Serine-like proteolytic enzymes correlated with differential pathogenicity in patients with acute *Acanthamoeba* keratitis

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Abstract

Acute ocular infection due to free-living amoebae of the genus *Acanthamoeba* is characterized by severe pain, loss of corneal transparency and, eventually, blindness. Proteolytic enzymes secreted by trophozoites of virulent *Acanthamoeba* strains have an essential role in the mechanisms of pathogenesis, including adhesion, invasion and destruction of the corneal stroma. In this study, we analysed the relationship between the extracellular proteases secreted by clinical isolates of *Acanthamoeba* and the clinical manifestations and severity of disease that they caused. Clinical isolates were obtained from patients who showed typical symptoms of *Acanthamoeba* keratitis. Trophozoites were cultivated axenically, and extracellular proteins were collected from cell culture supernatants. Secreted enzymes were partially characterized by gelatin and collagen zymography. *Acanthamoeba* trophozoites secreted proteases with different molecular masses, proteolysis rates and substrate specificities, mostly serine-like proteases. Different enzymatic patterns of collagenases were observed, varying between single and multiple collagenolytic activities. Low molecular weight serine proteases were secreted by trophozoites associated with worse clinical manifestations. Consequently, proteolytic enzymes of some *Acanthamoeba* trophozoites could be related to the degree of their virulence and clinical manifestations of disease in the human cornea.

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Introduction

The frequency of corneal infections caused by free-living amoebae of the genus *Acanthamoeba* has increased among the Brazilian population [1]. Previous studies have shown a closest relationship between contact lens wearers and *Acanthamoeba* keratitis (AK) cases, and risk factors for disease transmission involved corneal trauma followed by improper or inadequate lens care [1–3]. In the earlier stages of this devastating infection, adherence of trophozoites to the epithelial layer is followed by invasion and destruction of the anterior cornea; the later stages are clinically characterized by ulceration of the corneal epithelium, oedema and necrosis of the stroma [4]. As the parasitic interaction between amoebae and epithelial cells involves the cytopathic effects of pathogenicity factors of *Acanthamoeba* trophozoites, proteolytic enzymes secreted in the corneal surface should play a key role in this process [5,6]. The action of extracellular proteases includes damage to the collagen shield and degradation of glycoproteins, such as fibronectin and laminin, and other proteins, such as plasminogen, fibrinogen, fibrin and haemoglobin [7].

We describe the partial biochemical characterization of extracellular proteolytic enzymes secreted by *Acanthamoeba* trophozoites isolated from infectious sites, and correlate the occurrence of proteases with clinical manifestations. Furthermore, we report the presence of different proteinase activities related to pathogenicity mechanisms of protozoa in the corneal stroma.

Materials and Methods

Patients

The research was approved by the local ethics committee and was conducted in accordance with the tenets of the

Declaration of Helsinki. Informed consent was obtained before sample collection. *Acanthamoeba* strains were isolated from ten different patients diagnosed with infectious keratitis. All patients were contact lens wearers, and their ages varied between 20 and 52 years. Clinical symptoms were contact lens wear intolerance, pain, photophobia and tear production. The severity of the degree of corneal infection by different clinical isolates was categorized into two classes: severe and moderate (previously classified as potentially and rarely sight-threatening infections, respectively) [8].

Culture

Laboratory diagnosis was based on culture of corneal samples on agar examined with an inverted phase contrast microscope at ×200 magnification. Identification of free-living amoebae of the genus *Acanthamoeba* was based on trophozoite and cyst morphology according to the Page [9] criteria. The reference strain *Acanthamoeba castellanii* from the American Type Culture Collection (ATCC 30011) was used as a non-pathogenic control [10]. In order to achieve axenic cultivation of clinical isolates, a piece of agar culture containing amoebae, cysts and trophozoites from each corneal sample was picked up aseptically and transferred to a tissue culture flask containing 5 mL of Neff's [11] broth medium.

Supernatant analysis

Culture supernatants were taken from axenic trophozoites after 72 h of incubation at 25°C. In order to remove cell debris, supernatants were filtered through a sterile 0.22-μm cellulose membrane filter. Samples were extensively dialysed against physiological saline solution (sodium chloride 0.85%) for 72 h at 4°C. Protein contents were concentrated tenfold with Amicon Ultra-15 centrifugal filter devices with a molecular mass cut-off of 5 kDa (Millipore, Bedford, MA, USA). Protein quantification was performed by the method of Bradford [12], and the concentration was adjusted to 200 mg/L. Fifty microlitres of each concentrated protein solution was subjected to precipitation with methanol/chloroform prior to 10% SDS-PAGE under non-reducing conditions [13]. Proteins were stained with silver nitrate as described previously [14]. The apparent molecular masses of the proteins were estimated by comparison with protein molecular mass markers (Low and High Molecular Weight Markers; GE Healthcare Life Science, Piscataway, NJ, USA).

Gelatinolytic activity assays

Zymography assays were performed with 10 μL of each crude protein extract (200 mg/L). Samples were analysed in 7.5% acrylamide gels containing 0.1% gelatin as substrate (SDS-PAGE-gelatin) [15]. In order to ensure the correct size of proteins, prestained molecular mass marker was used as standard (Spectra Multicolor Broad Range Protein Ladder; Fermentas Inc., Hanover, MD, USA). After electrophoresis, the enzymes were renatured by rinsing the gels in 2.5% (v/v) Triton X-100 solution for 1 h in order to remove SDS, and this was followed by incubation at 37°C overnight in Tris-buffered saline (20 mM Tris-HCl, pH 7.5; 150 mM NaCl). Gels were stained with 0.25% Coomassie Brilliant Blue solution in 50% methanol and 10% acetic acid. In this assay, the gelatin proteolysis was detected as colourless bands on the otherwise blue gel.

Protease inhibition assays

In order to study the class of proteolytic enzymes, protein extracts from culture supernatants were mixed with protease inhibitors and incubated for 30 min at 37°C before zymography electrophoresis. The inhibitors used were phenylmethylsulphonyl fluoride (PMSF) (final concentration of 1 mM), an irreversible serine protease inhibitor, and EDTA (final concentration of 10 mM), a chelating metalloprotease inhibitor. Both inhibitors were purchased from Sigma Chemical Co. (St Louis, MO, USA) and were prepared according to the manufacturer's instructions. Zymography analysis in SDS-PAGE-gelatin was performed as described above, and the gels were visually compared with zymogram gels where the samples were not treated with protease inhibitors.

Collagen degradation assays

Type I purified collagen (bovine achilles tendon; Sigma Chemical Co.), at a concentration of 0.1%, was copolymerized with 7.5% SDS-PAGE gels. Zymography assays were performed as described above. Collagenase activity was observed as clear bands on a blue background.

Results

Acanthamoeba cysts and trophozoites were isolated from all corneal tissue samples analysed (Fig. 1). All AK patients were treated with 0.02% topical polyhexamethylene biguanide, a cationic antiseptic agent, combined with 0.1% propamidine, instilled hourly. Different protein patterns were observed with different clinical isolates (Fig. 2). The results shown in Fig. 2 indicate a single prominent protein band with a molecular mass of approximately 30 kDa, secreted by trophozoites of the ATCC 30011 strain (lane 2) as well as by those from patients 01 and 03 (lanes 3 and 5, respectively). Trophozoites from patients 04, 05 and 06 (lanes 6, 7 and 8, respectively) showed similar protein patterns, with molecular mass bands