·Review·

Review of clinical and basic approaches of fungal keratitis

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Abstract

• **Fungal keratitis (FK) is a serious disease which can cause blindness. This review has current information about the pathogenesis, limitations of traditional diagnosis and therapeutic strategies, immune recognition and the diagnosis and therapy of FK. The information of this summary was reviewed regularly and updated as what we need in the diagnosis and therapy of FK nowadays.**

• **KEYWORDS:** fungal keratitis; pathogenesis; diagnosis; treatment
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INTRODUCTION

Fungal keratitis (FK) is well known as a severe infected ocular disease that causes cornea opacity and blindness, and even loss of the eyeball [1-6]. In China, the primary cause is vegetable corneal trauma after agricultural work and the most common pathogens are *Fusarium solani* and *Aspergillus fumigatus*[1,3,6]. Although new therapies have been excogitated in clinical, FK remains a challenge to ophthalmologists because of the delay of diagnosis and shortness of standard guidelines of treatments. At the same time, the pathogenesis of FK has still not been clarified. Therefore, a better understanding of the pathogenesis of FK is imperative for diagnosis and effective therapy.

PATHOGENESIS OF FUNGAL KERATITIS

Detail pathogenesis of FK has not been clarified. Recent studies and advances have broadened the approach to FK. Previous studies [6-8] have revealed that immune system plays an essential role in the pathogenesis of FK. The corneal epithelium, the frontier of cornea against pathogen infection, plays an important role in innate immunity [2-3]. When the integrity of ocular surface has been breached due to damage or trauma, it will be more vulnerable to be infected by fungi and other microbes resulting to FK. Innate immunity is the first line of defense in the immunity defenses. The dynamic balance between the host defenses and the virulence of invasive fungus is critical in the development of FK. It can specifically identify the pathogen-associated molecular patterns (PAMPs) on the surface of fungus through pattern recognition receptors (PRRs), including C-type lectin-like receptors (CLRs), toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (leucine-rich repeat-containing receptors, NLRs), scavenger receptor (SR) family [2-15]. PRRs play a critical role in innate immunity, which can mediate the secretion of cytokines and chemokines and the infiltration of neutrophils, macrophages, T cells, and ultimately fungal clearance.

LIMATATIONS OF TRADITIONAL DIAGNOSIS AND THERAPEUTIC STRATEGIES

The diagnosis of FK remains challenging and elusive [16]. Culture and microscopic examination are still the reference standard for pathogens diagnosis [17-18]. However, they are time-consuming and may delay the treatment [18]. Currently, the rapid and reliable diagnosis of FK has attracted more and more attention, which including confocal microscopy, anterior segment optical coherence tomography (OCT), polymerase chain reaction (PCR). They are effective but expensive and not readily used in all facilities. If the diagnosis can be made in a short time, it will result in better outcome. Therefore, it is imperative to develop the quick, sensitive, non-invasive and more convenient ways for effective diagnosis[16-21].

In developing countries, because of the lack of effective antifungal agents, the treatment of FK is still a challenge[16,22-24]. Two main typical classes of antifungal agents are azoles (voriconazole, fluconazole, ketoconazole, posaconazole, itraconazole) and polyenes (natamycin, amphotericin B)[16,25]. Topical natamycin (5%) is considered to be the first-line therapy for superficial infection among antifungal agents and is the only available antifungal eye drop [24,26-27]. Natamycin is preferable choice in treatment against *Fusarium* and *Aspegillus*, especially in the early period [16,28]. Currently, other effective medical therapy has attracted attention,

include antimicrobial peptides (AMPs)[23], terbinafine[27], micafungin (MCFG)[29], caspofungin[24], immunosuppressant like tacrolimus (FK506)[30] and vitamine D receptor agonist (VDRA)[31]. Corneal collagen cross-linking (CXL) has been suggested to be a promising alternative option in treatment of infectious keratitis[25,32-36]. Surgical intervention is needed for those refractory to medical means to control severe and deep ulcer, like debridment, penetrating keratoplasty (PK), amniotic membrane transplantation (AMT), lamellar keratoplasty (LK), deep anterior lamellar keratoplasty (DALK)[16,26]. However, more randomized controlled trails should be needed to assess their efficacy.

IMMUNE RECOGNITION

Pattern Recognition Receptors The corneal epithelium is the fist line[3]. The adhesion of pathogens to epithelial or endothelial cells is the first step with surface open to accessible pathegens. This may be associated with the binding between pathogen ligands and host receptors[37-38]. After being invaded, PRRs of the host indentifies specifically PAMPs on fungal surface and triggers innate immunity[3-5,8-13,39-40]. PRRs include TLR, CLRs, NLRs, and SR family[3,8,40]. TLRs, expressed on immune cells, like neutrophils, monocytes, macrophages, dendritic cells, are the main PRRs, especially TLR2 and TLR4[12-13,41-43]. The interaction between PRRs and PAMPs plays an important role in innate immune response[5,11]. Dectin-1, one of the CLRs, can recognize fungal β-glucans and α-mannose[3,41,44]. Dectin-1 is expressed in macrophages, dendritic cells, neutrophils, and corneal eptithelial cells[3,12]. The recognition activates NF-κB by Syk-CARD9 and RAF pathways[3,41]. Dectin-1 can also increase the production of IL1-β and IL-18 through NLRP3 inflammasome pathway[41]. Syk pathway is the most important signaling ways of Dectin-1[40]. A study showed that in early period of fungal defenses, Dectin-1 and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) are important[5]. Dectin-1 takes part in anti-fungal activities through recognizing fungi, recruiting neutrophils and macrophages, releasing pro-inflammatory cytokines and starting an adaptive immune response[4,8,41]. Therefore, Dectin-1 plays an essential role in pathogenesis of FK. Surfactant protein D (SP-D), a member of CLRs, plays an important role in early innate immunity of *Fusarium solani* infection[7,45]. SP-D activates through TLR4-MyD88 signaling pathway. And through the interaction with TLRs, SP-D regulates immune function by down regulating the expression of cytokines[7,45]. Lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1), belonging to SR family and also structurally CLRs, expresses in normal corneal epithelium, macrophages, neutrophils, *etc*. The special structure enables LOX-1 to have many diverse activities, recognizing substances widely. LOX-1 plays a pro-inflammatory role in host defense during FK. However, further study is needed for more detail roles of LOX-1 in FK[10]. NOD2, a kind of NLRs, takes part in the innate immunity by increasing the proinflammatory cytokines[7,46]. A recent study proposed that NOD2 triggers the inflammatory responses in *Aspergillums fumigates* keratitis through the interaction with TLRs. There are a negative regulation described in the study, "tolerance" between diverse PRR families, avoiding excessive inflammatory responses[9]. Triggering receptor expressed on myeloid cells-1 (TREM-1) is a surface receptor and amplifies the inflammation induced by *Aspergillums fumigatus*. TREM-1 has been classified that functioned synergistically with TLRs and NLRs[6,39,47].

Cytokines, Chemokines and Cellular Immunity Macrophages play a critical role in innate immunity against fungal infections. It serves as antigen presenting cells as well as phagocytes. The activation of macrophages can promote the production of inflammation cytokines and chemokines, increase polymorphonuclear (PMN) infiltration, however, also can cause an excessive immune response and lead to more severe corneal infection[1,6,41]. Neutrophils are important and active in innate immunity. Once being infected, neutrophils are the earliest infiltrating the cornea against fungi. Macrophage inflammatory protein-2 (MIP-2), intercellular cell adhesion molecule-1 (ICAM-1) and some cytokines can attract neutrophils to the inflammatory site in the cornea[1,48-49]. A study also showed that γδ T cells may play a role in recruitment and activation of neutrophils[48]. γδ T cells are essential in innate immunity, they also take part in FK by secreting cytokines and chemokines. γδ T cells also involve in the repair in trauma by secretion of chemokines like CC chemotactic factor-3, 4, 5 (CCL-3, 4, 5)[48]. interleukin-1β, 6, 8 (IL-1β, 6, 8) are found in tears of patients with FK, suggesting that inflammatory cytokines are involved in FK[14,49]. IL-6, IL-8 and MCP-1 play a significant role in inflammatory response against fungal infection[49]. IL-1β and TNF-α increase in the early period of *Aspergillus* and *Fusarium* keratitis[50]. Increase of IL-17 has been detected in tears of filamentous fungi infection. IL-17, produced by neutrophils, plays a critical role in the severity of autoimmune and inflammatory infection, resulting in protection against fungal infection. Activation of IL-17 receptors on epithelial cells increases the production of cytokines which mediate recruitment of neutrophils to infected site[44,51-54]. Antifungal anctivity depends on CD18, not Dectin-1[55]. Matrix metalloproteinases-14 (MMP14) is upregulated in the early process of trauma forming. MMPs can mediate cellular adhesion and play an essential role in fungal infection[56]. IL-10 is considered to be a negative regulator of imflammation, inhibiting proinflammation cytokines. COX-2 also can regulate immunity. Th cells are essential in immune response. Th1 cells can improve the

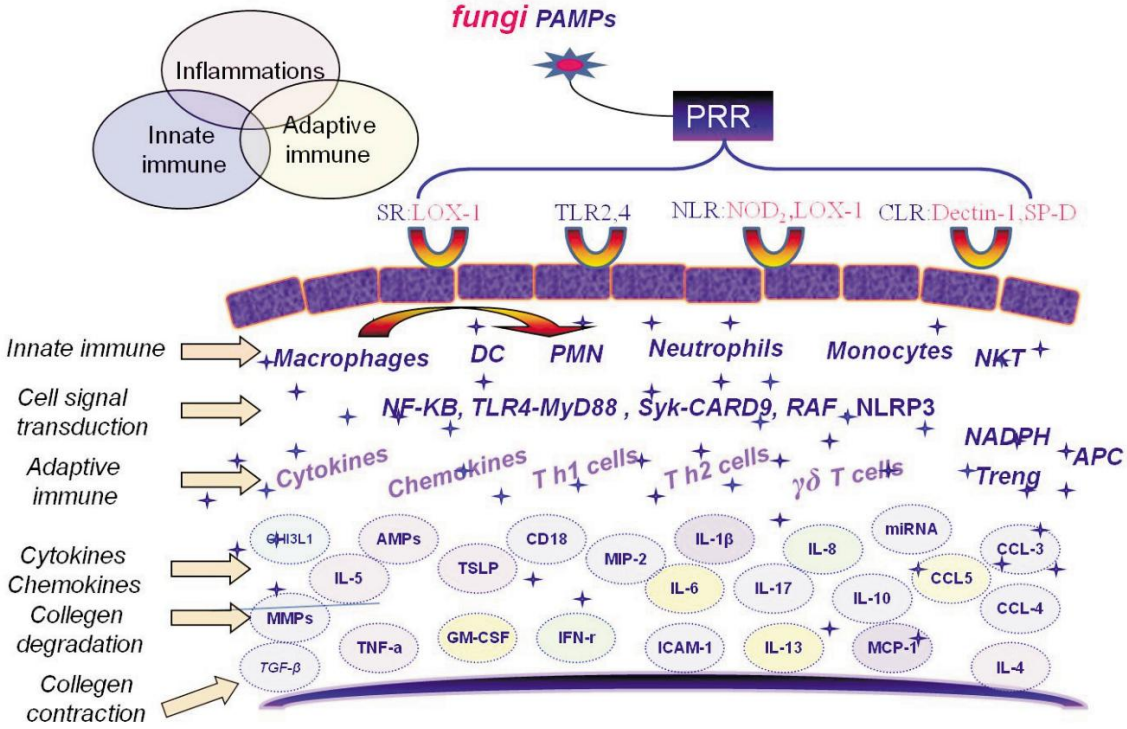

Figure 1 The mechanism of FK.

anti-fungal ability, however, Th2 cells stand in the opposite side. The balance between them may effect the outcome of the disease[57].

Humoral Immunity HCEC-derived thymic stromal lymphopoietin production (TSLP) can promote humoral immunity and take part in adaptive immune response of FK[58]. Vasoactive intestinal peptide (VIP), anti-inflammation neuropeptide, can decrease pro-inflammation cytokines, limit cellular immunity, inhibit the proliferation of macrophages and T cells, and contribute to healing in *Pseudomonas aeruginosa* keratitis[59-60]. Antimicrobial peptides (AMPs) are essential parts if innate and adaptive immunity and are considered to have wide multifunction. AMPs are chemotatic for some immune cells. AMPs provide protective effect in FK[61].

NEW TARGETS

Chitinase 3-like 1 (CHI3L1) is a multifunctional protein that plays a regulatory role in corneal innate immunity through mediating the production of chemokines AMPs, and anti-inflammatory factors. It has been implicated as a regulator of adaptive Th2 responses [62]. The role of miRNA in FK has not been classified. A recent study demonstrated that miRNAs play a regulatory role in trauma inflammation of cornea[63] (Figure 1) .

THE PROGRESSIONS OF DIAGNOSIS OF FUNGAL KERATITIS

Conventional microbiological methods remain to be the "gold standard" for the diagnosis of FK. Culture results are highly specific but insensitive and time-consuming. And expertise is required to identify the fungal specis isolates precisely [17-18,21]. Smear microscopy is a rapid and direct method. A recent study shows that the positive detection rate of smear is higher than that of culture [17]. *In vivo* confocal microscopy (IVCM) is a rapid method to diagnose FK and is non-invasive safe technique, becoming a kind of routine[19-20]. In addition, it has unique advantages that can monitor therapeutic response [19-20,64]. Currently, IVCM is the only means allowing to detect the depth of infection, helping determine the time for appropriate surgical treatment. However, smaller microbes, like bacteria and virus, are invisible. So IVCM is useless in these cases. Moreover, experienced technicians are necessary to accurate results[64]. Conflict views about IVCM have been proposed. One study did not suggest stand-alone use of IVCM while using a positive part as the reference. On the contrary, another study held that it provided precise evidence with the conventional microbiology as the gold standard, especially in deep corneal infiltrate [21]. PCR is a rapid and sensitive diagnostic method, which has been widely used in the diagnosis of infectious keratitis [17,21]. There is a disadvantage of PCR that the rate of false positive error is high because of the commensal comtaminants. Quantitation of microbes using quantitative PCR, and multiplexing of primer sets have been allowed in recent advances[65]. However, template deoxyribonucleic acid (DNA) extraction is needed. The process of DNA extraction is limited by the specimens. A direct PCR without template DNA extraction which used a special polymerase was developed to detect microbes [17]. A study used a improved DNA extraction method and fungal pathogens could be detected specifically within 8h[66].

TREATMENT OF FUNGAL KERATITIS

Medical Treatment So far, only the polyenes and azoles have been used commonly in the treatment of FK [24]. Biofilms of microorganisms are critical for resistant to antimicrobials [67]. Appropriate antifungal agents for FK are urgently needed.

Natamycin, with a broad spectrum and stong anti-fungi activity, is safe and effective at a very low concentration [68]. Currently, natamycin is considered to be the most effective topical medical agent against *Fusarium* and *Aspergillus*[16,27-28]. However, it has poor coverage against Candida species. Futhermore, natamycin can only be used topically resulting to limitation of deep stroma invasion [16]. It has been showed that voriconazole has a broad therapeutic window that covers not only filamentous fungi but also Candida. Therefore, it has been proposed as an alternative to natamycin. In cases refractory to topical natamycin, voriconazole was used as an adjunct to natamycin and showed good ocular penetration [16,27,69-70]. A study has shown that voriconazole was less effective in cases with infiltrates and hypopyon [71]. Natamycin is more effective than voriconazole in treatment of FK, especially *Fusarium* keratitis[72-75]. Natamycin has been proposed that it resulted in better visual acuity than outcome of voriconazole [76]. The susceptibility to natamycin was concerned with the size of ulcer and infiltrating. While there susceptibility to voriconazole was not associated with the outcome[77].

Amphotericin B is a drug choice for *Aspergillus* and Candida, while poor activity against Fusarium species [16,78]. The side effect of amphotericin B is that it is toxic to human cells at a higher dose. Therefore, it is not a first line drug in treatment of FK while other better agents are at hands [16]. Instrastromal injection of amphotericin B may be an adjunct for deep severe FK [79]. Intracameral amphotericin (ICAMB) can be a safe agent in FK refractory to local conventional therapy to better outcome. But ICAMB is not benefit when given alone[80-81]. A study proposed that combining intravitreal amphorericin B and voriconzole could be a new choice in endophthalmitis caused by filamentous FK[82].

Fluconazole is known because of its low side effect and good intraocular penetration [16]. The 0.2% fluconazole is effective in FK combined with 5% natamycin [83]. Subconjunctival injection of fluconazole has been found efficacious in patients unresponsive to conventional antifungal medical treatment of Candida and alternaria keratitis [16,84]. However, fluconazole has narrow coverage of filamentous fungi [16]. Oral ketoconazole combined with topical miconazole was effective in FK, but it did not add benefit to topical natamycin in deep FK [16,85]. A study said that posaconazole might be effective in Paecilomyces and Fusarium keratitis refractory to conventional treatment[86-87].

The way of injection is essential to efficiency of drugs. Intracameral and intrastromal antifungal medication have been proved to be effective[88-89]. Intrastromal injection should be used to the severe and recalcitrant keratitis[90].

It has been proposed that liposomal formulation of voriconazole and itraconazole has better anti-fungal activity and is effective in treatment of FK [91-92]. Voriconazole aqueous drops have higher penetration and can be used topically in FK[93].

Terbinafine is a efficient anti-fungal agent used in fungal skin diseases. It also inhibits the growth of fungi in cornea. It has been proved that topical terbinafine was effective in filamentous keratomycosis [25]. Tacrolimus (FK506), a novel immunosuppressant, can inhibit the inflammation caused by fungi [30]. Caspofungin eye drops seem to be a possible alternative for treatment of FK. But it still needs more randomized controlled trails[24]. Topical micafungin, inhibiting β-(1,3)-glucan synthesis, appear to be effective for FK[29].

Corneal Collagen Cross Linking CXL has been recently used as a promising and worthwhile treatment in refractory infectious keratitis, defined as PACK-CXL: photo activated chromophroe for keratitis [27,32-33,36,94]. PACK-CXL can be a available adjuvant method in the management of FK unsensitive to medical treatment [35,95-97]. CXL is a quick, efficient and less expensive means to eliminate pathogens which is non-pathogen-specific [34]. In deep stroma FK cases, CXL did not improve outcome and resulted in an increased risk of perforation [98-99]. Prospective studies are needed for further study of the anti-fungal function of PACK-CXL[100].

Therapeutic Surgical Intervention Penetrating keratoplasty (PK) is the most commom therapeutic surgery[16]. It has been suggested that early suigical management of PK was required [101]. PK is an effective method in corneal infectious and non-infectious diseases resisitant to other treatment[102]. It is critical to remove the infected tissue through surgery to vision [103]. Lamellar keratoplasty (LK) and deep anterior lamellar keratoplasty (DALK) are selected for focal invasion or infection that did not invasive into deep layers of cornea[16]. A study demonstrated that DALK using the big bubble technique appear to be effective in FK resistant to medical treatment [104]. Acellular porcine corneal stromas (APCSs) grafts are safe and effective during LK in FK[105]. Higher rate of graft rejection, infection recurrence, secondary glaucoma in therapeutic corneal transplantation, but it remains to be an effective method for refractory FK[16,106]. Amniotic membrane transplantation (AMT) is considered as an expedient to prevent PK secondary infection[16].

New Methods A study used a novel combination of cryotherapy and anti-fungal agents to treat ulcer of FK and found that cryotherapy was effective in treating ulcer. Rose Bengal-mediated photodynamic therapy (PDT) can inhibit the growth of fungi. It may contribute to useful treatment for

infectious keratitis [107]. With the role of VDR in innate immunity being discovered gradually, a new target of treatment can be explored for FK [31]. All-trans retinoic acids (ATRA) is proved to have anti-inflammatory and immunoregulatory effects [50]. However, it is difficult to put the new targets into use clinically. We still need to do more clinical trials.

CONCLUSION AND FUTURE PERSPECTIVE

Recent studies have revealed deeper understanding of the pathogenesis of FK. Current method of diagnosis and drugs are not effective enough, newer, more effective and promising means are urgent for better outcome. For instance, the immunotherapeutic modalities has attracted more and more attention. At the same time, clinical trials are needed to find new strategies that contribute to more effective control of the infection.

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