Pseudomonas aeruginosa Protease IV Produces Corneal Damage and Contributes to Bacterial Virulence

Lee S. Engel,1 James M. Hill,1,2 Judy M. Moreau,1 Linda C. Green,1,2 Jeffery A. Hobden,2* and Richard J. O’Callaghan1,2

PURPOSE. A Pseudomonas mutant deficient in protease IV has significantly reduced virulence in experimental keratitis. In the present study, the corneal toxicity of purified protease IV and its ability to augment the virulence of protease-IV-deficient bacteria were analyzed.

METHODS. The toxicity of purified protease IV was determined by intrastromally injecting the exoenzyme (20–200 ng) into the cornea. The effects of protease IV on the corneal virulence of the protease-IV-deficient strain, PA103-29::Tn9, were determined by injecting eyes with 1000 CFU of log phase bacteria plus either 200 ng active purified protease IV or 200 ng heat-inactivated protease IV. Changes in ocular disease, determined by slit-lamp examination, were measured at 3, 16, 22, and 27 hours after infection. Colony-forming units per cornea were quantified at 27 hours after infection.

RESULTS. Purified protease IV at doses from 50 to 200 ng induced epithelial defects within 3 hours of injection. Injection of 20 ng active protease IV or heat-inactivated protease IV (200 ng) had no effect on ocular tissue. Corneal virulence of the protease-IV-deficient strain was augmented by intrastromal injection with purified protease IV but not with heat-inactivated protease IV (P ≤ 0.0001). Neither active nor heat-inactivated protease IV altered the growth of bacteria in the cornea (6 log units; P = 0.81).

CONCLUSIONS. The important role of protease IV in corneal virulence was demonstrated by direct toxicity and by its ability to significantly augment the virulence of protease-IV-deficient Pseudomonas. (Invest Ophthalmol Vis Sci. 1998;39:662–665)

From the 1Department of Microbiology, Immunology, and Parasitology, and the 2Department of Ophthalmology, LSU Eye Center, Louisiana State University Medical Center School of Medicine, New Orleans.

*Present address: The Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI 48202.

Supported in part by United States Public Health Service grants EY08871, EY10974, and EY02377 from the National Eye Institute; an unrestricted grant from Research to Prevent Blindness, New York, New York; and a student research grant from the Greater New Orleans Cancer Association.

Submitted for publication July 21, 1997; revised October 8, 1997; accepted October 28, 1997.

Proprietary interest category: N.

Reprint requests: Richard J. O’Callaghan, Department of Microbiology, Immunology, and Parasitology, LSU Medical Center, 1901 Perdido Street, New Orleans, LA, 70112-1393.

P. aeruginosa, one of the most destructive of all the opportunistic pathogens, is a major cause of bacterial keratitis. P. aeruginosa keratitis spreads rapidly and is very damaging to corneal tissues.1–6 During keratitis, host and bacterial factors mediate damage to the cornea. Even with the most aggressive antibiotic therapy, P. aeruginosa keratitis can progress to corneal perforation. The extent of visual impairment relates proportionally to inflammatory cell infiltration, primarily polymorphonuclear leukocytes; activation of host proteolytic enzymes; corneal cell death; damage to endothelial cells; and corneal scarring.1

The pathologic potential of Pseudomonas appears to correlate with protease production. Twining et al.5 have shown that alkaline protease is an important corneal virulence factor. We have shown that the virulence of strain PA103-29 correlates with the activity of protease IV.4 We have also demonstrated that a protease-IV-deficient strain, PA103-29::Tn9, lacks corneal virulence in both a rabbit model and a mouse model of keratitis.3 Furthermore, we have determined that protease IV is a serine protease.6

The results of the present study demonstrated that nanogram quantities of purified protease IV were directly toxic to the cornea, mediating rapid destruction of the corneal epithelium and inflammation. The action of purified protease IV in these very low quantities was found to augment the corneal virulence of the protease-IV-deficient strain.

MATERIALS AND METHODS

Bacterial Strains

PA103-29 produces protease IV in tryptic soy broth (Difco, Detroit, MI) and is deficient in exotoxin A, elastase (lasB), and alkaline protease.3,4,6 PA103-29 was subjected to transposon mutagenesis, yielding a mutant deficient in protease IV (PA103-29::Tn9).3,4

Purification of Protease IV

Protease IV was purified to homogeneity from concentrated culture supernatants of strain PA103-29 by cation exchange and molecular-sieve column chromatography as previously described.6 Purified protease IV had a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a single peak by mass spectroscopy.

In Vivo Action of Purified Protease IV on Corneas

Corneas were intrastromally injected with active or heat-inactivated protease IV diluted in sterile tryptic soy broth at 20, 50, 100, or 200 ng per eye. Injection volumes were 20 μl. Protease IV was heat-inactivated at 60°C for 40 minutes, and the inactivation was quantified using a chromogenic substrate (Chromozym PL; Boehringer Mannheim Biochemicals, Indianapolis, IN).3,4

Rabbit Intrastromal Model

New Zealand White rabbits used in this investigation were treated and cared for in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rabbits (2.0–3.0 kg) were anesthetized as previously described.1,3,4 Corneas were intrastromally injected with 20 μl purified enzyme preparation, 1000 CFU logarithmic phase P. aeruginosa mixed with enzyme,
FIGURE 1. Effect of purified protease IV on the rabbit cornea. Active protease IV (200 ng) was intrastromally injected into rabbit corneas. (A) By 3 hours after injection, epithelial erosion was evident over the center of the cornea. (B) By 27 hours after injection, all epithelial damage was healed.

RESULTS

In Vivo Action of Protease IV

Protease IV (200 ng) injected intrastromally into the rabbit cornea produced a complete corneal epithelial erosion over the site of injection by 3 hours after injection (Fig. 1A). SLE scores of eyes injected with active protease IV were significantly higher than SLE scores of eyes injected with heat-inactivated protease IV at 3, 16, and 22 hours after injection ($P \leq 0.040$; Fig. 2). This damage was resolved by 27 hours after injection (Fig. 1B). Quantities of protease IV as low as 50 ng produced epithelial damage. Across a range of protease IV concentrations (20-200 ng), the amount of damage was directly proportional to the dose of protease IV injected (Table 1). Neither the injection of 20 ng active protease IV nor the injection of heat-inactivated protease IV (200 ng) resulted in significant ocular changes.

Augmentation of Virulence of PA103-29::Tn9 by Addition of Exogenous Protease IV

PA103-29::Tn9 does not produce protease IV, and an intrastromal injection of 1000 CFU log phase PA103-29::Tn9 into a rabbit cornea results in minimal corneal damage in 27 hours (SLE = 4.26) compared with its parent PA103-29 (SLE = 13.88; $P = 0.001$).

Eyes intrastromally injected with PA103-29::Tn9 plus 200 ng active protease IV were compared by SLE with eyes intrastromally injected with PA103-29::Tn9 plus heat-inactivated protease IV (200 ng), active protease IV alone (200 ng), or heat-inactivated protease IV alone (200 ng). SLE scores for the eyes infected with PA103-29::Tn9 plus active protease IV were higher than the SLE scores for all other groups at 16, 22, and 27 hours after injection ($P < 0.003$; Fig. 2). Figure 3 illustrates the difference at 27 hours after injection between eyes injected

Augmentation of Corneal Virulence of the Protease-IV-Deficient Pseudomonas by Exogenous Enzyme

The effect of purified protease IV on corneal virulence of PA103-29::Tn9 was studied using four groups: group 1, eyes injected with viable bacteria (1000 CFU) plus 200 ng active purified protease IV; group 2, eyes injected with viable bacteria plus 200 ng heat-inactivated protease IV; group 3, eyes injected with 200 ng active protease IV with no bacteria; and group 4, eyes injected with 200 ng heat-inactivated protease IV with no bacteria. Protease IV (200 ng) was mixed with 1000 CFU log phase Pseudomonas to a final volume of 20 μl, and this volume was injected into the cornea.

Evaluation of Ocular Pathogenesis in Rabbits

Inflammation was graded and scored by two or more masked observers using slit-lamp examination (SLE) as previously described. After SLE at 27 hours after injection, the corneas were excised and homogenized and the CFU quantitated and expressed as base 10 logarithm,$^1$ Results were analyzed by using a Statistical Analysis System (SAS, Cary, NC) as previously described.$^4$
TABLE 1. Epithelial Defects in Rabbit Corneas Caused by Pseudomonas Protease IV

<table>
<thead>
<tr>
<th>Condition of Purified Protease IV</th>
<th>Quantity of Protease IV Injected per Cornea* (ng/cornea)</th>
<th>Epithelial Defects (eyes with defects/total eyes)</th>
<th>Epithelial Defect Score† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>200</td>
<td>4/4</td>
<td>56 ± 16</td>
</tr>
<tr>
<td>Active</td>
<td>100</td>
<td>3/4</td>
<td>38 ± 22</td>
</tr>
<tr>
<td>Active</td>
<td>50</td>
<td>3/4</td>
<td>21 ± 11</td>
</tr>
<tr>
<td>Active</td>
<td>20</td>
<td>0/4</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Heat-inactivated†</td>
<td>200</td>
<td>0/4</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

*Purified protease IV in tryptic soy broth (20 μl) was injected intrastromally into rabbit corneas.
†The extent of the epithelial loss or damage was measured in millimeters of diameter and expressed as the percentage of corneal surface involved.
‡Protease IV was heat inactivated by incubating at 60°C for 40 minutes.

with PA103-29::Tn9 plus active protease IV (SLE = 9.38; Fig. 3A) versus eyes injected with the same bacteria and heat-inactivated protease IV (SLE = 2.53; \( P \leq 0.0001 \); Fig. 3B). The eyes injected with active protease IV alone had epithelial defects within 3 hours; these defects were no longer visible at 27 hours after injection. Eyes injected with heat-inactivated protease IV alone showed no significant changes from 0 to 27 hours after injection.

The protease-IV-deficient strain, PA103-29::Tn9, has been described previously as growing in the cornea as efficiently as its protease-IV-producing parent, PA103-29. The growth of the protease-IV-deficient strain, PA103-29::Tn9, in the cornea (CFU per cornea) was unaffected by the presence of protease IV; the CFU per cornea for the strain with active protease IV was equivalent to that of the strain with heat-inactivated protease IV (6.20 ± 0.58 log_{10} CFU versus 6.35 ± 0.57 log_{10} CFU, at 27 hours after injection; \( P = 0.81 \)).

DISCUSSION

We have reported that the production of protease IV correlates with ocular virulence. The mutant deficient in protease IV activity has minimal ocular virulence in both the rabbit intrastromal and the mouse scratch models of infection. In the present study, we report that protease IV was directly toxic to the cornea, causing epithelial erosion. The toxic action of small quantities (50 ng) of protease IV and the ability of this enzyme to significantly augment the virulence of a protease-IV-deficient mutant further established a significant role for this enzyme in keratitis. The toxicity mediated by the injection of protease IV suggested a direct dependency on the specific enzymatic activity of protease IV; heat-inactivated protease IV failed to stimulate ocular changes. The augmentation of virulence of PA103-29::Tn9 mediated by exogenously added protease IV could not be attributed to greater bacterial growth in the presence of active protease IV; inoculation of bacteria with either active or heat-inactivated protease IV resulted in the same number of CFU per cornea. The number of bacteria in corneas with exogenously added protease IV at 27 hours was the same as that reported previously for corneas infected with this mutant but without any additives at 27 hours (6.32 and 6.69 log_{10} CFU, respectively).

The role of protease IV in corneal virulence is probably analogous to that described for alkaline protease. We have shown that protease IV can directly damage the corneal surface. The protease IV restoration of corneal virulence to the protease-IV-deficient strain, PA103-29::Tn9. Rabbit eyes were intrastromally injected with 1000 CFU of strain PA103-29::Tn9 with either 200 ng active protease IV (A) or 200 ng heat-inactivated protease (B). Photographs were taken at 27 hours after infection.
and can stimulate the virulence of protease IV-deficient Pseudomonas. Howe and Iglewski, using a mouse keratitis model, demonstrated that the application of purified alkaline protease at a very high concentration (1.3 mg per eye) augments the virulence of a Pseudomonas strain producing minimal quantities of protease. Gupta et al. have demonstrated that treatment of mouse corneal tissue with alkaline protease (50 ng) increases the binding of Pseudomonas to the epithelial surface.

The significant increase in virulence demonstrated by PA103-29::Trn9, when combined with active protease IV, could be caused by the action of the protease on either the host, the bacteria, or both. Protease IV could activate corneal metalloproteinases or affect host immune responses. In contrast or in addition to this activity, protease IV could act on another bacterial virulence factor, converting that protein to a potent damaging factor. Recent findings of Twining et al. (personal communication) could be significant in understanding the role of protease IV in ocular disease. The protease IV production of numerous Pseudomonas strains was analyzed, and it was found that all ocular isolates produced an exoxygen protease that appeared identical to protease IV. The implications of these findings on the development of new therapies for Pseudomonas keratitis require further study.

References


---

**Evaluation of the Human Arrestin Gene in Patients with Retinitis Pigmentosa and Stationary Night Blindness**

Kimberly C. Sippel, John D. DeStefano, Eliot L. Berson, and Thaddeus P. Dryja

**Purpose.** To establish the DNA sequence of the coding regions of the human arrestin locus and to determine whether defects in this sequence are present among patients with retinitis pigmentosa (RP) or types of stationary night blindness in addition to Oguchi disease.

**Methods.** The human genomic locus encoding arrestin was cloned in bacteriophage and Pl vectors. The sequence of the intron DNA flanking each exon was determined from these clones. Single-strand conformation polymorphism analysis and direct genomic sequencing techniques were used to screen 272 unrelated patients, comprising 177 patients with autosomal dominant RP, 85 with recessive RP, and 10 with stationary night blindness.

**Results.** The arrestin gene is divided into 16 exons ranging in size from 10 bp to 194 bp, with die open reading frame spanning exons 2 through 16. The authors identified several discrepancies between the genomic sequence the authors obtained and the previously published cDNA and genomic sequences. In the set of patients with dominant RP, the authors found one of three heterozygous missense changes (Arg84Cys, Thr125Met, and Val378Ile) in each of four unrelated patients; none of these changes cosegregated with disease in the respective families. In the set of patients with recessive RP, the authors found one of two heterozygous missense changes in each of two unrelated patients with recessive RP (Pro264Leu and Arg284Cys). One of the patients was the offspring of a consanguineous marriage; because the Arg284Cys change in him was heterozygous, it is unlikely to have been the cause of his RP. Cosegregation studies could not be performed on the patient with the Pro264Leu change. The authors confirmed the existence of two previously described polymorphisms (Ile67Val and a multiallelic polymorphism at codon 403), and the authors identified several silent polymorphisms and rare sequence variants. No sequence changes, other than polymorphic changes also found in some patients with RP, were identified in the patients with stationary night blindness.

**Conclusions.** We found no evidence that mutations in arrestin are a cause of RP or stationary night blindness other than Oguchi disease. According to the genomic sequence obtained, a region in exon 8 that has been postulated to represent the site of interaction between arrestin and rhodopsin is 100% conserved between humans and all other mammals studied to date. (Invest Ophthalmol Vis Sci. 1998;39:665–670)