Roles of Adherence and Matrix Metalloproteinases in Growth Patterns of Fungal Pathogens in Cornea

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ABSTRACT

Purpose: To investigate the roles of adherence and matrix metalloproteinases (MMPs) in growth patterns of major fungal pathogens in cornea.

Methods: Ninety-six eyes in 96 rabbits were equally divided into four groups receiving inoculation of fungal conidia of Aspergillus fumigatus, Candida albicans, Fusarium solani, and Penicillium citreo-viride, respectively, to induce fungal keratitis. Corneas in each group were obtained at 2, 8, 16 hr, and 1, 2, 3, 5, and 8 days after inoculation and were subjected to scanning electron microscopy, histopathological examination, and gelatin zymography. Eight saline-inoculated eyes in another eight rabbits served as controls.

Results: All eyes in the fungus-inoculated groups developed fungal keratitis. The binding of conidia to corneal epithelial basement membrane was initiated earlier in the A. fumigatus and C. albicans groups than in the F. solani and P. citreo-viride groups. Destruction of basement membrane began at 1 to 3 days. Histopathologically, infiltration of inflammatory cells was more evident in the A. fumigatus and C. albicans groups than in the F. solani and P. citreo-viride groups at 3 days. The hyphae of A. fumigatus and C. albicans traversed the cornea in a plane perpendicular to the stromal lamellae, whereas the hyphae of F. solani and P. citreo-viride lay parallel to the corneal lamellae. MMP-9 and MMP-2 were found in all infected corneas. At 3 days, proteolysis was most active; the level of MMP-9 was higher in the A. fumigatus and C. albicans groups than in the F. solani and P. citreo-viride groups at 3 days. The hyphae of A. fumigatus and C. albicans traversed the cornea in a plane perpendicular to the stromal lamellae, whereas the hyphae of F. solani and P. citreo-viride lay parallel to the corneal lamellae. MMP-9 and MMP-2 were found in all infected corneas. At 3 days, proteolysis was most active; the level of MMP-9 was higher in the A. fumigatus and C. albicans groups than in the F. solani and P. citreo-viride groups. There were positive correlations among the number of binding conidia, degree of inflammation, and level of MMP-9 (p < 0.05).

Conclusions: The adherence ability, chemotaxis to neutrophils, and MMP-9 expression level differ in eyes with different fungal pathogens, which may contribute to the different growth patterns of fungi in cornea.

KEYWORDS adherence; fungal keratitis; growth pattern; matrix metalloproteinase

INTRODUCTION

More than 70 species of fungi have been reported as pathogenic to human cornea. Although geographical distribution of fungal pathogens differs, Fusarium, Aspergillus, and Candida predominate worldwide. In China, Penicillum...
is also among the commonest species. Clinically, corneal lesions caused by different fungal species have different characteristics, and the response to medical and surgical treatment differs as well. Growth patterns of fungal pathogens in cornea, which have been examined histopathologically, may contribute to various clinical features. However, due to the poor knowledge of the fungal keratitis mechanism and pathological characterization of fungal species in cornea, it remains difficult for clinicians to treat fungal ulcers effectively.

Most of our knowledge concerning the mechanism of fungal infection comes from infections at lung, kidney, skin, and so forth. Adherence ability to host tissue and proteases are suggested as important virulence factors. Aspergillus fumigatus and Candida albicans have been proved to adhere to many components of extracellular matrix (ECM); there is a positive correlation between binding ability and pathogenicity. Moreover, proteolytic enzymes, which could degrade ECM components and induce conidia adherence, are helpful in mediating tissue invasion.

However, little is known about the roles of adherence and proteases in the pathogenesis of fungal keratitis. Rao et al. investigated adherence of C. albicans to the rabbit corneal surface in vitro and found that blastospores of C. albicans rarely bound to the intact corneal epithelium but consistently adhered to the stroma with epithelial denudation, which indicated that adherence to corneal stroma may be an important initial event of keratomycosis. The extracellular protease is known to initiate ECM degradation in bacterial keratitis and other noninfectious corneal pathologies. Zhu et al. identified extracellular proteases in the culture (in vitro) of an Aspergillus flavus isolate from cornea. Gopinathan et al. reported the presence of two members of matrix metalloproteinases (MMPs) family, MMP-9 and MMP-2, in corneas infected by A. flavus and Fusarium solani, finding that the expression of MMP-9 correlated positively with the number of polymorphonuclear cells in the infected tissues. However, the quantitation of MMPs expressed in fungus-infected corneas has not been investigated; neither was the possible difference in MMPs expression among different fungal species.

In this study, we attempted to characterize the adherence process and expression of MMPs and the histopathological changes in experimental fungal keratitis caused by commonly encountered corneal fungal pathogens. The mechanism of fungal growth patterns in cornea was also investigated.

**MATERIALS AND METHODS**

**Inocula**

Aspergillus fumigatus, Candida albicans, Fusarium solani, and Penicillium citreo-viride were selected for this study because they are commonly encountered corneal pathogenic species at our institution, one of the largest referral eye centers in China. The strains used in this study were AS 02272, ATCC 2.2086, AS 3.1829, and AS 3.226 (China Committee of Culture Collection for Microorganisms, Beijing, China), respectively. These isolates were cultured on potato glucose agar tubes at 28°C for 5 days and flushed with 1 ml of sterile saline solution. A hemocytometer was used to count the number of colony-forming units (CFU) in the solution, and the concentration was titrated to $10^8$ CFU per ml.

**Animals**

One hundred four New Zealand albino rabbits of both sexes were used, each weighing 2 to 2.5 kg. All animals were treated according to the Association for Research in Vision & Ophthalomyology Statement on the Use of Animals in Ophthalmy and Vision Research. One eye of each rabbit received 0.3% ofloxacin eye drops (Tarivid; Santen, Osaka, Japan) four times daily for 3 days. Eight eyes served as controls, and the other 96 eyes were equally divided into four groups. In addition, 104 rabbit corneal grafts were prepared.

Rabbits were anesthetized with intramuscular ketamine and xylazine. Proparacaine hydrochloride (0.5%) was used topically for corneal anesthesia. After placing a 6-mm-diameter pyroxylin filter paper on the central cornea for 30 s, the epithelium within the 6-mm area was removed. A full-thickness donor corneal graft was placed on the recipient bed and sutured to the rim of the cornea with 6 interrupted 10-0 nylon sutures. Each eye in the four experimental groups was injected with 50 μl of inoculum of A. fumigatus, C. albicans, F. solani, and P. citreo-viride, respectively, into the space between graft and recipient bed. The controlled eight eyes were inoculated with 50 μl of sterile normal saline. Immediately after the surgery, 0.3% ofloxacin eye ointment (Tarivid; Santen) was administered.
All grafts were removed at 2 days after inoculation. At 2, 8, 16 hr, and 1, 2, 3, 5, and 8 days, three rabbits from each fungus-inoculated group and one from the control group were randomly chosen for clinical scoring using slit-lamp microscopy, after which the rabbits were sacrificed. The corneas were trephined (12 mm), excised, and rinsed in sterile saline for 5 min before bisected longitudinally. When scanning electron microscopy or histopathological examination was performed, one random portion of the cornea was used; when enzymatic characterization was performed, one-half of the cornea was needed.

**Clinical Evaluation**

All inoculated eyes were photographed before the rabbits were sacrificed. One observer maskedly examined these photographs and scored the severity of keratomycosis. In the category of opacity area, score 1 to 4 was given respectively when <25%, 26–50%, 51–75%, >75% of the cornea was opaque. Regarding the opacity density, score 1 was slight cloudiness with discernable outline of iris and pupil; score 2, cloudy but visible outline of iris and pupil; score 3, cloudy and not uniform opacity; score 4, uniform opacity. As to the surface regularity, score 1 was slight surface irregularity; score 2, rough surface with some swelling; score 3, significant swelling with crater or serious descemetocele formation; score 4, perforation or descemetocele. A score of 0 in each of the three categories was given to the normal cornea. The score of each eye for each category was tallied at 1, 3, 5, and 8 days after inoculation to yield a total score.

**Scanning Electron Microscopy**

Three corneal samples in each fungus-inoculated group were fixed in 2.5% glutaraldehyde, dehydrated, and dried at critical point for scanning electron microscopy at 2, 8, 16 hr, and 1, 2, and 3 days after inoculation. Each corneal sample was divided into 9 parts by drawing a “#” sign on the cornea, and the upper-left, upper-right, middle, lower-left, and lower-right areas were selected. Twelve microscopic fields at a magnification of 1500 were randomly chosen in each area, and the number of conidia in the fields was calculated.

**Histopathology**

At every time point, three corneal samples in each fungus-inoculated group and one control sample were formalin-fixed and paraffin embedded. Continuous 5-μm sections got periodic acid–Schiff (PAS) or hematoxylin and eosin (H&E) staining. The presence of inflammatory cells, type of cells, and degree of inflammation were observed. The corneal inflammation was scored 1 to 3 for mild (the background stroma and keratocyte nuclei were clearly seen), moderate (the keratocyte nuclei were obliterated with visible stroma), and severe (complete obliteration of keratocyte nuclei and corneal stroma was available). Moreover, we defined the growth patterns of fungal pathogens as horizontal when most of the hyphae grow at an angle <45 degrees to the plane of stromal lamellae, perpendicular when >45 degrees.

**Enzymatic Characterization**

Three corneal samples in each fungus-inoculated group and one control sample were collected for enzymatic characterization at 16 hr, and 1, 3, 5, and 8 days after inoculation. Samples were stored at −70°C after a 5-min rinse in saline. Before assay, samples were thawed and homogenized in 100 μl of RPMI-1640 (Sigma, St. Louis, MO, USA) containing 4% sodium dodecyl sulfate (SDS). After the corneal proteins were extracted, samples were centrifuged at 8000 × g for 10 min at 4°C, and the resultant supernatants were diluted in sample buffer (125 mM Tris-HCL, pH 6.8, 20% glycerol, 2% SDS, and 0.04% bromphenol blue) and incubated at 37°C for 45 min for electrophoretic separation. The gelatin zymography used in this study was a modification of the method used by Kernacki et al. Proteases were resolved on 8% SDS-polyacrylamide gels containing gelatin (type A from porcine skin, Sigma) at a final concentration of 0.15%. Twenty μl of each sample was loaded onto each lane. The gels were shaken at room temperature in a solution of 2.5% Triton-X 100 for 45 min to remove the SDS after electrophoresis at 4°C and then were incubated at 37°C in incubation buffer (50 mM Tris-HCL, pH 7.8, 10 mmol/L CaCl2) with or without 10 mM ethylenediaminetetraacetic acid (EDTA-Na) for 24 hr. The position of the proteases could be identified as white bands in the stained gelatin background after the gels were stained with Coomassie Brilliant Blue R-250 (Sigma). Kodak Gel Image Scanning and Analysis System was used to calculate the mean absorbance value of each white band. Broad-range SDS-PAGE protein standards (Bio-Rad, Adherence and MMPs in Fungal Growth Patterns
Richmond, CA, USA) were used as molecular weight markers.

**Statistical Analysis**

SPSS 10.0 software was used for statistical analysis. Standard analysis of variance was used to compare MMP-9 and MMP-2 expression level among the fungus-inoculated groups. Pearson correlation coefficient was calculated to determine if there were correlations among conidia adherence ability, density of inflammatory cells, and expression levels of MMP-9 and MMP-2.

**RESULTS**

**Clinical Features**

All eyes in the fungus-inoculated groups developed invasive fungal keratitis successfully. The clinical features manifested most evidently at 3 days after inoculation and varied with fungal species (Fig. 1). In the *A. fumigatus* group, the ulceration appeared whitish and dense with satellite lesion or immune ring at peripheral area and spread quickly; large hypopyon was present in most of the infected eyes. In the *C. albicans* group, the ulceration was gray-white, dry, and homogeneously elevated with a defined edge. In the *F. solani* group, the infected cornea was edematous, and the lesion got thickened gradually and spread extensively. In the *P. citreo-viride* group, the lesion was superficially spreading and translucent with a small amount of fibrous exudate in the anterior chamber. At 8 days after inoculation, infections in most of the eyes lightened, except *A. fumigatus*-inoculated eyes in which total corneas were involved even with perforation. Corneal neovascularization was present at the periphery of most corneas.

The total score of each eye at the four time points was summed up respectively in the *A. fumigatus, C. albicans, F. solani,* and *P. citreo-viride* groups. At 3 days, the average clinical scores of the four groups were $8.67 \pm 0.58, 7.67 \pm 1.15, 6.67 \pm 0.58,$ and $3.67 \pm 0.58$, respectively. There was statistically significant difference ($p < 0.01$) in the severity of disease between any two groups. *A. fumigatus* and *C. albicans* caused more severe corneal disease than *F. solani* and *P. citreo-viride*.

In the control group, the corneas re-epithelialized at 3 days and remained clear throughout the observation period.

**Scanning Electron Microscopy**

The basement membrane was exposed in the area debrided of corneal epithelium. Adherence of conidia to epithelial basement membrane could be seen at 8 hr after inoculation in the *A. fumigatus* and *C. albicans* groups and at 16 hr in the *F. solani* and *P. citreo-viride* groups. The surface of the conidia was rough with thorn-like prominency, and silk-like material connected it to the epithelial basement membrane. The number of conidia adhering to basement membrane at 16 hr (Fig. 2) was $42.5 \pm 8.8, 38.3 \pm 6.4, 20.1 \pm 6.9,$ and $7.9 \pm 2.8$, respectively, in eyes with *A. fumigatus, C. albicans, F. solani,* and *P. citreo-viride*. The conidia of *A. fumigatus* and *C. albicans* adhered more densely to basement membrane than *F. solani* and *P. citreo-viride*. There

**FIGURE 1** The rabbit eyes in groups of *A. fumigatus* (A), *C. albicans* (B), *F. solani* (C), and *P. citreo-viride* (D).

**FIGURE 2** Scanning electron microscopic photographs of the conidia of *A. fumigatus* (A), *C. albicans* (B), *F. solani* (C), and *P. citreo-viride* (D) adhering to the epithelial basement membrane. Original magnification, \( \times 1500 \).
was significant difference between *F. solani* and *P. citreo-viride* (*p* < 0.01), but not between *A. fumigatus* and *C. albicans* (*p* = 0.247). Moreover, there was a significant positive correlation between the number of adherent conidia and clinical scores (*r* = 0.953, *p* = 0.047).

The destruction of corneal epithelial basement membrane occurred at 1 day in the *A. fumigatus* group, 2 days in the *C. albicans* group, and 3 days in the *F. solani* and *P. citreo-viride* groups. With partial or extensive damage to basement membrane, the collagen fibers of the stroma were exposed. The membrane was formed on the surface of conidia, which were connected with the corneal stroma (Fig. 3).

**Histopathology**

**H&E Staining**

Inflammatory cell infiltrates and tissue injury, which were caused by the epithelial debridement and injected inocula, peaked at 3 days after inoculation and varied in the four groups with fungal species (Fig. 4). Most of the inflammatory cells were neutrophils. In the *A. fumigatus* group, there were dense inflammatory cell infiltrates, extensively destroyed stromal collagen structure, and complete obliteration of keratocyte nuclei. In the *C. albicans* group, the inflammatory cells were present in the superficial and midlayers of the stroma with a dense infiltration around the hyphae; stromal collagen structure was destroyed, and a part of keratocyte nuclei disappeared. In the *F. solani* group, the inflammatory cells were evenly distributed in the stroma; stromal integrity was maintained despite mild edema, and most of the keratocyte nuclei were clearly seen. In the *P. citreo-viride* group, a small number of inflammatory cells were scattered in the stroma; stromal integrity was maintained, and keratocyte nuclei were clearly seen. With the decrease of inflammatory cell infiltration, blood vessels could be seen in the stroma at 8 days.

The corneal inflammation score was 2.8 ± 0.5, 2.2 ± 0.5, 1.2 ± 0.5, and 1.4 ± 0.5, respectively, in eyes with *A. fumigatus*, *C. albicans*, *F. solani*, and *P. citreo-viride* at 3 days. There was no significant difference between the *A. fumigatus* and the *C. albicans* groups (*p* = 0.063).
or between the *F. solani* and the *P. citreo-viride* groups (*p* = 0.541). Moreover, there was a positive correlation between the degree of inflammation and the number of adherent conidia (*r* = 0.955, *p* = 0.045).

The stromal structure in the control group was normal. A few neutrophil infiltrates resulting from epithelial debridement appeared in the superficial layer of the peripheral cornea at 1 day and disappeared at 3 days.

**PAS Staining**

At 3 days after inoculation, thick and short hyphae of *A. fumigatus* were observed growing perpendicularly to the stromal lamellae, some of which penetrated the Descemet membrane (Fig. 5A); by day 8, hyphae became less. The spores of *C. albicans* lined up in chains like rosary beads and formed fine, tightly packed, nonbranching pseudohyphae; at 3 days, the pseudohyphae penetrated one-third of the corneal stroma in depth, growing perpendicularly to the stromal lamellae (Fig. 5B); there was no significant change by day 8. The hyphae of *F. solani* were long, septate, and branched at less than 45 degrees; by day 3, the hyphae reached one-fifth of the corneal stroma in depth, with a trend to grow horizontally to the lamellae (Fig. 5C); by day 8, hyphae were still limited to the superficial stroma. Hyphae of *P. citreo-viride* reached only one-tenth of the corneal stroma in depth at 3 days, with a trend to grow horizontally to lamellae (Fig. 5D); by day 8, no hyphae could be detected in the corneas. There was a positive correlation between the number of adherent conidia and depth of hyphal penetration (*r* = 1.000, *p* = 0.042).

No fungi were found in the control group.

**Gelatin Zymography**

MMP species were identified as bands of substrate, which were cleared within gelatin zymography gels. Bands of gelatinolytic activities of 92 kDa and 65 kDa, corresponding to the activities of MMP-9 and MMP-2, were identified in the infected corneal samples harvested at 1, 3, 5, and 8 days after inoculation, and all were susceptible to inhibition of 10 mM EDTA. In normal corneas and the controlled corneal samples in this study, there was only a little MMP-2 expression. The expression of MMP-9 and MMP-2 in the fungus-inoculated corneas at different time points was calculated and expressed as absorbance value (Fig. 6).

The expression of MMP-9 was directly proportional to the degree of inflammation (*r* = 0.964, *p* = 0.036), the number of adherent conidia (*r* = 0.991, *p* = 0.001), the clinical evaluation scores (*r* = 0.956, *p* = 0.044), and the depth of hyphal penetration (*r* = 1.000, *p* = 0.042).

There was no significant correlation between the expression of MMP-2 and MMP-9 (*r* = 0.239, *p* = 0.761); neither was the expression of MMP-2 and the degree of inflammation (*r* = −0.833, *p* = 0.167) or clinical scores (*r* = −0.645, *p* = 0.257).

**DISCUSSION**

In previous animal models, the inoculum was injected directly into the corneal stroma and effectively induced fungal keratitis; however, the stromal lamellae were disrupted, which may preclude us from examining the details of fungal growth and conidia adherence to...
corneal epithelial basement membrane. O’Day et al. created an animal model by placing an inoculum on the corneal surface and covering it with a contact lens. However, to investigate enzymatic changes in cornea, steroids, which can stimulate fungal hyphal growth, were required to enhance protease production. To better mimic the natural behavior of fungi in cornea and ensure a high success rate of inoculation, we inoculated the cornea after the epithelium was debrided in this study, which allowed the inoculum to lodge onto the stromal surface. Suturing a donor graft onto the corneal surface prevented the inoculum from being flushed away by tears and provided a warm and moisturizing environment for fungi to grow. Fungal keratitis was successfully developed in all the fungus inoculated eyes.

In a previous study, we examined the histopathology of 108 clinical cases of fungal keratitis, finding that different species behave differently in cornea. A further study showed that Fusarium grew parallel to the plane of corneal lamellae, whereas Aspergillus hyphae traversed the corneal stroma vertically. In this study, we investigated the adherence potential and MMP expression in corneal fungal infection models, which may be related to the fungal growth patterns such as hyphal penetration and degree of inflammation.

We verified that conidia adherence to epithelial basement membrane was an initial pathogenesis in fungal keratitis. The roles of MMP-2 and MMP-9 in keratomycosis were investigated in this study as well. There were reports about their roles in bacterial keratitis and other noninfectious corneal pathologies. MMP-9 can degrade basement membranes, type I and IV collagen; MMP-2 can degrade type IV, V, VII collagen and degenerative type I, II, or III collagen. MMP-9 mainly contributes to the degradation of basement membrane and stromal collagen, whereas MMP-2 is responsible for the maintaining of corneal integrity. In this study, the expressions of MMP-9 and MMP-2 were detectable in all infected tissues at 1 day after inoculation and had a rapid increase. The flood tide was consistent to clinical infection, and the expression of MMP-9 was directly proportional to the clinical evaluation scores in the four groups with different fungal species infections, which indicated that MMP-9 may be the major protease in fungal invasion and corneal destruction. In this study, it also showed that neutrophils can help to increase proteolytic activities in fungal-infected corneas, which is similar to the previous reports. The initial step of fungal infection may be exertion of chemotaxis to neutrophils after conidia adhering to corneal epithelial basement membrane. Neutrophils can synthesize MMPs, especially MMP-9. Species with strong adherence ability may exert chemotaxis to neutrophils more effectively, and the expression of MMP-9 in cornea becomes high, which may result in destruction of corneal collagen and penetration of hyphae into the lamellae. Such fungi appear to penetrate the cornea in a plane perpendicular to the stromal lamellae. On the contrary, species with low MMP-9 expression cannot cause significant stromal lamellae degradation, and hyphae appear to traverse the cornea in a plane that corresponds to the horizontal lamellae of the stroma. Therefore, growth patterns of various fungal species appear closely correlated with the adherence ability, chemotaxis to neutrophils, and the expression of MMP-9 in cornea. Prevention of conidia adherence to cornea, moderate inhibition of inflammation, and administration of metalloproteinase inhibitors may be of great clinical significance in preventing fungal ulcers and lightening the severity of corneal injuries by changing the growth patterns of fungal pathogens.

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