·Basic Research·

Dectin -1 expression at early period of *Aspergillus fumigatus* infection in rat´s corneal epithelium

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Abstract

• AIM: To investigate the expression of dendritic cell-associated C-type lectin-1 (dectin-1) at the early period of *Aspergillus fumigatus* infection in rat´s corneal epithelium.

• METHODS: A total of 72 Wistar rats were randomly divided into three groups: A, B and C. The right eyes were chosen as experimental eyes. Group A was control group. Rats in group B were not inoculated with *Aspergillus fumigatus*. Group C was taken as *Aspergillus fumigatus* keratitis model. Rats in group B and C (six from each group) were executed randomly at 4, 8, 16 and 24 hours after experimental model being established to assess the expression of dectin-1 mRNA through real-time PCR. Another six rats in group B and C were executed randomly at 24 hours to assess the expression of dectin-1 protein through immunohistochemistry.

• RESULTS: The results of real-time PCR indicated that dectin-1 mRNA expression was low in corneal epithelium of normal rats´. There was no significantly difference of dectin-1 mRNA expression in group A and B (P>0.05). The expression of *Aspergillus fumigatus* infected corneal epithelium increased gradually after 8 hours in group C. The synchronous expression of group A and C had significant difference (P<0.01). Immunohistochemisty discovered that dectin-1 receptor existed in normal rat´s corneal epithelium . Dectin-1 protein increased after 24 hours in group C. There was a significant difference of synchronous expression in group B and C(P<0.01).

• CONCLUSION: Dectin -1 exists in rat´s corneal epithelium and its expression significantly increases at the early period of *Aspergillus fumigatus* infection. Dectin-1 is a pattern recognition receptor that expresses in corneal epithelium and involves in immune response to *Aspergillus fungal* keratitis.

• **KEYWORDS:** keratitis; *Aspergillus fumigatus* ;dectin-1;rat

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INTRODUCTION

Fungal keratitis is one of the most serious infectious corneal disease and can more easily lead to blindness than some other corneal diseases. *Aspergillus fumigatus* is one of the main pathogen of Fungal keratitis on a global scale [1]. The occurrence of Fungal keratitis is the result of the role of fungal virulence factors and host defense factors. As the first line to defense microbial infection, innate immune system of the ocular surface activate and launch the immune defense response to identify and remove pathogens, through pattern-recognition receptors (PRRs) expressed by innate immune cells recognize pathogen associated molecular patterns (PAMPs)[2]. Dectin-1 is a member of C-type lectin super family originally found in dendritic cells. It can identify β-glucan, and then mediate a variety of fungal innate immune response and regulate the production of cytokine and chemokine [1]. In addition to expressing in dendritic cells, dectin-1 also exists in immune- related non-immune cells such as normal human epidermal cells [3] and II airway epithelial cells [4]. But up to now, if it expressed in corneal epithelium especially in corneal epithelial cells is still unknown. We also want to know, can the corneal epithelial cells recognize the pathogenic fungi with dectin-1 at corneal epithelium? In this study, we investigated the dectin-1 expression at the early period of *Aspergillus fumigatus* infection in rat's corneal epithelium and preliminarily explored the relationship between dectin-1 in corneal epithelium and anti-fungal immunity.

MATERIALS AND METHODS

Materials *Aspergillus fumigatus* strains (NO3.0772) was bought from China General Microbiological Culture

Collection Center; Sabouroud culture was purchased from American Sigma company; Rabbit anti-rats dectin-1 multi-clonal antibody, Histostain® PLUS kit and DAB kit were purchased from Beijing Biosynthesis Biotechnology Co., Ltd; Trizol Reagent was purchased from Invitrigen; PrimeScript® RT reagent Kit With gDNA Eraser (Perfect Real Time) was purchased from TaKaRa; Primers and probes were purchased from TaKaRa.

Animals Wistar rats (both male and female) were purchased from Qingdao Institute of Drug Control (Qingdao, China), weighed between 200-300g. Those with corneal disease were excluded after slit-lamp examination. The remains were treated in accordance with the guide lines provided in the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. A total of 72 Wistar rats were randomly divided into 3 groups: 12 in group A, 30 in group B and 30 in group C. The right eyes were chosen as experimental eyes. Model establishment and evaluation criteria method was in accordance with Li *et al*'s [5]. Group A was as control group. Group B was untreated group, which the rat was the completed model but was not inoculated with *Aspergillus fumigatus*. Rats in group C were taken as *Aspergillus fumigatus* keratitis models. Six rats in group A were executed randomly for real-time PCR and six for immuno-histochemistry. Six rats in group B and C were executed randomly at 4, 8, 16 and 24 hours respectively after the experimental model being established for real-time PCR and six in group B and C were executed randomly at 24 hours for immunohistochemistry. The eyeballs were removed under sterile condition. The method to obtain rat corneal epithelium for real-time PCR was in accordance with Zhang *et al*'s [6]. The corneal epithelium was treated with 500μL RNAiso Reagent, then was stored in -80℃ refrigerator for real-time PCR. Fungal hyphae could be found by PAS stain in each observation point. 10% potassium hydroxide (KOH) wet mount was used to show pathogenic fungi. The same fungal strain with which rats were inoculated could be cultivated from histological scraping.

Real-time PCR To minimize the experimental error, total RNA was synchronously extracted from the corneal epithelium samples obtained at different observation points using Trizol reagent according to the manufacturer's protocol. Determination of RNA concentration, reverse transcription and real-time quantitative PCR reactions were executed in succession. Dectin-1 sequence: Sense TGGACGAAGATG GATATAC; Antisense CAAGCACAGGATTCCTA; Probe CTACCGCAATGGACCGCC. Internal reference was rat GAPDH housekeeping genes: Sense CCCCCAATGTATCC GTTGTG; Antisense GTAGCCCAGGATGCCCTTTAGT; Probe TCTGACATGCCGCCTGGAGAAACC. The reverse transcription steps were to adopt the recommended steps of PrimeScript RT reagent Kit With gDNA Eraser. Dectin-1 target sequences were amplified for 40 cycles. The fluorescence values were recorded after each cycle. The amplification curve was formed after 40 cycles. The cycle number (Ct values) was reported when fluorescence reached its threshold.

Immunocytochemistry Corneal paraffin sections, the thickness of 2μm, were conventional dewaxed to water. S-P method was applied to stain dectin-1 in corneal tissue. PBS buffer instead of primary antibody was used as negative control, and lung biopsy immunohistochemical staining was as positive control. Corneal tissue appeared brown particles for positive criteria. Field of vision was randomly selected and saved under 200 times view, the mean optical density of dectin-1 staining was analyzed with VIDAS-21 (Japan SANYO) the computer color image analysis system.

Statistical Analysis All data were presented as mean±SD (n=6). The data was analyzed with SPSS17.0 statistical package. Single factor analysis of variance was used to collectively comparison. The LSD test were used to pairwise comparisons. The differences between group B and C were analyzed by T test. P<0.05 was considered to be statistically significant.

RESULTS

Expression of Dectin-1 mRNA GAPDH stably expressed in corneal epithelium. The integrity of reverse transcriptase cDNA indicated a successful PCR reaction. The expression of dectin-1 mRNA was detected in each group. The expressions between group A and B had no significant difference (F=1.325, P=0.288). The expression of dectin-1 in *Aspergillus fumigatus* infected corneal epithelium increased gradually after 8 hours in group C. The difference of synchronous expressions in group A and C was statistically significant (F=74.679, P=0.000). The five samples were pairwise compared (Table 1). There was significant difference of dectin-1 expressions in group A and C at observation points 8, 16 and 24 hours (P<0.01) and had no significant difference at 4 hours (P=0.063) (Figure 1). And that on 8 and 16 hours in group C was not significantly different (P=0.125), but on 24 hours and other observation points, the differences were significant (all P<0.01).

Immunocytochemistry Results of Dectin-1 The results of Brown staining indicated that dectin-1 expressed in rat's corneal epithelium (Figure 2). The dectin-1 expressions in group A and B were weakly positive with no significant difference (t=1.3881, P=0.197) and that in group C was positive. The positive particles were mainly located in cytoplasm and membrane of corneal epithelial cells. There was significant difference (t=-27.983，P=0.000) of dectin-1 expressions group A and C (Table 2).

Table 1 LSD test to pairwise comparison

	Difference between two means	*P*
A *vs* C, 4 hours	-0.481	0.063
A *vs* C, 8 hours	-1.292	0.000
A *vs* C,16 hours	-1.684	0.000
A *vs* C, 24 hours	-3.896	0.000
C, 4 hours *vs* C, 8 hours	-0.811	0.003
C, 4 hours *vs* C, 16hours	-1.203	0.000
C, 4 hours *vs* C, 24 hours	-3.415	0.000
C, 8 hours *vs* C, 16 hours	-0.392	0.125
C, 8 hours *vs* C, 24 hours	-2.604	0.000
C, 16 hours *vs* C, 24 hours	-2.212	0.000

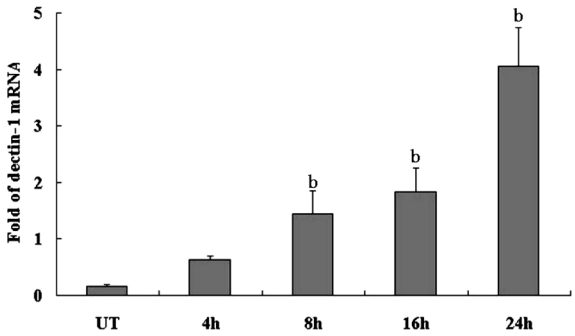

Figure 1 Real −time PCR results of dectin −1 The dectin-1 mRNA expression were low in normal rat corneal epithelium. The expression increased gradually in *Aspergillus fumigatus* infected corneal epithelium. $^{b}P<0.01$ *vs* group A.

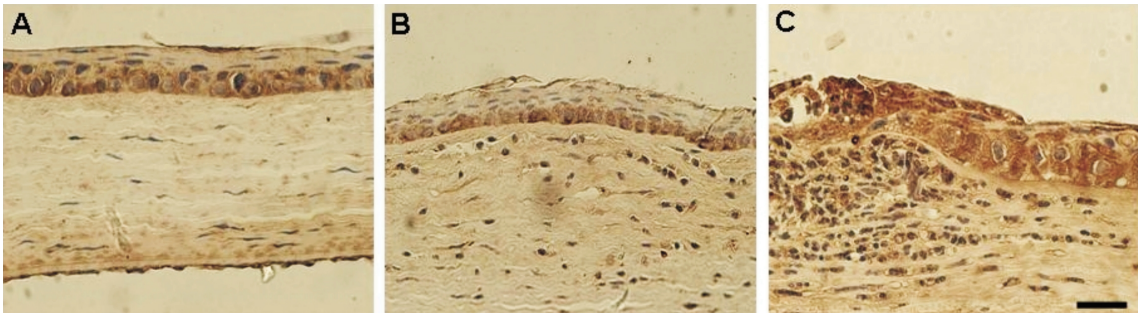

Figure 2 Dectin−1 expression evaluated by immunohistochemical staining (×400) A: Normal cornea; B: Negative control cornea; C: 24 hours after *Aspergillus fumigatus* infection. Bar:100μm.

Table 2 Average *A* values of dectin-1

Groups	*n*	Average *A* value
A	6	0.364±0.090
B	6	0.357±0.093
C	6	0.560±0.158

DISCUSSION

A variety of pattern recognition receptors participate the pathogenesis of fungal keratitis and Toll-like receptors (TLRs) have the most extensive research [7-9]. In recent years, with the increasing of the reports of C-type lectin receptors (CLRs), the role of CLRs in the pathogenesis of fungal keratitis get more attention[10,11]. CLRs can recognise pathogen associated molecular pattern and also can lead to immune tolerance combined with soluble antigen and maintain homeostasis of endogenous glycoprotein[12]. In addition, CLRs may cooperate with TLRs to fine-tune inflammatory responses[13].

In this research, we preliminary study the relationship between fungal infection and the expression of dectin-1, an important member of C-type lectin receptor and a type II transmembrane protein [14]. It consists of a single extracellular C-type lectin-like domain (CTLD), linked by a stalk and transmembrane region to a cytoplasmic tail containing an immuno-receptor tyrosine-based activation motif. The receptor exists as two major isoforms, generated by alternative splicing, that differs by the inclusion of the extracellular stalk region and which possesses slight functional differences. Dectin-1 specifically recognises β1, 3-linked glucans, carbohydrates found in the cell walls of fungi, and in plants and some bacteria[15]. Dectin-1 recognises several fungal species, including a number of human pathogens such as Candida, Aspergillus, Pneumocystis and Coccidioides, and plays an important role in host defending against these pathogens. In addition to expression in dendritic cells, dectin-1 also exists in immune-related non-immune cells such as normal human epidermal cells and II airway epithelial cells.

Early studies suggested that corneal epithelial PRRs exist only in local scattered dendritic cells and macrophages. Recent studies reveal as the first line of defense to resist fungal infection in the ocular surface corneal epithelial cells can express a variety of PRRs. Corneal epithelial cells assist anti-fungal infection with PRRs and play an important role in the immune response. Our research indicates that corneal epithelium of normal rats' low-levelly expresses dectin-1 receptor. The expression of dectin-1 rapidly increases after *Aspergillus fumigatus* infection. The results suggest that dectin-1 not only expressed in corneal epithelium especially in corneal epithelial cells, also involved in the process of corneal tissue resistance to fungal infections. Other findings indicate that the corneal epithelial cells has functional TLR2 and TLR4, and activation of TLR2 and TLR4 through

NF-kappaB may contribute to pathogenesis of keratomycosis[16]. In addition, corneal epithelial cells also express Nod1/Nod2 receptor involved in the innate immune response to fungal infection [17]. These studies further indicate that corneal epithelial cell is an important component of corneal immune except as a physical barrier.

In summary, dectin-1 is not only the inherent structural component of rat corneal epithelial cells also significantly increases at the early period of *Aspergillus fumigatus* infection in cornea. The increased expression is only one of the evidences that dectin-1 involves in anti-fungal immunity. A functional test is needed to further analyze the role of dectin-1 in the innate immune. Rat fungal keratitis model is undoubtedly a good tool for the dectin-1 related functional test.

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