**Introduction**

Chronic ulcers are a major health problem and an increasing burden to health care providers. The ulcers have various underlying causes, such as venous or arterial insufficiency, diabetes, vasculitis, or excessive pressure (1). Venous leg ulcers represent a condition characterized by unregulated and excessive inflammation. Initial pathogenetic steps involve activation of endothelial cells by up-regulation of various cell adhesion molecules (such as I-CAM) on endothelial cells, and their corresponding ligands on leukocytes, thus leading to extravasation of inflammatory cells into the wound area (2). The high influx of leukocytes and subsequent release of proteases, such as neutrophil elastase and gelatinases leads to high proteolysis in the surroundings of chronic ulcers (3-5). These findings, together with the observation that keratinocytes display an altered, non-migratory, phenotype in venous ulcers leads to a failure of re-epithelialization (2). It is known that all chronic ulcers are constantly colonized or infected by various bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Proteus mirabilis* (6). During recent years, clinical and experimental data support the view that these, and other pathogens, may contribute to the non-healing state of chronic ulcers (7).

HBP belongs to a family of serine proteases with an overall structure homologous to the elastase fold. The protein lacks enzymatic activity due to the exchange of two essential amino acids in the catalytic triad (8). HBP is a multifunctional protein that is responsible for a number of host response mechanisms to infection such as antimicrobial activity, recruitment and activation of monocytes, mobilization of T cells, and induction of detachment and homotypic aggregation of endothelial cells and secreted products of *Pseudomonas aeruginosa* were found to induce release of HBP from human neutrophils. Our data suggest a possible link between bacterial presence and HBP-release in chronic ulcers. Thus, targeting HBP offers an interesting option for reduction of endothelial barrier dysfunction in chronic ulcers.
fibroblasts. Furthermore, endocytosis of HBP by monocytes enhances lipopolysaccharide induced TNF-α production (9), and more importantly, HBP is able to induce a Ca²⁺-dependent cytoskeletal rearrangement and intercellular gap formation in endothelial-cell monolayers in vitro, and increased macromolecular efflux in microvessels in vivo (10). Hence, HBP could represent an interesting mediator involved in host response as well as initiation of vascular leakage in chronic ulcers. Addressing this question, we here demonstrate that HBP is indeed present in chronic leg ulcer fluid, and that the levels are significantly increased in comparison to those detected in acute, non-infected, wound fluid. Furthermore, bacteria such as P. aeruginosa, common in chronic ulcers, induce release of HBP from neutrophils.

Materials and methods

Suppliers

LL-37, LLGDFFRKSKKEKIGKEFKRIVQRKDFLNLVPRTES (MW 4492) was synthesized by Innovagen AB (Lund, Sweden). Lactoferrin was obtained from Sigma (Sweden). Mouse monoclonal antibody 2F23C3 and rabbit antiserum (409A) to recombinant HBP were prepared and purified as described earlier (11).

Patients and wound fluid collection

This research project was approved by the Ethics committee, Lund University Hospital. Informed consent was obtained form the patients. Wound fluid was collected from 12 patients with chronic venous leg ulcers with ulcer duration for more than 3 months. The age ranged from 72-84 years with a mean age of 79.6 years. The venous insufficiency was routinely determined by a handheld Doppler (5-MHz probe) or by color duplex examination. The patients had a systolic index of > 0.8. Patients showing signs of general or local infection, or patients with diabetes or immunological disorders were excluded. Op-Site dressings were applied on the wound and wound fluid was collected by gentle aspiration underneath the films after 2 hours (3). Wound fluids were centrifuged at 10000 rpm in an Eppendorf centrifuge, aliquoted and stored at -20°C until further use. In the control group, we included nine patients (mean age 65 yrs). Wound fluid was obtained from surgical drainages following mastectomy. Fluid was collected for 24 hours, 24 to 48 hours after surgery. The wound fluid was thereafter immediately transported on ice and centrifuged for 10 minutes at 10000 rpm. The supernatant was carefully removed and thereafter stored at -20°C.

Biopsies

In 14 patients, (representing a separate cohort from the chronic ulcer patients above), 4 mm biopsies were taken from the wound edges of chronic venous leg ulcers. Control biopsies were taken from healthy skin of the thigh. The tissues were fixed overnight in 4% paraformaldehyde, dehydrated sequentially in ethanol, and subsequently embedded in paraffin. Tissue sections (5 µm) were cut in a microtome, mounted on slides and airdried over night in a 37°C incubator.

Bacterial cultures

Bacterial cultures were obtained from patients as described previously (12). The patients were not treated with antibiotics prior to and during the study. The bacteria used in this study were P. aeruginosa (11 strains), S. aureus (9 strains), E. faecalis (4 strains), and P. mirabilis (3 strains). 6 of 9 strains of S. aureus were derived from patients with atopic dermatitis, all other isolates were obtained from patients with chronic venous leg ulcers.

HBP-release

The bacterial strains were cultured over night in Todd-Hewitt medium. The bacterial medium was centrifuged at 3000 rpm for 10 min, and the supernatants were carefully removed and stored at -20°C. Peripheral venous blood from a healthy volunteer was diluted 1/5 in PBS and incubated with 500 µl of bacterial medium at 37°C for 30 min. Unconditioned Todd-Hewitt-medium was used as a control. For determination of total HBP of neutrophils, Triton X-100 (0.1%) was added to the blood and the released HBP was considered as 100%. Thereafter, samples were centrifuged at 10000 rpm for 10 min. The supernatants were carefully removed and stored at -20°C. The concentration of HBP was determined by a sandwich ELISA as described elsewhere (13).

For statistical analysis, the Wilcoxon test was applied. HBP values from the chronic ulcer group were significantly higher (p=0.0021) than those originating from the control group.

Immunohistochemistry

Sections were deparaffinized by routine procedures, and endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 10 minutes. After rinsing in distilled water, followed by PBS, sections were permeabilized with Tween 20 (0.05%) in PBS. To unmask epitopes we used heat-induced epitope retrieval. Slides were transferred to 0.01M sodium citrate buffer, pH 6.0, briefly boiled in a microwave oven (at 900 W) and then for 6 min (at 90 W). Thereafter sections were rinsed in PBS. After preincubation with normal goat serum (1%) for 30 minutes at room temperature, incubation was performed with primary antibody diluted 1:8000 in PBS (containing 0.05% Tween 20, 0.2 % bovine serum albumin) over night at 4°C. Antibody detection was performed with the standard avidin-biotin-complex (ABC) detection system using DAB as the chromogenic substrate (Vectastain ABC, Vector Laboratories, Burlingame CA, USA). Sections were counterstained with Mayer’s Hemalum (Diagnostica Merck, Darmstadt, Germany).
mounted with Pertex (Histolab Products AB, Gothenburg, Sweden) and examined and photographed (Olympus BHS photomicrographic system).

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis**

Electrophoresis was performed using the Ready Gel system (Biorad, USA) on 16.5% pre-cast polyacrylamide gels with a 4% stacking gel (Tris-Tricine Ready Gel, Biorad). For analysis of HBP, LL-37 and lactoferrin degradation, the polypeptides (2 µg) were solubilized in 10 mM Tris, pH 7.4 and incubated with purified *P. aeruginosa* elastase (60 µM; ~50 ng) for 6 hours at 37°C. HBP was also incubated with growth culture supernatants (10 µl) from *E. faecalis* strains (expressing gelatinase), *P. mirabilis* strains (expressing a 50 kDa proteinase) (14), or human neutrophil elastase (~100 ng) for 6 hours at 37°C. The incubations were stopped by boiling (for 5') and samples were supplemented with Tricine sample buffer (Biorad). After fixing, the gels were stained with 0.25% Coomassie brilliant blue, destained and dried between two cellophane sheets in a Gel dryer (Biorad) and scanned (Duoscan T1222; Agfa). Proteins were also transferred onto nitrocellulose membranes for 30 min at 100 mA. The membranes were blocked with PBS containing 5% (w/v) dry milk powder and 0.05% (w/v) Tween-20, pH 7.4. Immunoprinting of the transferred proteins was done according to Towbin, et al. (15). Polyclonal antibodies against HBP protein, diluted 1:1000 in the blocking buffer, were used. Bound antibodies were detected using peroxidase-conjugated secondary antibodies against rabbit IgG (dilution 1:3000) followed by a chemiluminescence detection method. Pictures were processed using the Adobe photoshop software (Adobe).

**Bactericidal assays**

For antimicrobial assays *P. aeruginosa* were grown to mid-log phase in TH-medium. Bacteria were washed and diluted in 10 mM Tris-HCl, pH 7.5, containing 5 mM glucose. When indicated, wound fluid (acute or chronic) or serum was added to a final concentration of 20%. Bacteria (50 µl, 2 x 10^9 colony forming units/ml) were incubated with HBP at 0, 2, 10, 20, 40, and 100 µg/ml. Incubations were carried out at 37°C for 4 h. To quantify the bactericidal activity, serial dilutions of the incubation mixture were plated on TH agar, incubated at 37°C over night, and the numbers of colony forming units were determined.

**Results**

Wound fluids from 12 patients with chronic venous ulcers were collected for 2 hours under a polyurethane dressing (3) and the HBP concentration was analysed. Non-infected wound fluids, collected after mastectomy, were used as controls. The HBP levels in the chronic ulcer fluids were significantly higher (p value of 0.0021 using the Wilcoxon test) than those detected in the acute wound fluids (Fig. 1). Mean values for the two groups were 28.2 µg/ml (n=12) and 4.2 µg/ml (n=9), respectively.

Immunohistochemistry of chronic ulcer tissues from a separate group of 14 patients with chronic ulcers (typical findings are displayed in Fig. 2) identified HBP in the inflammatory infiltrate, including macrophages and leukocytes. This corresponds to previous findings demonstrating that macrophages internalise neutrophil-derived HBP (16). In 5 of the 14 patients, staining was also detected in the pericellular environments (exemplified in E and F, Fig. 2). In addition, the chronic ulcer tissues displayed typical morphological changes including an excessive inflammatory infiltrate, epidermal acanthosis, dermal capillary proliferation and presence of fibrin cuffs (Fig. 2).

In 9 of the 12 patients from which wound fluid was sampled, bacterial swabs were performed. As indicated in Table 1 and in agreement with previous studies, various bacteria, such as *S. aureus*, *P. aeruginosa*, *E. faecalis* and *P. mirabilis* were identified in the chronic ulcers. In order to study the effects of bacteria on the release of HBP, human blood was subjected to culture supernatants from various chronic ulcer-derived *P. aeruginosa* isolates (Fig 3). In blood, HBP is only found in neutrophils (17), hence the results clearly demonstrated that *P. aeruginosa* culture medium induced a significant release (40-100%) of total HBP present in neutrophils. Common virulence factors of *P. aeruginosa*, exotoxin A, lipopolysaccharide and elastase, did not release HBP. Other bacteria were also tested for...
HBP-releasing activity. Culture supernatants of wound-derived *P. mirabilis* (3 isolates) and *E. faecalis* (1 of 4 isolates) were also able to release HBP. *S. aureus* bacteria (9 strains) isolated from the skin of atopic dermatitis patients (6 strains) or from chronic ulcers (3 strains) exerted no HBP-releasing activity (not shown). Stimulation of purified neutrophils by *P. aeruginosa* supernatants also yielded similar results (not shown).

We have previously demonstrated that proteases of common wound bacteria, such as *P. aeruginosa*, *P. mirabilis* and *E. faecalis*, rapidly degrade LL-37, a significant antimicrobial peptide released by neutrophils and keratinocytes during wound healing (14). Chronic ulcer fluid, but not wound fluid from acute wounds, also had this capacity and these findings corroborate with the fact that LL-37 is lacking in the wound bed of chronically infected leg ulcers (18). In Figure 4A, we demonstrate that HBP, in contrast to LL-37 and lactoferrin, is highly resistant to the action of *P. aeruginosa* elastase, a potent metalloproteinase. Likewise, HBP was not degraded by *E. faecalis* gelatinase or *P. aeruginosa* elastase. In Table 1, we show the bacterial growth in CU and corresponding HBP-levels in wound fluid.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Type of bacteria</th>
<th>HBP concentration µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S. aureus, β-streptococci group G</td>
<td>93.8</td>
</tr>
<tr>
<td>2</td>
<td>S. aureus, <em>E. faecalis</em></td>
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</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>S. aureus, <em>P. aeruginosa</em>, β-streptococci group A</td>
<td>23.6</td>
</tr>
<tr>
<td>5</td>
<td>S. aureus, <em>P. aeruginosa</em>, <em>E. faecalis</em></td>
<td>22.1</td>
</tr>
<tr>
<td>6</td>
<td><em>P. aeruginosa</em></td>
<td>19.2</td>
</tr>
<tr>
<td>7</td>
<td><em>E. faecalis</em>, <em>E. coli</em>, <em>K. oxytoca</em></td>
<td>8.5</td>
</tr>
<tr>
<td>8</td>
<td>S. aureus, <em>E. faecalis</em></td>
<td>7.1</td>
</tr>
<tr>
<td>9</td>
<td><em>E. coli</em>, <em>P. mirabilis</em>, <em>E. cloacae</em></td>
<td>1.6</td>
</tr>
</tbody>
</table>
mirabilis 50 kDa protease, two proteases that rapidly cleave LL-37 (14). In addition, HBP was unaffected by human neutrophil elastase (not shown). These findings indicate that HBP is stable in highly proteolytic environments, an observation consistent with the noted high HBP-concentrations in wound fluid from chronically infected ulcers (Fig. 1). Western blot analysis using polyclonal antibodies against HBP, demonstrated the presence of intact HBP in wound fluids from two patients infected by *P. aeruginosa* (Patients 5 and 6, see Table 1) (Fig. 4B). This excluded the possibility that the HBP ELISA recognized fragments of HBP in the wound fluids. HBP has well-known antimicrobial properties. Therefore, it was intriguing to us that bacteria, such as *P. aeruginosa*, still frequently colonise and infect chronic ulcers. In order to address this, we examined the bactericidal effects of HBP in low-salt buffer, acute wound fluid and human serum (Fig. 4C). The results showed, that HBP efficiently killed *P. aeruginosa* in low-salt conditions. In wound fluid and serum, no antibacterial activity was demonstrated, corre-

Figure 3: *Pseudomonas aeruginosa* conditioned medium release HBP from human blood. Human blood was incubated together with conditioned medium of *P. aeruginosa* cultures. The background release of HBP in PBS and Todd-Hewitt broth is indicated. The bacteria induced 40-100% release relative the HBP levels obtained by complete solubilisation by Triton X100. A representative experiment (of three) is shown.

Figure 4: HBP is stable in proteolytic environments and wound fluid and serum completely abolishes the antimicrobial effect of HBP. (A) 2 µg each of HBP (HBP), LL-37 (LL-37), and lactoferrin (LF) were incubated for 6 hours with *P. aeruginosa* elastase (+) at 37º C. Buffer was used as control (–). The material was analysed by 16.5% SDS-PAGE (Tris-Tricine gels). Both LL-37 and lactoferrin were degraded by *P. aeruginosa* elastase, whereas HBP was unaffected. Molecular weight markers (in kDa) are indicated to the left. (B) Wound fluid from 2 patients with *P. aeruginosa* infection (U1 and 2, patients 5 and 6, respectively) was separated by SDS-PAGE (10%) and HBP visualized by western blotting using polyclonal antibodies against human HBP. Acute wound fluids were used as controls (C1 and 2). Purified recombinant HBP (HBP) is included for comparison and appears with a slightly higher apparent molecular weight because of its different glycosylation profile (leftmost lane). (C) For analysis of the effect of wound fluid on the antimicrobial effect of HBP bacteria were washed and diluted in 10 mM Tris-HCl, pH 7.5, containing 5 mM glucose. Wound fluid or human serum were added to a final concentration of 20% (wound fluid; O, serum; ▲). Buffer was used as control (●). 50 µl bacteria (2 x 10⁶ colony forming units/ml) were incubated with HBP at the indicated concentrations. To quantitate the bactericidal activity, serial dilutions of the incubation mixture were plated on TH agar, incubated at 37º C over night, and the numbers of colony forming units were determined. Wound fluid and serum abolished the antimicrobial effect of HBP.
sponding with previous reports indicating that serum inhibits the antibacterial action of HBP (19). Likewise, chronic ulcer fluid, dextran sulfate, and dermatan sulfate blocked the antibacterial activity of HBP (not shown).

**Discussion**

The earliest stage of normal wound repair involves a transient inflammatory phase characterized by endothelial activation, neutrophil infiltration and excessive protease activity, which is followed by macrophage and keratinocyte migration, matrix synthesis, and angiogenesis. In contrast to acute wounds, chronic venous ulcers fail to follow this timely pattern of events but persist in a chronic inflammatory state with neutrophil numbers and enzyme activities similar to those detected during the first phase of acute wound healing (2, 20). Endothelial activation represents a crucial step in the initiation as well as perpetuation of inflammation in chronic skin ulcers, and hence, precedes the secretion of leukocyte-derived proteinases and ultimately, high tissue degradation (2, 5). Therefore, it is worth noting that among the various substances released by activated neutrophils, HBP is mainly responsible for endothelial activation and induction of vascular leakage (10, 21). Thus, the demonstration of significantly increased HBP levels in wound fluid from chronic ulcers suggests that high HBP levels, leading to endothelial hyperpermeability and neutrophil recruitment (10) may represent an early pathogenic step during the development of ulcers. The finding that *P. aeruginosa* released significant amounts of HBP from neutrophils ex vivo suggests that bacteria may contribute to the significantly increased HBP levels detected in chronic ulcer fluid. Interestingly, *S. aureus* products did not release HBP. Thus, our results could explain the clinical observation that classic inflammatory signs such as high exudation, swelling, and erythema often accompany *P. aeruginosa* infected wounds. There was no clear correspondence between bacteria isolated from the ulcers and HBP levels (Table 1). However, it must be pointed out that we did not account for several factors, such as presence of bacteria deeper down in the connective tissues, presence of anaerobic bacteria (22), bacterial numbers, or the exudation rate of the ulcer.

HBP can be added to the growing list of proinflammatory factors that appear to be significantly increased in chronic ulcerations (2). Intriguingly, although intracellular HBP was detected in all chronic ulcer tissues, extracellular HBP was detected in 5 of the 14 biopsies. Several explanations underlying this apparent variability are possible. First, biopsies were only taken from the superficial parts of the wounds whereas it is plausible that HBP is generated and released from neutrophils (17) or endothelial cells (23) deeper down in the wound bed. Second, the fixation and staining procedures could lead to depletion of extracellular HBP. Finally, extracellular HBP could be rapidly internalized by macrophages (16). The finding that HBP was not antimicrobial in the presence of wound fluid, underscores the role of HBP as a proinflammatory molecule. Interestingly, recent data indicate that many peptides and proteins, initially ascribed antimicrobial functions *per se*, have been found to exert multifunctional and diverse roles, including chemotaxis and angiogenesis (24).

From a clinical perspective, modulation of protease activity represents one therapeutic option in chronic ulcers (25-27). Inhibition of HBP-release or action could represent an interesting alternative. Therefore, it is interesting to note that inhibitors of HBP-function, such as use of blocking antibodies, negatively charged polysaccharides (such as dextran sulphate), or aprotinin, indeed attenuates neutrophil-evoked increases on endothelial hyperpermeability (10). Interestingly, pulmonary leakage of plasma and blood cells due to *Streptococcus pyogenes* infection could be prevented by interference with neutrophil activation and release of HBP (21).

In conclusion, we have identified significantly increased levels of HBP, a potent proinflammatory factor, in chronic ulcer fluids. HBP was released from human neutrophils subjected to secreted products of *P. aeruginosa*. These data suggest a novel mechanism by which *P. aeruginosa* may aggravate inflammation in chronic leg ulcers and our findings could be helpful in the development of novel therapeutic strategies.

**Acknowledgements**

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**Abbreviation**

HBP, Heparin binding protein

**References**