). The left panel shows proteins stained with Coomassie blue. The right panel shows ^{35}S-methionine labelled fibroblast proteins.

and 94.5% (mean of duplicates) for the 15 min and the 4 h incubated samples, respectively.

Fibroblast-derived radiolabelled proteins were analysed and 86% and 83% of the radioactivity was released by PBS (15 min and 4 h incubations). The majority of the proteins were extracted from the filter by PBS, however, 2, possibly 3, fibroblast proteins seemed to be adsorbed to the filter and released by GdnHCl (arrows; Fig. 1, radiolabel).

The major aim of the present study was to develop methodology for the study of the major plasma-derived proteins of wound fluid, such as antiproteinases (exemplified in this study by antichymotrypsin, see below), acute phase reactants and other soluble proteins, such as plasma fibronectin. These components normally occur in sufficient amounts in plasma to allow detection by standard protein staining protocols, such as Coomassie Brilliant Blue (10, 11). In the control experiment, material corresponding to 0.9 µl plasma was analysed by SDS-PAGE and densitometric scanning. The detection limit for Coomassie staining of polyacrylamide gels is ~100 ng/band (11), and hence, proteins of concentrations >0.1 µg/µl should be detected. Taken together, these results indicate that the filter showed essentially no detectable interference with this group of proteins.

Sampling and analysis of wound fluid

Filters were placed in the centre of the wounds and were usually fully saturated with wound exudate after 4 h. No discomfort during the sampling period was reported by any of the 5 patients studied. The filters were transferred to tubes and extracted as described above. Usually, the maximal binding capacity/filter was about 500 µl of fluid. The serine-, thiol- and metallo-proteinase inhibitors were added to block possible proteolytic activity.

In one experiment (Fig. 2), analysis showed the filters to be essentially completely extracted by PBS. The filters were reextracted by PBS followed by 0.5 M NaCl, 2 M NaCl buffer and 4 M GdnHCl buffer. The material was recovered and analysed by SDS-PAGE. In correspondence with previous results (Fig. 1, left panel) no residual material was bound to the filters after the PBS extractions.

In further experiments, human plasma and wound fluid from 1 of the patients were compared. 10 µl plasma and 800 µl extract were separated according to charge by ion exchange HPLC on MonoQ, and the fractions were analysed by SDS-PAGE (Fig. 3). Wound fluid and plasma contained bands of similar molecular weight and elution pattern on HPLC MonoQ indicating that the majority of proteins in wound fluid are identical to those found in plasma. However, additional bands of molecular weight 20–50 kDa were detected in wound fluid. These bands were also found in further experiments involving the other 4 patients (results not shown).

To validate the method further, wound fluid extracts containing 20 µg of protein (as determined by the Bradford method, usually approximately 20–40 µl PBS extract) were separated on 3–12% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with antibodies against antichymotrypsin. As can be seen (Fig. 4), a single band of 70 kDa was detected.

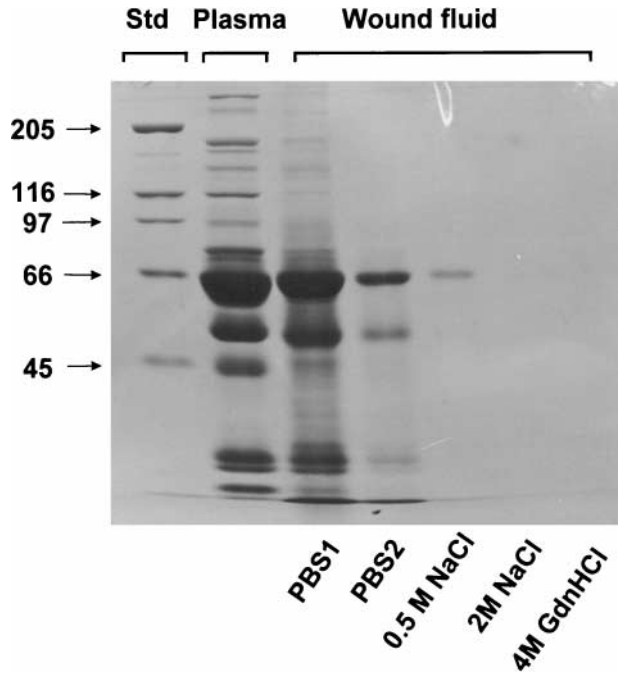


Fig. 2. Analysis of proteins in wound fluid and plasma. Filters were extracted twice with PBS and then re-extracted using buffers containing 0.5 M NaCl, 2 M NaCl and 4 M GdnHCl, respectively. The material obtained together with the corresponding plasma sample was analysed by SDS-PAGE (8% gel). Numbers on the left indicate molecular weights for the standard proteins in kDa.

DISCUSSION

Using inert hydrophilic filters for sampling performed on wounds during a defined period of time, with subsequent extraction using low salt and proteinase inhibitors, thus yields a representative starting material for the further analysis of events at the wound surface. The protein concentration in wound fluid was 10–32 g/l (mean 23 g/l), which corresponds to the reported concentrations for interstitial fluid and joint fluid (~20 g/l). Normal values for human plasma are 65–82 g/l. Separation according to relative charge on HPLC MonoQ followed by subsequent analysis on SDS-PAGE, showed the plasma and wound fluid-derived material to share many constituents. The additional low molecular weight products could represent either degradative products or endogeneously produced wound-specific components synthesized by the wound-bed cells and/or by resident bacteria. High molecular weight components (>150 kDa) were also detected (see Fig. 1, PBS extract). The products were in the size range of fibronectin, as previously described by Grinnell & Zhu (1). The wound fluid extracts can be further analysed by various techniques, such as immunoblotting. In the present study, the material was assayed for the presence of antichymotrypsin. The 69 kDa serin-proteinase inhibitor, usually found in plasma (concentration approximately 0.4 g/l), was also detected in wound fluid. This protein is the major physiological inhibitor of cathepsin G (12). It has been shown that fibronectin as well as tenascin-C degradation in chronic wounds depends on the relative levels of leukocyte elastase (1, 13). Fibronectin degradation was inhibited by the two protease inhibitors α_1-proteinase inhibitor and α_2-macro-

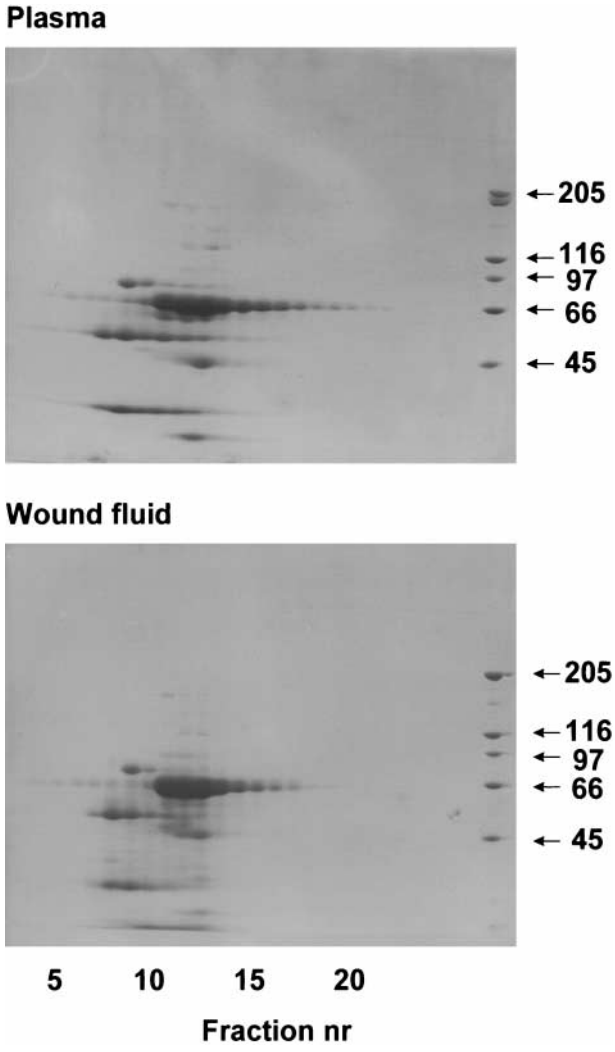


Fig. 3. Electrophoresis of plasma and wound fluid proteins separated on HPLC MonoQ. Material from filter extracts and corresponding plasma was separated by ion-exchange chromatography, precipitated and analysed by SDS-PAGE (3–12% gel). Standard proteins are indicated on the right (kDa).

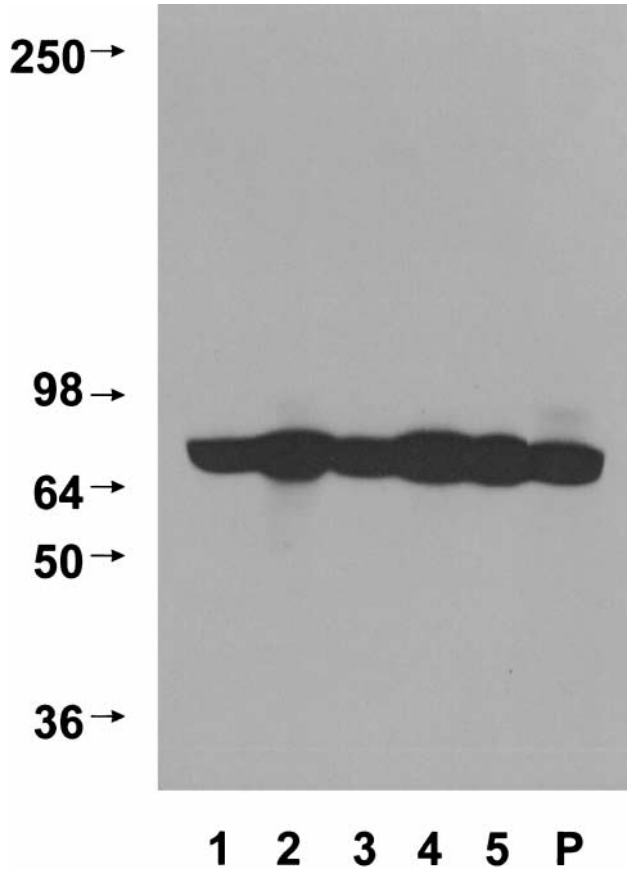


Fig. 4. Identification of anti-chymotrypsin in wound fluid by immunoblotting. Samples (20 μg of protein) were electrophoresed (3–12% SDS-PAGE), transferred to nitrocellulose membranes and assayed for antichymotrypsin using a polyclonal antiserum. 1–5; patient 1–5, P; plasma (patient 2).

globulin (1, 14). Antichymotrypsin could act in concert with these antiproteinases.

Finally, it should be noted that the approach described in the present study was designed for identification of the major components of wound fluid, such as plasma-derived antiproteinases, complement and structural proteins (such as fibronectin). In the control experiment, plasma-derived material showed low interference with the filters. Recoveries were about 95%, and the protein patterns after analysis by SDS-PAGE of filter-extracted plasma and control plasma were identical. With respect to fibroblast extracellular products, the filter material appeared to bind some components with molecular weights of 150–200 kDa. The reason for this is unclear, but could be due to interactions between these proteins and the filters. The data thus showed that the "risk" for interactions (binding and/or degradation) between plasma-derived proteins and the filter material should be low for most proteins. However, molecules, such as contact system proteins, known to be activated by interaction with negatively charged or polar surfaces should be further studied. Also, no attempts have been made to characterize other factors, such as cytokines, in the wound fluid extracts or their possible interference with the filter material. The approach presented here is currently used for qualitative studies of wound fluid components. Semiquantitative studies might be possible; relative amounts of individual proteins can be estimated, using the actual protein and/or a reference protein as a standard (11).

In summary, the present method was shown to be suitable for the study of the major wound fluid components. The method combines low interference with the wound, standardized sampling conditions (time and area) and rapid inhibition of protease activity after sampling, allowing a representative material to be obtained for further studies. Since the method does not require that patients be immobilized during the sampling procedure, it is suitable for studies of patients referred to and regularly visiting a leg ulcer unit such as ours.

ACKNOWLEDGEMENTS

The study was supported by grants from The Swedish Foundation for Strategic Research and the Harald and Greta Jeansson, Magnus Bergvall, Alfred Österlund and Kock Foundations. I am grateful to Professor Ove Bäck for valuable suggestions during revision of this manuscript. I also thank Professor Lars-Åke Fransson (MFR13X567) for support during the research.

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