Cytopathic effect of *Acanthamoeba* on human corneal fibroblasts

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**Purpose:** *Acanthamoeba* keratitis is associated with keratocyte depletion in humans. We investigated how *Acanthamoeba* isolated from corneas affected by *Acanthamoeba* keratitis interacted with human corneal stromal cells in vitro.

**Methods:** *Acanthamoeba* were isolated from 6 patients with *Acanthamoeba* keratitis and genotyping was done. Whether the isolated *Acanthamoeba* could invade the corneal stroma was assessed with denuded corneal stroma ex vivo. The cytopathic effect of *Acanthamoeba* on cultured corneal fibroblasts from donor corneas was quantitatively evaluated by the MTT assay after culture under various conditions. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and Annexin V staining were employed to detect apoptotic cells among the corneal fibroblasts co-cultured with *Acanthamoeba*.  

**Results:** All 6 *Acanthamoeba* isolated from the patients with *Acanthamoeba* keratitis were shown to have the T4 genotype by 18S rDNA sequence analysis. *Acanthamoeba* invaded the denuded corneal stroma in the ex vivo experiments and had a cytopathic effect on human corneal fibroblasts after direct adhesion, but not via chemical mediators. A cytopathic effect was detected with all 6 *Acanthamoeba* and corneal fibroblasts mainly died by apoptosis, as evidenced by Annexin V staining.

**Conclusions:** *Acanthamoeba* isolated from patients with *Acanthamoeba* keratitis had a cytopathic effect on human corneal fibroblasts, mainly via induction of apoptosis after direct adhesion. Our findings may provide some clues to the pathophysiology of corneal keratocyte depletion in patients with *Acanthamoeba* keratitis.

*Acanthamoeba* are free-living cyst-forming protozoans that can cause painful keratitis with the potential loss of vision [1,2]. *Acanthamoeba* keratitis (AK) is closely associated with use of contact lenses, but can also occur in non-contact lens wearers after corneal trauma or exposure to contaminated water [3-5]. *Acanthamoeba* are classified into 15 genotypes (T1-T15), among which the T4 genotype has been identified as the major cause of AK [6,7].

The human corneal epithelium contains immunoprotective dendritic cells as the first line of defense against corneal infection [8]. Creation of corneal epithelial damage before the application of *Acanthamoeba*-infected contact lenses is essential for the development of AK in experimental models [9,10]. Because AK is rare in comparison with the very large number of contact lens wearers, the occurrence of corneal epithelial damage seems to be a precondition leading to AK. Therefore, the interaction between *Acanthamoeba* and keratocytes could provide clues to the mechanism underlying the development of AK. *Acanthamoeba* has a cytopathic effect on various cells [11-16], and keratocyte depletion by invading trophozoites has been detected by histological examination of human corneas with AK [17], but it is still unknown whether *Acanthamoeba* has a direct or indirect effect on human corneal fibroblasts.

In the present study, we isolated *Acanthamoeba* from the corneas of patients with AK and obtained activated keratocytes (i.e., corneal fibroblasts) from human donor corneas. Then we investigated whether *Acanthamoeba* had any effect on cultured corneal fibroblasts. Our findings suggested that *Acanthamoeba* has a direct adverse influence on the survival of corneal fibroblasts.

**METHODS**

*Isolation of Acanthamoeba:* *Acanthamoeba* isolates were obtained from the corneal scrapings of 6 patients with AK at the Medical Center East of Tokyo Women’s Medical University. The isolates were grown on non-nutrient agar plates with heat-killed *Escherichia coli* as a source of nutrients and were designated as follows: E44, E46, E51, E52, E57, and E58. This research was done according to the tenets of the Declaration of Helsinki and was approved by the institutional review board of Tokyo Women’s Medical University. Written informed consent was obtained from the patients with AK.
Isolation and culture of human corneal fibroblasts: Human corneas for research were obtained from the Northwest Lions Eye Bank (Seattle, WA) and the tissue outside the sclerocorneal button was removed. The endothelial layer of the cornea with Descemet’s membrane was removed as a sheet, and then the corneal epithelium was removed mechanically. Denuded corneas were treated with collagenase (2 mg/ml in low glucose D-MEM; Wako, Osaka, Japan) at 37 °C until a single cell suspension of corneal keratocytes was obtained. Then human corneal fibroblasts were cultured from the corneal keratocytes in D-MEM with 10% fetal bovine serum (FBS) at 37 °C under 5% CO2 in 6-cm diameter dishes (Thermo Fisher Scientific, Roskilde, Denmark), glass-bottomed culture dishes (MatTek Corporation, Ashland, MA), and 24-well plates (Thermo Fisher Scientific). Cells were used for the present experiments after two to four passages.

Isolation and sequencing of Acanthamoeba DNA: Acanthamoeba were grown in PYGC medium (10 g proteose peptone, 10 g yeast extract, 1 g glucose, 5 g NaCl and 1 g L-Cysteine in 1,000 ml of 5 mM phosphate buffer pH 7.0) [18] at 30 °C for 3–4 days in 25 cm2 culture flasks, and then were harvested and washed with phosphate-buffered saline (PBS). DNA was extracted using the QIAmp DNA mini kit® (Qiagen, Valencia, CA) according to the manufacturer’s instructions. To identify each Acanthamoeba strain, a fragment of the 18S rDNA gene was amplified using two Acanthamoeba-specific primers (IDP1 and JDP2) [19,20]. A 25 µl reaction mixture including 1 µl of extracted DNA was prepared and PCR was performed with a thermal cycler (GeneAmp PCR System; Applied Biosystems, Carlsbad, CA) using 45 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 30 s.

The amplified fragments of 18S rDNA were visualized by 1.5% agarose gel electrophoresis with ethidium bromide staining and compared to DNA size markers. Amplicons were collected and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was done to detect DNA fragmentation in apoptotic cells using an In situ Apoptosis Detection Kit (TaKaRa, Shiga, Japan). Corneal fibroblasts cultured without Acanthamoeba were used as the negative control, while corneal fibroblasts in dishes with Actinomysin D, Streptomyces sp. (MERCK, Darmstadt, Germany) were the positive control.

Ex vivo invasion of Acanthamoeba into corneal stroma: Denuded corneas without epithelial or endothelial cells were placed endothelial side up in the wells of a 24-well plate containing D-MEM. Acanthamoeba (1×104; >95% trophozoites) were added to the center of each cornea and incubated at 37 °C under serum-free conditions. Two days later, the corneas were fixed in 10% formalin and stained with hematoxylin and eosin (HE) solution for light microscopy. Besides, Acanthamoeba were checked not to transform into cyst form in 37 °C condition.

Cytopathic effect of Acanthamoeba on corneal fibroblasts: After human corneal fibroblasts were grown to form confluent monolayers, the culture dishes were washed three times with PBS to remove the medium containing FBS and then serum-free D-MEM was added. Next, Acanthamoeba (1×104 E44, >95% trophozoites) were carefully added to the center of each 6-cm diameter dish and incubated at 37 °C for 2 days.

To test whether the cytopathic effect of Acanthamoeba was due to direct adherence to corneal fibroblasts or to soluble factors such as chemical mediators, we used insert culture dishes with 0.4 µm pores (Transwell; Corning Life Sciences, Corning, NY), and corneal fibroblasts were cultured in the outer plate. Acanthamoeba could not pass through these pores under any culture conditions (data not shown). We used 6-well plates with insert culture dishes to investigate the morphological change of corneal fibroblasts. We prepared four times as many Acanthamoeba (4×106) as 24-well plates (1×105) based on our experiments, because the well size of 6-well plate is about four times as much as one of 24-well plate. The number of 1×104 Acanthamoeba used in 24-well plate was the highest number used in our experiments. Corneal fibroblasts were harvested by using 0.05% trypsin. The optical density of each well was read using a Cell Proliferation Kit I (MTT assay; Roche Molecular Biochemicals, Mannheim, Germany) at 655 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA) to quantify viable cells.

Next, Acanthamoeba (1.3×107 to 1×109) were added to 4 wells of a 24-well plate and incubated at 37 °C for 2 days. Dishes without Acanthamoeba were used as the negative control. After incubation, corneal fibroblasts were harvested by using 0.05% trypsin and the optical density was determined with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. This assay was done in the 6 different isolates of Acanthamoeba using quadruplicate samples (1×104 of E44, E46, E51, E52, E57, and E58).

TUNEL assay: Corneal fibroblasts were cultured in 5 ml flasks (Thermo Fisher Scientific) until confluence and 1×104 Acanthamoeba (E44) were added. After 4 days, cells were collected and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was done to detect DNA fragmentation in apoptotic cells using an in situ Apoptosis Detection Kit (TaKaRa, Shiga, Japan). Corneal fibroblasts cultured without Acanthamoeba were used as the negative control, while corneal fibroblasts in dishes with Actinomysin D, Streptomyces sp. (MERCK, Darmstadt, Germany) were the positive control.

Quantification of apoptotic cells: Acanthamoeba (1×104) were added to human corneal fibroblasts and cultured at 37 °C for up to 5 days under serum-free conditions. On days 0, 1, 2, 3, 4, and 5 of incubation, the corneal fibroblasts were harvested using 0.05% trypsin-EDTA (Invitrogen, Tokyo, Japan) and were centrifuged at 200× g for 5 min at 4 °C. After removal of the supernatant, cells were labeled with Annexin V-FITC and propidium iodide (PI) by using an Annexin V-FITC apoptosis detection kit (Beckman Coulter, Brea, CA).
according to the manufacturer’s instructions. Fluorescence from FITC and PI was detected under a fluorescence microscope (Axioplan 2 Imaging; Micro-optik, Deursen, Netherlands) at 518 nm and 620 nm, respectively. FITC/Annexin V-positive cells were counted in ten fields because these cells were regarded as being in the initial stage of apoptosis. FITC/Annexin V-negative and PI-negative (unstained) cells were defined as viable cells. While FITC/Annexin V-positive cells showing green fluorescence were regarded as early apoptotic cells, FITC/Annexin-positive and PI-positive cells showing red and green fluorescence were regarded as necrotic cells, because PI-positive cells include both necrotic cells and cells that have gone through apoptosis. The percentage of apoptotic cells among total cells was calculated.

Statistical analysis: Statistical comparisons between two groups were performed by the Mann–Whitney U-test. For multiple comparisons among groups, one-way ANOVA was used and then a post-hoc least significant difference test was performed. Statistical significance was set at p<0.05.

RESULTS

Genotyping of Acanthamoebae isolates: The isolates were termed E44, E46, E51, E52, E57, and E58. Based on 18S rDNA sequence analysis, all of these Acanthamoebae isolates belonged to the T4 genotype, but their sequences varied. Table 1 summarizes the results of genotyping and the BLAST search findings. The sequences of the isolates showed 98~100% correspondence with those of clinical isolates reported previously.

We summarized the results of each isolates. E44, 46, 51, 52, 57, and 58. Those isolates were analyzed based on 18S rDNA and compared with isolates reported previously. “Strain” shows isolates that had high homology (“Homology”) with isolates examined in our hospital. “Tissue source” shows the organ where “Strain” isolates were obtained. “Genotype” shows each isolates’ genotype in 18S rDNA classification. All isolates were T4 genotype. F; female, M; male, CDC; Centers for Disease Control and prevention, ATCC; American Type Culture Collection.

| Isolate | Sex | Age (y) | Strain [GeneBank] | Tissue source | Homology | Genotype |
|---------|-----|---------|------------------|---------------|----------|----------|
| E44     | F   | 35      | ATCC 50497 [U07410] | cornea        | 100%     | T4       |
| E46     | M   | 28      | ATCC 30461 [AY026243] | cornea        | 99%      | T4       |
| E51     | F   | 34      | AC 29 [AB554228]   | cornea        | 99%      | T4       |
| E52     | M   | 59      | CDC V390 [AY703004] | cornea        | 99%      | T4       |
| E57     | F   | 47      | CDC V062 [AY702989] | cornea        | 100%     | T4       |
| E58     | M   | 17      | CDC V029 [U07402]   | cornea        | 98%      | T4       |

Effect of Acanthamoeba on denuded human corneal stroma: We used the E44 isolate throughout our experiments because it was the first Acanthamoeba isolated at our hospital that had the typical morphological and proliferative features of Acanthamoebae. To test whether E44 could inva

Figure 1. Ex vivo invasion of Acanthamoeba into corneal stroma. Acanthamoebae were added to denuded human corneal stroma. Acanthamoebae were placed on the denuded corneal stroma (endothelial side up) and incubated at 25 °C for 2 days. Hematoxylin and eosin staining shows Acanthamoebae (arrowheads) located in fine collagen fibrils. Arrow shows the direction of corneal epithelium. Scale bar=10 μm.
Acanthamoebae in the inside the corneal stroma (Figure 1), indicating that the E44 isolate of Acanthamoeba could attach to the corneal surface and invade the corneal stroma through fine collagen fibrils. Similar findings were detected in different three donor corneas.

Cytopathic effect on corneal fibroblasts: To investigate the effect of Acanthamoeba on corneal fibroblasts, isolates were added to the center of 6-cm dishes containing human corneal fibroblasts and were stained with Giemsa solution after culture. In this experiment, Acanthamoebae were placed carefully on the center of the dish not to spread to the periphery. It was found that fibroblasts had disappeared from the center of the dishes and this central area with no staining (Figure 2A, right) corresponded to the site where Acanthamoebae were placed. Although corneal fibroblasts on the peripheral area should have been exposed to soluble factors Acanthamoebae produced, there was no apparent change in the outside area of corneal fibroblasts at least 2 days after Acanthamoebae addition, suggesting that direct adhesion of Acanthamoebae to corneal fibroblasts is essential for the cytopathic effect of Acanthamoebae and soluble factors Acanthamoebae produced does not affect the fate of corneal fibroblasts.

To test whether the cytopathic effect of Acanthamoeba was due to direct adherence to corneal fibroblasts or to soluble factors such as chemical mediators, we used insert culture dishes with 0.4 µm pores, and corneal fibroblasts were cultured in the outer plate. Acanthamoebae could not pass through these pores under any culture conditions. MTT assay showed there was no significant difference of optical density value in the outer dishes with corneal fibroblasts with or without insert culture dishes bearing Acanthamoebae. Significant low optical density value was detected in Acanthamoebae direct adhesion group, compared with insert culture dishes bearing Acanthamoebae. Significant low optical density value was detected in Acanthamoebae direct adhesion group, compared with insert culture dishes bearing Acanthamoebae. Significant low optical density value was detected in Acanthamoebae direct adhesion group, compared with insert culture dishes bearing Acanthamoebae. (n=6) Amoeba; Acanthamoeba, Inserter; insert culture dish. C: Phase contrast microscopy shows many corneal fibroblasts are detached and Acanthamoebae adhere to corneal fibroblasts and the dish surface. Arrowheads show active Acanthamoebae co-cultured with corneal fibroblasts. D: Confluent human corneal fibroblasts are seen. Acanthamoebae in the insert culture dishes with 0.4 µm pores are not observed. Similar findings were obtained with repeated two sets of experiments. Representative data are shown. Scale bar=10 µm.
significantly in a dose-dependent manner after co-culture with *Acanthamoeba* (Figure 3A). We analyzed corneal fibroblast viability over time using 1×10^4 *Acanthamoeba*, because larger numbers of *Acanthamoeba* were too toxic for the fibroblasts. As shown in Figure 3B, the viability of corneal fibroblasts cultured with *Acanthamoeba* was significantly decreased from day 2 compared with the control cultures. Next, we evaluated the cytopathic effect of various T4 genotypes isolated from patients with AK. Significant decreases of corneal fibroblast viability were detected with all *Acanthamoeba* strains tested as compared to culture without *Acanthamoeba* (Figure 3C), suggesting that *Acanthamoeba* isolates from AK patients have a similar cytopathic effect on human corneal fibroblasts.

**DISCUSSION**

All 6 *Acanthamoeba* isolates from the corneas of our AK patients had the T4 genotype according to 18S rDNA sequence analysis, as shown in Table 1. Our present findings clearly demonstrated that all of the *Acanthamoeba* isolated from AK patients had a cytopathic effect on human corneal fibroblasts and that this cytopathic effect was due to direct adhesion to corneal fibroblasts rather than soluble factors, suggesting that the keratocyte depletion demonstrated by histological examination of human corneas with AK [17] is induced by direct adhesion of *Acanthamoeba* to activated corneal keratocytes (i.e., corneal fibroblasts). DNA fragmentation was detected in corneal fibroblasts cultured with *Acanthamoeba* and the fibroblasts were mainly depleted by apoptosis, as evidenced by Annexin V staining to detect early apoptosis. In vitro observation showed active *Acanthamoeba* trophozoites phagocytosing fragments of corneal fibroblasts (unpublished observation 2010). These findings may reproduce those occurring in AK induced by pathogenic *Acanthamoeba*.

Our in vitro study suggested that activated corneal fibroblasts may be extremely vulnerable to *Acanthamoeba* infection in vivo. However, AK is localized to the central area of the cornea and does not expand to the periphery or the conjunctiva. This may be because all layers of the peripheral corneal stroma contain monocytes [21] and the substantia
propria of the conjunctiva has several leukocytes (mainly macrophages) [19]. Moreover, corneal fibroblasts have the potential to produce abundant chemokines that attract macrophages and neutrophils [21-23]. Clinically, vascular invasion of AK lesions dramatically suppresses disease activity, implying a critical role of leukocytes from the blood vessels in combating AK. Thus, differences in the immunoprotective microenvironment of the ocular surface may confine AK lesions to the central area of the avascular cornea, but severe visual impairment due to corneal scarring is a common problem. These findings suggest that local application of host leukocytes and use of chemokines to attract neutrophils and macrophages may be treatment options for AK that is uncontrolled by current therapies.

Sequencing of nuclear 18S rDNA [6,7] is a useful method of classifying Acanthamoebae accurately. Fifteen types of Acanthamoebae (T1 to T15) have been identified, but isolates obtained from AK are mainly of the T4 genotype. All 6 isolates obtained at our hospital were of the T4 genotype and all isolates had a cytopathic effect on corneal fibroblasts (Figure 3C). However, the extent of the cytopathic effect varied, suggesting pathophysiological diversity of Acanthamoebae with the T4 genotype. Therefore, we retrospectively examined the correlation between the cytopathic effect in vitro and clinical severity of AK caused by each isolate, but no correlation was detected in our series (unpublished observation). This may be because clinical severity of AK depends on various factors, such as the stage at the first visit to hospital, previous treatment, and use of topical corticosteroids due to misdiagnosis. However, detailed classification of Acanthamoeba by DNA typing may contribute to prediction of the prognosis and selection of appropriate treatment for each isolate in the near future.

The following limitations of our study should be noted. Cultured human corneal fibroblasts are not the same as keratocytes in the normal corneal stroma, because corneal fibroblasts are activated in response to inflammation [24,25]. Considering that AK causes inflammation of the cornea, corneal fibroblasts rather than corneal keratocytes may be more suitable for analysis of interactions between corneal stromal cells and Acanthamoebae. In this study, Annexin V expression was used to detect early apoptosis and PI-positive cells were regarded as necrotic cells. Therefore, we could not determine the actual proportion of necrotic cells, because PI-
positive cells resulting from apoptosis were not completely excluded. However, we at least did not overestimate the number of apoptotic cells. Further studies need to be performed to determine the exact percentage of apoptotic cells. Acanthamoebae express a trans-membrane protein with the characteristics of a cell surface receptor, which is called mannose-binding protein (MBP) and mediates adhesion to the surface of the cornea. Following MBP-mediated adhesion to host cells, the amoebae produce a contact-dependent metalloproteinase and several contact-independent serine proteinases [26]. Kinnear [27] reported indirect cytopathic effects of Acanthamoeba on corneal fibroblasts using insert culture dishes, suggestive of contact-independent serine proteinases. This may be because approximately 400× higher number of Acanthamoeba were employed in the study than our experimental setting. Considering that Acanthamoeba used in our experiment was enough number to kill directly activated corneal fibroblasts and Acanthamoeba are sparse in the corneal stroma of patients with confocal microscopic observation [28], indirect cytopathic effects of Acanthamoeba on corneal fibroblasts can be at least ignorable in an actual clinical setting.

In summary, we showed that Acanthamoeba from AK patients had a cytopathic effect on human corneal fibroblasts by direct adhesion rather than soluble mediators. This cytopathic effect on corneal fibroblasts was mainly due to apoptosis. Our findings provide some clues to the pathophysiology of corneal keratocyte loss in human AK.

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