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RNA interference (RNAi) for the silencing of extracellular serine proteases genes in *Acanthamoeba*: Molecular analysis and effect on pathogenecity

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Abstract

Silencing of extracellular serine protease genes was undertaken by interference RNA (RNAi). Chemically synthesized, small interfering RNA (siRNA) were highly specific and efficient in silencing the catalytic domain of extracellular serine proteases of *Acanthamoeba*. In order to confirm the silencing phenomenon, the extracellular serine protease activities in RNAi-treated parasites were compared to non-treated parasites, using zymography profiles, *Acanthamoeba*-conditioned medium (ACM) protease activity, cytotoxicity assays and extracellular serine protease mRNA levels analysis. Zymography profiles showed a decrease in the extracellular protease levels in the moderate pathogenic and pathogenic strains, after treatment with siRNA. These results were supported after the ACM protease activity and CPE assays were performed in all studied isolates, showing a lower protease activity or cytotoxicity both in the pathogenic and moderate pathogenic strains treated with RNAi. These results support that extracellular serine proteases are directly involved in the pathogenesis and virulence of *Acanthamoeba*.

Keywords: *Acanthamoeba*; RNAi; Pathogenecity; Serine protease

1. Introduction

Acanthamoeba are opportunistic protozoan parasites that pervade in a variety of environments and subsist as one of the most widely distributed organisms [1]. Genus *Acanthamoeba* consist of both pathogenic and non-pathogenic isolates. If the appropriate host conditions and the correct access occurs, pathogenic *Acanthamoeba* can cause life-threatening granulomatous encephalitis (GAE) or a more common eye keratitis frequently associated with contact lens use [1,2].

Since *Acanthamoeba* keratitis was first recognized in 1973, several hundred cases have been reported worldwide. This is due to the increasing population of contact lens wearers and immunocompromised patients, although reported cases in immunocompetent patients and non-contact lens wearers have also increased. The pathologies caused by *Acanthamoeba* are not notifiable diseases and therefore the true incidence among the worldwide population is difficult to ascertain [3–5].

Previous studies have shown that *Acanthamoeba* mediated host cell cytotoxicity requires the adhesion of *Acanthamoeba* to the host cells, the secretion of proteases (mainly serine and cysteine proteases) and phagocytosis. It has been demonstrated that amoebic proteases may be important in tissue invasion, migration and host pathology [6–9].

Extracellular proteases from trophozoites of *Acanthamoeba* induced damage to collagen shields in, in vitro and in vivo, cornea models [10,11]. Nevertheless, the pathogenesis and pathophysiology of *Acanthamoeba* infections remains unclear and understanding the mechanisms of *Acanthamoeba*

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pathogenesis is crucial for the development of therapeutic intervention.

A strategy for reducing RNA expression termed RNA interference (RNAi) has shown great promise in genetic analysis of many organisms [12]. RNAi is an evolutionary conserved phenomenon, based on a common mechanism throughout the eukaryotic kingdom, in which a cycle of RNA degradation is triggered by the transitory presence of a double-stranded RNA (dsRNA). This leads to the sequence-specific destruction of endogenous RNAs that match the dsRNA [13–15]. Since RNAi activity eliminates mRNAs, arising from both alleles or from repetitive genes, it is ideally suited for asexual diploids like most of the amoebae. However, in trypanosomatides, which are diploids with an experimentally difficult sexual cycle, RNAi is a powerful tool for both forward and reverse genetic analysis [16].

Two different approaches are currently used to enhance gene silencing: feeding, in the case of organisms that feed on bacteria producing a dsRNA [17,18] and soaking, which consists of the addition, of chemically synthesized siRNAs, to the cell culture medium that diffuse into the cytoplasm [19,20].

In this study, the use of iRNA mechanisms to inhibit the catalytic domain of extracellular serine proteases of *Acanthamoeba*, which may be involved in the pathogenesis of *Acanthamoeba*, is evaluated using different clinical and environmental isolates.

2. Material and methods

2.1. Culture of Acanthamoeba

Amoeba controls from American Type Culture Collection (ATCC) and Culture Collection of Algae and Protozoa (CCAP) were grown without shaking in 712 PYG medium (ATCC) at 25 °C. Clinical and environmental *Acanthamoeba* isolates previously classified [21,22] (Table 1), were processed as previously described [22,23]. Briefly, isolates were cultured in 2% Neff's saline non-nutrient plates, onto which a layer of heat-killed *Escherichia coli* (JM109) had been spread, at 25 °C. These plates were monitored for out-growth of *Acanthamoeba* microscopically and blocks containing *Acanthamoeba* were removed and the amoebae cloned by dilution. *Acanthamoeba* isolated in this way were then transferred into axenic cultured by placing the amoebae into 712 PYG medium (ATCC).

For the extracellular protease activity, cytotoxicity and zymography assays, *Acanthamoeba* trophozoites (10^7) were incubated at 37 °C in a 5% CO_2 incubator for 24 h. The parasites were then removed from the medium by centrifugation ($100 \times g$ for 5 min), and the supernatant, termed *Acanthamoeba*-conditioned medium (ACM), was used for the assays as previously described [11].

2.2. Gene silencing methodology

siRNA targeting the catalytic domain of extracellular serine proteases of *Acanthamoeba* was synthesized by Ambion Ltd. (Huntingdon, UK), based on the region previously described (Genebank Accession No.: AF221523) [24]. The siRNA duplex with the following sense 5′-CACGGCACUCACGUU and GUGCCGUGAGUGCAA-5′ anti-sense sequences was used.

siRNA was added to *Acanthamoeba* cultures at a density of 2×10^7 parasites ml^{-1} at a concentration of 15 μg ml^{-1}. Cultures were grown for 15 h. As a control, a scrambled sequence siRNA, that was absent from the *Acanthamoeba* spp. genome, was chosen. The scrambled siRNA duplex sequence (5′-CAAGCUGACCCUGAAGUUC for the sense strand and GUUCGACUGGGACUUCAAG-5′ for the antisense strand) was based on the gene encoding green fluorescent protein and was used at 15 μg ml^{-1}.

2.3. Purification of RNA from Acanthamoeba isolates and real time RT-PCR

Acanthamoeba spp. (2×10^7 parasites ml^{-}) RNA was extracted using the RNAqueous©-4PCR kit following the

Table 1
Acanthamoeba strains used in this study

No.	*Acanthamoeba* sp.	Strain	Source	Pathogenecity
1	*A. astronyxis*	ATCC[a] 30137	Soil (USA)	Non-pathogenic
2	*A. castellanii*	ATCC 30010	Soil (USA)	Moderate pathogenic
3	*A. polyphaga*	ATCC 30461	Human cornea (USA)	Moderate pathogenic
4	*A. polyphaga*	CCAP[b] 1501-18	Soil (USA)	Moderate pathogenic
5	*A. polyphaga*	MN-7	Mesenteric node (Spain)	Pathogenic
6	*A. polyphaga*	CLC-8	Contact lens case (Spain)	Pathogenic
7	*A. palestinensis*	CCAP 1501/3c	Fresh water (USA)	Non-pathogenic
8	*A. astronyxis*	TWT-024	Tap water (Spain)	Non-pathogenic
9	*A. polyphaga*	TWT-033	Sea water (Spain)	Non-pathogenic
10	*A. polyphaga*	AK-4	*Acanthamoeba* keratitis (Spain)	Pathogenic
11	*A. divionensis*	ATCC 50238	Type strain clone (UK)	Moderate pathogenic

[a] American Type Culture Collection (ATCC).
[b] Culture Collection of Algae and Protozoa (CCAP).