INVESTIGATIVE REPORT

Differential Proteinase Expression by *Pseudomonas aeruginosa* Derived from Chronic Leg Ulcers

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Pseudomonas aeruginosa **colonizes 20–30% of all venous leg ulcers. Hypothetically,** *P. aeruginosa* **could release proteases and cytotoxic substances in the environment of chronic ulcers, thus negatively affecting the wound-healing activity in this patient group. Here we show that** *P. aeruginosa* **isolates from leg ulcers exhibit a highly variable expression of the proteinases elastase and alkaline proteinase. We propose that bacterial phenotype should be taken into account in future studies on the clinical outcome of leg ulcers colonized by** *P. aeruginosa.* **Key words: bacteria; virulence factor; wound healing.**

Skin wound healing requires the action of various growth factors and proteases in the substitution of injured skin with new tissue. Proteolytic degradation of the extracellular matrix is indispensable for the normal wound-healing process (1). Venous leg ulcers represent a condition characterized by uncontrolled proteolysis, as indicated by increased levels of neutrophil elastase and gelatinase (2–7). Furthermore, protease inhibitors such as α_2-macroglobulin, α_1-antitrypsin and inter-α-inhibitor appear to be degraded in chronic wounds (3, 6–8), and hence do not counterbalance proteolytic enzymes, resulting in increased degradation of matrix components (7).

P. aeruginosa is found in 20–30% of chronic venous ulcers (9, 10). It is known that virulence factors of *P. aeruginosa*, such as elastase (11, 12), alkaline proteinase (13), protease IV (14) and exotoxins (A, S, T, U and Y) (15) mediate tissue damage during *P. aeruginosa* infections in various sites of the body. For example, in patients with burns and cystic fibrosis, the pathogen causes severe morbidity and mortality (16, 17). Among critically ill patients, nosocomial pneumonia due to *P. aeruginosa* is a leading cause of mortality (18). *P. aeruginosa* keratitis may lead to irreversible damage to the cornea (19).

Hypothetically, bacteria such as *P. aeruginosa* could release proteases and cytotoxic substances in the environment of leg ulcers, thus further compromising the wound-healing activity in this patient group. Studies aimed at linking different microbes to delayed wound healing have been inconclusive, some indicating a role (9, 20–22), others not (10, 23). Interestingly, *P. aeruginosa* was associated with large wounds (9, 10) and delayed healing (20, 21).

The variation in clinical outcome in the above studies could be related to variations of virulence factors of given bacteria,

such as *P. aeruginosa*. Whether such a variation exists or not is currently unknown. To address this question, we determined the presence and expression of genes coding for the two *P. aeruginosa* proteinases elastase and alkaline proteinase in *P. aeruginosa* isolates derived from patients with chronic leg ulcers.

MATERIALS AND METHODS

Materials

Taq polymerase buffer and Taq platinum polymerase were products from Life Technologies. Todd-Hewitt medium was purchased from Difco and dNTPs from Boehringer Mannheim. Oligonucleotides were synthesized by Innovagen AB, Lund. Azocasein, glycerol and coomassie blue were products from Sigma. Tris hydrochloride was from ICN Biomedicals. Acrylamide/bisacrylamide solution (30/0.8) was purchased from Scotlab. Other chemicals and reagents, as well as some of the equipment, have been described previously (6).

Patients, isolation of bacteria and preparation of P. aeruginosa conditioned medium

Bacterial cultures were obtained from 77 patients with chronic ulcers. *P. aeruginosa* growth was detected in ulcers of 17 patients (22%). The mean age (of the latter group) was 77 years, mean ulcer duration 27 months, mean ulcer size; 85 cm². Fifteen patients had ulcers of venous origin (ankle-brachial index >0.8), 2 patients had venous and arterial insufficiency, as assessed by routine clinical examination. In patient nos. 10 to 17, repeated bacterial cultures were obtained during subsequent visits. The patients were not treated with antibiotics prior to and during the study. Bacteria were isolated from bacterial sampling discs as previously described (10). For preparation of *P. aeruginosa* conditioned medium, Todd-Hewitt medium was inoculated and bacteria were grown overnight to stationary phase. The bacteria were centrifuged, and the supernatant was sterile-filtered and stored at −70°C.

PCR analysis of P. aeruginosa proteinase genes

Genomic DNA was prepared from the isolates (24). Oligonucleotides, PCR conditions, as well as restriction site analysis or sequencing of the DNA coding for *P. aeruginosa* elastase and alkaline proteinase, are described elsewhere (Schmidtchen, unpublished). For detection of the genes coding for these *P. aeruginosa* proteinases, we used the primer pairs 5′-TGGGAAGGCCGGATCCACGCCGAGGCG, 5′-CAGCCGGGACCGAATTCCTTACAACGCGCT, yielding a product of 942 bp (elastase gene) and 5′-GTCGACCAGGCGG-CGGAGCAGATA, 5′- GCCGAGGCCGCCGTAGAGGATGTC, yielding a 993 bp PCR-product (alkaline proteinase gene). Analysis of the resulting PCR products was performed on 1% agarose gels and the DNA visualized by staining with ethidium bromide.

Zymography

P. aeruginosa extracellular products (5–10 μl of conditioned medium) were mixed with sample buffer (0.4 M Tris HCl, 20% glycerol, 5% sodium dodecyl sulphate (SDS), 0.03% bromophenol blue, pH 6.8)

and electrophoresed on 10% polyacrylamide gels (1 mg bovine gelatine per ml gel). To remove SDS, gels were incubated with 2.5% Triton X-100. Incubation was then performed for 18 h at 37°C in buffer containing 50 mM Tris HCl, 200 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 7.5. Gels were stained with Coomassie blue G-250 in 30% methanol, 10% acetic acid for 1 h and de-stained in the same solution without the dye. Gelatinase-containing bands were visualized as clear bands against a dark background.

Protease activity assay

Total protease activity was determined by the azocasein method essentially as described (25). Conditioned media from the *P. aeruginosa* cultures (30 μl) were added to 50 μl azocasein substrate (2% azocasein in 10 mM Tris HCl, 8 mM $CaCl_2$, pH 7.4). The reaction mixture was incubated for either 3 h or 20 h. Thereafter, 240 μl 10% trichloroacetic acid was added and the samples mixed and allowed to stand for 15 min to ensure complete precipitation of undigested material. Tubes were centrifuged at 10,000 rpm (microfuge) for 10 min and 240 μl of the supernatant was transferred to tubes containing 280 μl 1.0 M NaOH. The absorbance at 440 nm was determined. One unit of enzyme activity was defined as the absorbance at maximal digestion of 1 mg azocasein (26).

RESULTS

Amplification of DNA coding for P. aeruginosa elastase and alkaline proteinase in bacterial isolates

Primer pairs amplifying parts of the DNA coding for *P. aeruginosa* elastase and alkaline proteinase (for primer sequences, see Materials and Methods), respectively, all yielded the expected fragment sizes when used in the amplification of DNA from the 26 *P. aeruginosa* isolates, as determined by agarose electrophoresis (data not shown). This experiment proved the existence of both proteinase genes in all the isolates.

Zymographic analysis of P. aeruginosa proteases

Gelatinase activity in conditioned media from *P. aeruginosa* overnight cultures (stationary phase) was determined. The total bacterial count in the cultures usually varied between 0.2 and 0.4×10^9/ml; 5–10 μl of conditioned media (corresponding to 2.4×10^6 bacteria) was applied on the zymograms. The major enzymes detected had molecular weights of ~150 kDa and 50 kDa, and have previously been identified as elastase and alkaline proteinase, respectively (Schmidtchen, unpublished). The ~150 kDa enzyme was identified by immunoblotting and aminoterminal sequencing as a multimeric form of *P. aeruginosa* elastase (33 kDa). Furthermore, the experiments showed that the enzyme co-migrated on zymograms with purified elastase. The 50 kDa enzyme co-migrated perfectly with purified alkaline proteinase (50 kDa), and was recognized by antibodies against alkaline proteinase (Schmidtchen, unpublished). Some isolates, such as those from patient nos. 1, 5 and 15, contained high amounts of *P. aeruginosa* elastase. Others, from patient nos. 2, 3, 4, 6, 9, 12 (first culture) as well as 16 and 17, displayed little or no elastase activity. The *P. aeruginosa* isolate from patient no. 14 contained an as yet unidentified protease of molecular weight ~100 kDa. *P. aeruginosa* strains isolated repeatedly (2–3 times) from the same patient displayed similar protease patterns (nos. 10, 11, 13, 14, 15, 16 and 17). Interestingly, the ~100 kDa protease was detected in both isolates from the same patient (Fig. 1). However, the activity of the second isolate was significantly lower (Fig. 2). In one patient, no. 12, elastase was only expressed in the second isolate (Fig. 1).
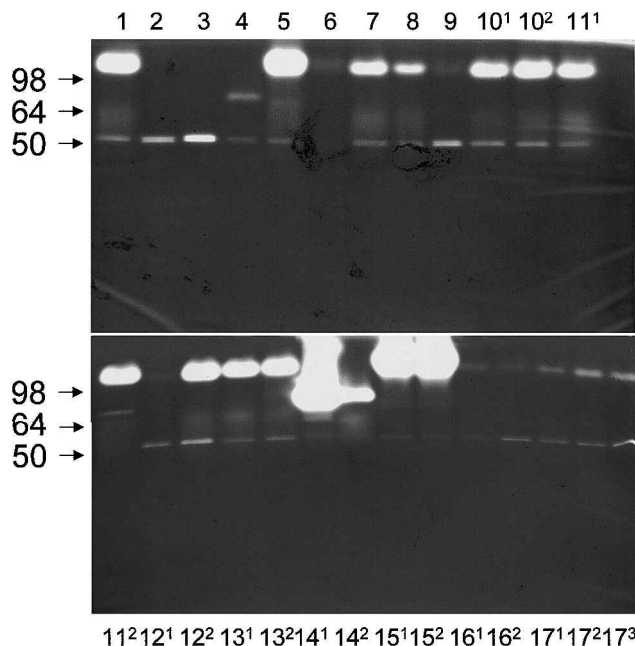

Fig. 1. Zymographic detection of *Pseudomonas aeruginosa* proteases. *P. aeruginosa* cultures were grown to stationary phase over-night. The sterile-filtered conditioned medium was then analysed for gelatinolytic activity by zymography (10% polyacrylamide gels with 1 mg/ml gelatin). Each lane was loaded with a volume of conditioned bacterial medium corresponding to 2.4×10^6 bacteria. Molecular weight standards are indicated to the left. Patient isolates are indicated at the top and bottom.
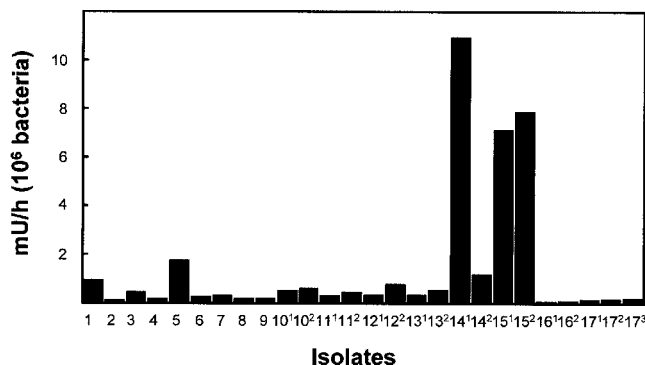

Fig. 2. Caseinolytic activity of *Pseudomonas aeruginosa* extracellular products. Total caseinolytic activity was measured with the use of azocasein (2% solution). Thirty microlitres of conditioned medium from *P. aeruginosa* cultures was incubated with the substrate (1 mg azocasein) for a time period of 3 h (for samples with high proteolytic activity) or 20 h (for samples with low proteolytic activity). Absorbance values up to 1.5 were accepted for the calculations. The activity in units/10^6 bacteria/hour is indicated on the y-axis.

These experiments were repeated 2–3 times (2 times for isolates from patients 14, 15 and 17, otherwise 3 times) using inocula from the bacterial stocks (stored at −70°C). No significant difference in the zymogram expression patterns was noted.

Caseinolytic activity of P. aeruginosa isolates

P. aeruginosa elastase and alkaline proteinase degrade azocasein (25). Protease activity was determined for each isolate.

One unit was defined as the activity, which hydrolyzes 1 mg of azocasein/h (26). The protease activity was expressed as units/10^6 bacteria/hour. As shown in Fig. 2, the protease activities varied significantly between the different isolates. Isolates from patients 14 (first isolate) and 15 (both isolates) displayed the highest activity; other isolates contained variable but clearly detectable activity (nos. 1, 5, 10, 11, 12, 13), whereas some displayed very little proteolytic activity (nos. 2, 4, 8, 9, 16, 17). In general, there was a reasonably good correspondence between the zymograms and the azocasein assay.

DISCUSSION

P. aeruginosa has been implicated in various human diseases, and may cause keratitis, pneumonias and burn wound infections (16–19). *P. aeruginosa* proteases induce degradation of complement, kininogens, cytokines, growth factors, immunoglobulins and various extracellular matrix components (see 27 for review). In this study, we show that *P. aeruginosa* isolates from chronic ulcers differ in the expression of two virulence factors, the proteinases elastase and alkaline proteinase. Our data on *P. aeruginosa* protease expression corroborate previous studies. In strains from other locations, similar differences in virulence factor expression have been described. For example, *P. aeruginosa* elastase, exotoxin A and S were shown to vary significantly in various clinical isolates (28, 29). Likewise, in *P. aeruginosa* isolates from patients with cystic fibrosis, variable expression patterns of elastase and alkaline proteinase were detected (30). Interestingly, we noted that isolates obtained after repeated cultures from the same ulcer displayed similar protease expression patterns. This *in vitro* finding suggests that the level of protease expression may be stable over a period of time in leg ulcers *in vivo*. However, because of the limited number of patients in this study, this matter needs further investigation. The fact that all the isolates contained the genes for elastase and alkaline proteinase confirms their roles as important *P. aeruginosa* virulence factors. The protease expression changes could be related to epigenetic mechanisms, such as transcriptional control, or due to rearrangements within the structural genes, or their corresponding upstream regions.

A correlation between severity of infection and *P. aeruginosa* protease levels has been observed (31, 32). In this study, however, no correlation between clinical severity (ulcer size and duration) and protease expression was noted (not shown). It must be stressed, though, that variables such as the total time period of *P. aeruginosa* colonization (before and after isolation), changes in ulcer treatments, as well as protease production by other bacteria were unaccounted for here.

In conclusion, we have reported that the significant pathogen *P. aeruginosa* derived from chronic ulcers exhibits a highly variable protease expression. This finding raises the interesting possibility that phenotypic variation of various virulence factors, such as proteases, may occur in various bacteria from chronic ulcers. Indeed, preliminary results indicate that bacteria such as *Proteus mirabilis* (Schmidtchen A, unpublished results) and *Peptostreptococcus magnus* (Andersson M-L, pers. comm.) exhibit similar variations in the production of proteases. We therefore propose that future clinical studies on ulcer healing delay should include analysis of bacterial proteases and other virulence factors, such as production of exotoxins and biofilms (33). Since analyses of bacterial isolates

may not fully reflect the true *in vivo* situation, future studies should also include the determination of virulence factors *in situ*.

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