·Basic Research·

Effects of COX -2 inhibitor NS -398 on IL -10 expression in rat fungal keratitis

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Abstract

• AIM: To investigate the expression of interleukin-10 (IL-10) and the effect of NS-398 (COX-2 inhibitor) on the expression of IL-10 in fungal keratitis in rats, and analyze its effects on anti-fungus immunity.

• METHODS: Ninety Wister rats were randomly divided into 3 groups. Group A was blank control group (10 eyes). Group B was fungal keratitis group (40 eyes). Group C was fungal keratitis group treated with NS-398 (40 eyes). PAS staining, 100g/L potassium hydroxide (KOH) smear and fungal culture confirmed the successful establishment of fungal keratitis model. After the central epithelium was scraped, Fusarium solani colonies were applied and contact lens was put on the right cornea of group B and C, and plane contact lens was put on the left cornea of control eyes. Phosphate buffered saline (PBS) eyedrops were given for group B and NS-398 eyedrops for group C. The expression of IL-10 on corneas of group B and C on the 1st day, 3rd days, 7th days, and 14th days were detected by immunohistochemistry and semi- quantitative reverse transcription- polymerase chain reaction (RT-PCR).

• RESULTS: Histopathologic examination showed neutrophil infiltration and severe tissue necrosis in ulcer cornea. PAS staining confirmed the existence of hyphae and spores in the superficial layer of stroma. In the blank and control groups almost no expression of IL-10 was detected at any observing points. In group B the expression of IL-10 increased at first and decreased thereafter. Its expression also showed significant difference at any observing points ($P<0.01$). Compared with group B, the expression of IL-10 in group C showed no difference on the 1stday, decrease on the 3rd day, but a significant increase on the 7th day and 14th day.

• CONCLUSION: IL-10 takes part in the occurrence and development of fungal keratitis. NS-398 can upgrade the expression of IL-10 in fungal keratitis in the later period of the ulcer. Meanwhile, pathologic observation showed a slightly corneal opacity. IL-10 may play an important role in the process of cornea anti-damage repair.

• KEYWORDS: fungal keratitis; immune regulation; IL-10; NS-398
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INTRODUCTION

The cases of ocular fungal infection increased significantly in the last decade in China [1]. Because unreasonable or non-standardized usage of antibiotics and corticosteroids and lacking of drugs sensitivity to certain fungi, it may be controlled ineffectively at the early stage, resulting in serious impact on visual function. Therefore, it is of great significance to do further study on the occurrence and development mechanism of it. When infection occurs, there is a fight between fungal organism and host immune system, and it is always accompanied with autoimmune injury in this process. IL-10 is a critical negative regulator of inflammation and has an immunosuppressive activity [2]. It acts as a balance factor and inhibits proinflammatory cytokines synthesis in many tissues [3]. COX-2 has an effect of immune regulation. COX-2 inhibitors can enhance the expression of IL-10 [4]. In our research we established the model of fungal keratitis, and treated it by NS-398 (specific inhibitor of COX-2). Then we analyzed the expression of IL-10 in infected group and NS-398 treated group, which will further clarify the role of immune regulatory factors in fungal keratitis. This may enhance our understanding of pathogenesis and provide theoretical basis for immunotherapy.

MATERIALS AND METHODS

Materials A total of 90 Wister rats (both male and female) were purchased from Qingdao Institute of Drug Control (Qingdao, China). All rats weighed between 200g-300g. Animals with corneal disease were excluded by slit lamp examination. Ninety eyes were randomly divided into 3

groups: 10 eyes for Group A (blank control group), 40 for Group B (fungal keratitis group), 40 for Group C (fungal keratitis group treated with NS-398). We chose the right eyes as the experiment one in groups B and C, the left eye with contact lenses as the control one. Levofloxacin eyedrops was given 3 times one day, and loxacin ointment was used every night before experiment. 10% chloral hydrate 3mL/kg for intraperitoneal injection, 0.4% oxybuprocaine hydrochloride eyedrops for surface anesthesia. Cleaning of conjunctival sac was done by using Yasuji iodine (0.5%) for routine disinfection. Central epithelium of cornea was scraped about 3mm-4mm in diameter, and the surface of cornea was scratched with a gauze sterile needle into stromal layer under microscope. Then the surface of cornea was smeared with colonies of Fusarium (about 3mm-4mm in diameter), and covered with contact lenses. Finally, 5-0 black silk suture closed eyelid. Nothing was done for the left cornea, except for laying a plane contact lens before closing the eyelids. The contact lenses were removed after 24 hours. Group B was given PBS eyedrops, and group C was given NS-398 eye drops, 100μmol/L, 4 times a day until the eyes were enucleated.

Methods After eyelid suture was removed, scraping of some corneal tissue between lesions and normal cornea was done for 100g/L potassium hydroxide smear observation, and also sent for fungus culture. Cornea was monitored daily with the slit lamp and pictures were taken with digital camera. Animals were killed respectively by overdose of chloral hydrate at the 1st, 3rd, 7th and 14th day. The eyeball was removed under sterile conditions. The cornea was divided into two parts; one half was fixed with 40g/L formaldehyde solution for PAS staining and immunohistochemical observation, the other half was preserved into Epoxy (EP) tube that had been treated with diethypyrocarbonate (DEPC) water and high pressure sterilization, then stored in -80℃ refrigerator.

After the eyelid sutures were removed, the contact lens was found to be on the cornea. After removing it, we found the cornea was edematous and rough. Slit lamp examination showed that the corneas in blank control and control groups were in good condition at each time point. Fungal model group showed corneal edema and rough surface at the first day. On the third day, it showed a grayish necrosis in the center with severe tissue congestion in the surrounding area. Seven days after operation, the degree of necrosis was aggravated. The central area of ulcer was slightly protruding from the surface. After 14 days, the ulcer almost subsided; we can see gray haze and scarring of the cornea with large amount of new blood vessels from the limbus growing towards the center of cornea. Drug treatment group showed the same course, but there was less corneal opacity and neovascularization on the 14th day than that in group without drug treatment.

Potassium hydroxide smear examination under the microscope showed a large number of mycelia and spores, the hyphae elongated as branches, and we also can see sickle or crescent shaped conidia. The hyphae and spores was purple stained, and has different shape in lesion area by PAS staining; the hyphae grew in horizontal, oblique or vertical direction, mainly in the shallow surface ulceration and necrosis of matrix layer, but less in the deep stroma. Pink and white fungus colony formed in Sand castle's medium, at 25℃ in CO_2 incubator for 10 days.

IL -10 immunohistochemistry Cornea specimens were fixed for 24 hours , then graded by ethanol dehydration and xylene; they were dipped in wax overnight and embedded in paraffin. Then they were trimmed and made into serial sections of 2μm thickness. These specimens were mounted on ordinary glass slide and the positively charged anti-off slide, stained for PAS and immunohistochemical observation. Immunohistochemical staining was done by SP method. We used normal lung tissue as a positive control, whereas the negative control was used by 0.01mol/L PBS instead of primary antibody, and the remaining steps are the same. Then it was observed and photographed under the microscope. We defined positive criteria as cytoplasm and/or cell membrane showed brown particles. Randomly-selected 5 views from each slice at high magnification were taken and the average optical density of IL-10 staining was analyzed using a VIDAS-21 image analysis system. Five sections in each group were selected for quantitative analysis.

IL-10 mRNA RT-PCR RNA of cornea were extracted by Trizol, *A* values of total RNA prepared were between 1.8-2.0 measured by UV spectrophotometer. We began RT-PCR reaction after adjusting the RNA concentration. The primer of IL-10 and selected β-actin were designed as internal reference. IL-10 upstream 5'-TGCCTTCAGT CAAGTGAAGAC-3 ', downstream 5'-AAA CTCATTCAT GGCCTTGTA-3', amplification product length is 346bp; β-actin upstream 5'-ATCATGTTTGAGACCTTCAAC-3', downstream 5'-CATCTCTTGCTCGAAGTCCA-3' PCR product length is 317bp. Primers were synthesized by Shanghai Biological Engineering Technology Engineering Service Co., Ltd. We used the PrimeScript RT-PCR Kit of Takara Company for RT-PCR and operated according to the manual. Total reaction volume was up to 20μL. Conditions: reverse transcription: 42℃ 30min, 95℃ 5min, PCR amplification: 94℃ 30s, 55℃ (IL-10), 55℃ (β-actin) 30s, 72℃ 40s 38 cycles (IL-10), 38 cycles (β-actin), loop end of

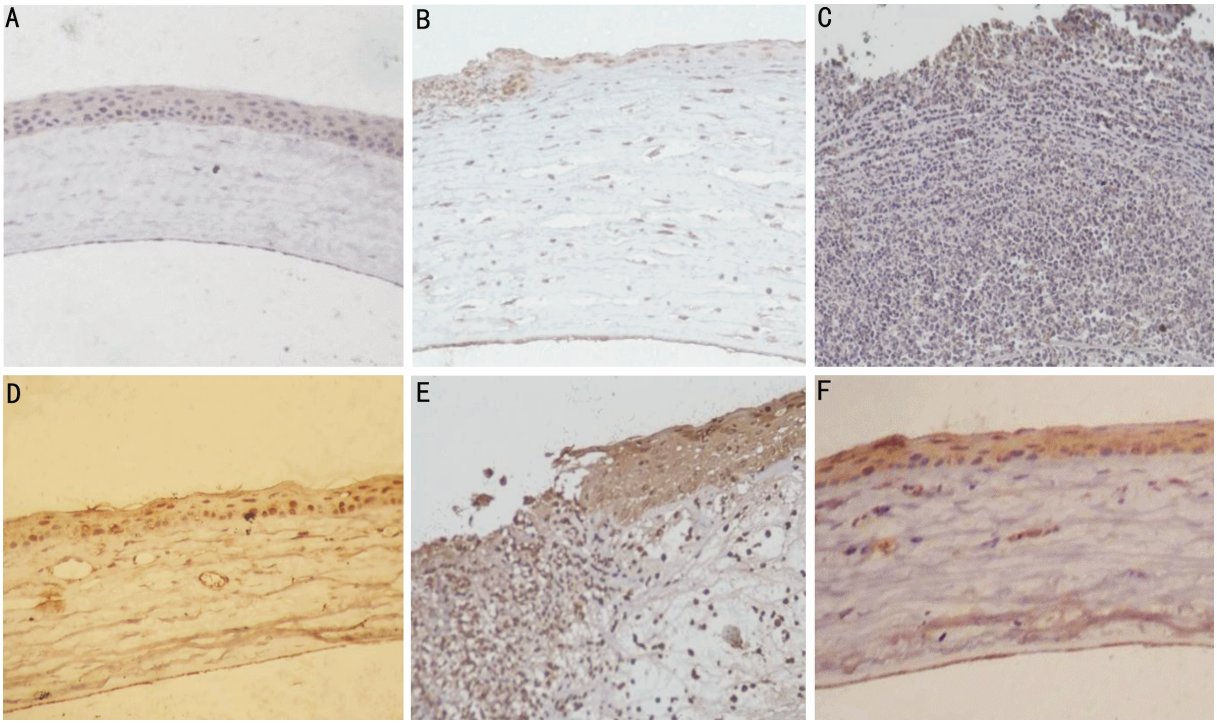

Figure 1 IL-10 expression in rat cornea (SP×200) A: Normal SP×200; B: 1st day of infection SP×200; C: 7th day of infection SP×200; D: 14th day of infection SP×200; E: 7th day of treatment SP×200; F: 14th day of treatment SP×200

the terminal extension at 72℃ 10min. PCR products were under electrophoresis with 15g/L agarose gel, voltage 120V, staining with 0.5μg/mL ethidium bromide 30min later, the gel imaging was observed by UV image system. The picture for the electrophoresis gel imager was taken and analyzed by Quantity One analysis quantitative software. The IL-10 and β-actin points VIS absorption degree (A) value was measured and the ratio of $A_{\text{IL-10}}/A_{\beta\text{-actin}}$, the relative integral absorbance (relative integral absorbance, RIA) was calculated, and the result was used as the relative expression of IL-10mRNA in each group.

Statistical Analysis Analysis was done by using CS11.58 statistical software. The data were indicated by mean±SD. Statistical analysis of the relative expression of IL-10 was made in each group and variance analysis was used to do overall comparison of each group. We used t-test between the two groups and q -test in groups. P <0.05 was considered as statistically significant.

RESULTS

IL-10 Expression IL-10's positive staining appeared in the cytoplasm of macrophages, alveolar epithelium of lung tissue as the positive control, and the results showed no color in inflamed cornea as a negative control with PBS. IL-10 in the blank control and experimental control group showed almost no expression of the cornea (Figure 1A). In fungal model group, IL-10 expressed mainly in the epithelium and stromal layer, the IL-10 began to express one days after the operation, and on the third and seventh days, there was a significantly increased expression and infiltration of inflammatory cells, the tissue showed epithelial lesion and severe necrosis, on the fourteen day the expression was significantly weakened, lamellar structure of matrix was disordered and angiogenesis was appeared (Figure1B, C, D). The expression of IL-10 was increased in treatment groups compared to model groups on the third and seventh days (Figure1E, F). The average optical density number of IL-10 positive of cells was measured with the computer image analysis system. The average absorbance value of the two groups at each time point was showed in Table 1.

Table 1 IL-10 absorbance in fungi keratitis (Mean±SD, A^{-3}, n=5)

t/d	Fungal model	NS-398
1	0.2464±0.0022	0.2520±0.0045
3	0.7021±0.0021	0.6012±0.0034[b]
7	0.6734±0.0037	0.8421±0.0029[b]
14	0.1363±0.0046	0.8045±.0.0025[b]

[b]P<0.01, *vs* fungal model

IL -10 mRNA Expression The stable expression of reference gene--β-actin can be detected in each group. electrophoresis of PCR product indicated the length of PCR product for β-actin gene fragment was 317bp, confirmed the integrity of cDNA from reverse transcription, and the PCR reaction was successful. No expression of IL-10mRNA was detected in the blank control group and control group. The results of gel electrophoresis for PCR product was analyzed by the UV image imaging system. There were significantly

different expression of IL-10mRNA in the control group and model group at different time points (P<0.01, Figure 2). From Figure 2 we can know IL-10 began to express on the first day after modeling, with times went on the expression was continue increasing, the strongest expression was appeared on the third day, on the seventh day the expression began to decrease but still expressed weakly on the fourteen day. No significant difference in the model group and drug treatment group on the first day (P>0.05).On the third day the latter showed decrease than the former, but increased on the seventh and fourteenth day, with significant difference (P<0.01, Figure 3).

DISCUSSION

There are different types of fungi in nature. About 70 species of them may cause corneal infections[5]. The positive rate of fungi culture in conjunctival sac among healthy people was about 2%-25%. In our study, we established the animal model by Fusarium solani which is the common pathogenic strains. We found the early stage of fungal keratitis was mainly suppurative necrosis, then scar at the late stage of infection. The imbalance between pathogenic fungi virulence and host defense has been recognized as an important pathogenesis. Fungal keratitis usually occurs in patients who were on a long-term steroid or immunosuppressive therapy. It suggested that the immune status has a relationship with fungal susceptibility. In the early stage of infection, innate immunity was activated rapidly, large number of inflammatory cells began infiltrating, but in the middle and late stage, T lymphocytes increased gradually and stayed in the dominant position [6]. By our knowledge, cellular immunity has been in a dominant position when resistant to Candida albicans and Aspergillus fumigatus infection. Clinical observations indicated that Candida albicans infections usually occurred in individuals with immune dysfunction [7]. In the cellular immune response, the helper T cells (T helpers, Th) play an important part in the induction, reaction and effect stage of immune response. It has two subtypes, Th1and Th2. Th1 cells can enhance the body's anti-fungal ability, but Th2 has the opposite effect. Balance between Th1 and Th2 may be regulated by a variety of cytokines, which may influence the outcome of infections. When macrophages were stimulated by pathogens, Th produced cytokines of Th1-type such as IL-12 which activates the immune system to kill the pathogens. Th1-type response's protective effect may play the major role in this period. With time goes on, Th2 type cytokines such as L-10, IL-4 appeared largely, at the same time the inflammatory responses of Th1-type was reduced, finally, Th1/Th2 reach to their new balance[8].

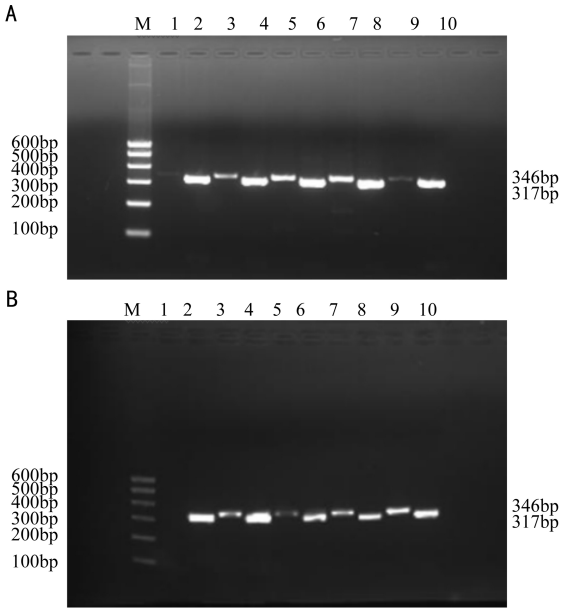

Figure 2 IL-10 mRNA expression A:Fungal group; B: Treatment group

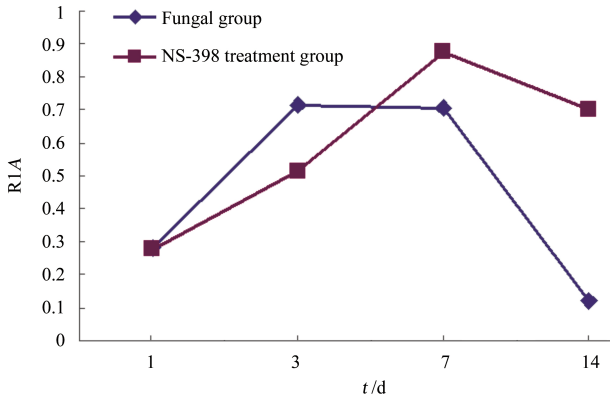

Figure 3 IL -10 mRNA expression in rat fungal keratitis showing NS-398 can increase the expression of IL-10 at late stage of the disease

In this study, we chose IL-10 as the immunological parameter because IL-10 is the negative regulatory factor of immune system, and also played an important role on of the differentiation of Th cell subsets. It is mainly produced by Th2 type T lymphocytes, but can also be secreted by macrophages and B cells. IL-10 inhibits the secretion of Th1-type factors by macrophages, such as IL-1, IL-6, TNF-α, and played an immunosuppressive effect by inducting the response of Th2-type indirectly. At the same time, as an important regulator of the maturation of dendritic cells, IL-10 can also impair hypersensitivity by inhibiting the maturation of dendritic cells, and may maintain this state by generating regulatory T cells. IL-10 could inhibit mature DC from secretion of IL-12, and reduce its ability to promote Th1 response. Previous studies of Candida albicans [9,10] found that IL-10 can inhibit the phagocytosis of macrophage, monocyte and neutrophil,

promote the activation of Th2 cells, and weaken the body's ability to resist fungus by inhibiting the proliferation of Th1 cells. We found that the expression of IL-10 has been associated with the progression of this disease. Normal cornea has almost no expression of IL-10, but the expression can be detected in fungal keratitis. It arose on the first day, and expressed stronger on the third and seventh day, at the same time pathological observation indicated the significantly damage of tissue, and large number of hyphae and spores grew and invaded into deep matrix. These results were the same as some of domestic research [6], and suggested that the pathological changes may be related to the reduction of phagocytosis of inflammatory cells by inhibited the effect of IL-10 to secrete the protective cytokines of Th1 type.

COX-2 is a sensitive inflammatory cytokines. It is widely involved in the development of eye tissue, inflammation process, generation of stress response and tumors and so on. It can make the imbalance of immune function, which may help the COX-2 to make inflammatory injury of body. Yokoyama *et al* [11] found that the expression of IL-4 and IL-10 increased significantly in the heart transplantation model of rat pretreated by the selective COX-2 inhibitor-NS-398, and CD4 + regulator cells can also be induced; and the results suggested that COX-2 may take part in inflammatory injury by breaking the balance of immune system.

In our study, the expression of IL-10 was significantly increased in NS-398 treatment group compared with fungi model group at the seventh days and the fourteen days after infection. The pathological observation showed scarring and turbidity performance of ulcer lesion in NS-398 treatment group are much more better than fungal group in the late stage of infection, which suggested that the expression of IL-10 are related to the destruction of inflammation and suppression of scar formation at the late stage of infection. In the study of experimental autoimmune encephalomyelitis, Ni *et al*[4] confirmed that the COX-2 inhibitor rofecoxib and celecoxib could enhance the expression of IL-10. Further experiments showed that COX-2 inhibitor has no significant effect on the process of antigen-presenting cells to secrete IL-10, and then speculated that the increase secretion of IL-10 was from T cells. In the study of herpes simplex keratitis (HSK) which has mainly T lymphocyte infiltration, the opacity and the formation of new blood vessels of cornea can be reduced by giving an injection of recombinant IL-10; and the researchers speculated that IL-10 may reduce damage by inhibiting the synthesis of Th1 type cytokines and proliferation of monocyte[12]. We can speculate from our study that the reduction of scar in the late stage may be because that hyphae and spores has been eliminated basically and cellular immunity played an important part in the lesions, at this time we used NS-398 to increase the expression of IL-10 which inhibits the secretion of Th1 type cytokines, and then played role on anti-inflammatory, anti-immunity, and prevented the opacity of cornea from excessive inflammatory damage.

We observed that the expression of IL-10 was negatively correlated with disease progress, and its expression in NS-398 group was higher than those in model group at the seventh days and the fourteen days after infection. We hypothesized that the high levels of IL-10 may have a relationship with tissue repair in the late stage; IL-10 may be involved in the process of tissue repair and resistant to injury by its roles of immune regulation. This observation gives us the inspiration for clinical treatment of fungal keratitis according to their stages. However, the specific mechanism of IL-10 expression mediated by NS-398 treatment is still not clear, further research needs to be done. In addition, there are a variety of cytokines and complex immune response participation in fungal keratitis; and further study should be done to recognize the relationship of those immune factors and confirm their interaction mechanism.

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