·Basic Research·

Inhibitory effect of CCR3 signal on alkali-induced corneal neovascularization

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Abstract

• AIM: To investigate the effect of CC chemokine receptor 3 (CCR3) signal on corneal neovascularization (CRNV) induced by alkali burn and to explore its mechanism.

• METHODS: Specific pathogen-free male BALB/C mice (aged 6-8 weeks) were randomly divided into CCR3-antagonist treated group (experimental group) and control group. CRNV was induced by alkali burn in mice. The time kinetic CCR3 expression in injured corneas was examined by reverse transcription polymerase chain reaction (RT-PCR). CCR3-antagonist (SB-328437 at different concentration of 125μg/mL, 250μg/mL, and 500μg/mL) was locally administrated after alkali injury. The formation of CRNV was assessed by CD31 corneal whole mount staining at two weeks after injury. Monocyte chemotactic protein 1 (MCP-1), monocyte chemotactic protein 3 (MCP-3) expressions in the early phase after injury were quantified and compared by RT-PCR. Macrophage intracorneal accumulation in the early phase after injury was evaluated and compared by immunohistochemistry.

• RESULTS: Alkali injury induced the time kinetic intracorneal CCR3 expression. 500μg/mL of CCR3-antagonist treatment in the early phase but not the late phase resulted in significant impaired CRNV as compared to control group (P <0.05). CCR3-antagonist treatment in the early phase significantly reduced the intracorneal MCP-1 and MCP-3 enhancement compare to control group at day 2 and day 4 (P <0.05). Moreover, the number of intracorneal macrophage infiltration in the experimental group was reduced than those in control group at day 4 (P <0.05).

• CONCLUSION: CCR3 signal is involved in alkali-induced CRNV. CCR3-antagonist can inhibit alkali-induced CRNV by reducing the intracorneal MCP-1 and MCP-3 mRNA expression and the intracorneal macrophage infiltration.

• KEYWORDS: corneal neovascularization; CCR3; monocyte chemotactic protein 1; monocyte chemotactic protein 3; macrophage

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INTRODUCTION

The cornea is characterized by the absence of blood vessels under physiological conditions, which is preserved by the balance between pro-angiogenic and anti-angiogenic molecules, and is required for optical clarity and visual acuity. When the pathological causes such as inflammation, corneal infections, misuse of contact lenses, chemical burn and so on break the balance of pro-angiogenic and anti-angiogenic molecules, the vessels were then growing into the transparent corneal from corneal limbus, which lead to severe impaired vision [1]. It is necessary to develop effective measures to prevent and treat corneal neovascularization (CRNV) based on understanding of its pathogenesis at the cellular and molecular levels. Accumulating evidence indicates that chemokine and chemokine receptor, such as CXCR2/IL-8, MIP-2, CXCR4/CXCL12,CCR1/CCL3, CCR2/MCP-1, CCR5/MIP-1, MIP-1β, RANTES, and CX3CR1/CX3CL1, were involved in the development of CRNV [2].

CC chemokine receptor 3 (CCR3), the G-protein-coupled receptor, was well known for its role in promoting eosinophil and mast cell trafficking. However, in 2009, Takeda *et al* [3] reported that CCR3 and its ligands eotaxin-1, -2 and -3 were specifically expressed on endothelial cells in human choroidal neovascularization (CNV) tissue with age-related macular degeneration (AMD). Neither eosinophils nor mast cells were present in human CNV. CNV was

suppressed by CCR3 blockade, which was due to direct inhibition of endothelial cell proliferation, and independence of macrophage and neutrophil recruitment. Moreover, several independent studies suggested that CCR3 might be responsible for CNV in human with AMD [4-6]. On the other hand, a recent study reported that CCR3 was not important in CNV development of gelatinous protein mixture model[7]. Liclican *et al* [8] observed that CCR3 gene expression was markedly increased 2 days after suture injury compared with normal corneas. More importantly, topical PGE2 treatment increased CCR3 expression compared with suture alone. Therefore, more investigative work regarding the potential role of CCR3 signal on CRNV needs to be done.

In the early phase of alkali injury induced CRNV, there were a lot of inflammatory cells infiltrated into the corneal tissue. We previously observed in the early phase after alkali injury induced experimental CRNV, leukocyte including neutrophils and macrophages recruited to the injured corneas, and then produced serials of the chemokines and pro-inflammatory factors [9]. Moreover, we reported that CCR2-expressing macrophage may be pro-angiogenic, and we also demonstrated that Monocyte chemotactic protein (MCP-1), a specific ligand for CCR2 can induce peritoneal macrophages to express vascular endothelial growth factor (VEGF), which is a key molecular promoting the development of neovascularization [10]. The monocyte chemotactic protein 3 (MCP-3), belongs to the MCP subgroup can promote chemotaxis of immune cells, is a nonspecific lig and of CCR2 and CCR3. Okada *et al*[11] reported that MCP-3 can also promote the chemotaxis of macrophages.

Based upon these studies, it is tempting to speculate that CCR3 may be involved in the development of CRNV. In this study, we compared the molecular and cellular pathological changes in CCR3-antagonist treated mice with control mice. We found the relationship between CCR3, MCP-1, and MCP-3 and macrophage infiltration in alkali-induced CRNV[12].

MATERIALS AND METHODS

Reagents and Antibodies A small molecule CCR3 selectivity antagonist (SB 328437) was purchased from Calbiochem (Darmstadt, Germany), which inhibits eotaxin, eotaxin-2 and MCP-4-mediated chemotaxis (IC 50 values: 32, 25 and 55nM, respectively). SB 328437 was dissolved in dimethyl sulfoxide and used at concentrations of (125μg/mL, 250μg/mL, 500μg/mL). Avertin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Rat anti-mouse CD31 (MEC13.3) mAbs were purchased from BD Pharmingen (San Diego, CA). Alexa Fluor 488 donkey anti-rat IgG was purchased from Invitrogen Life Technologies (Carlsbad, CA). Rat anti-mouse F4/80 (clone A3-1) monoclonal antibodies were purchased from Serotec (Oxford, United Kingdom). RNA easy Mini Kit, Omniscript RT Kit, and RNALate were obtained from Qiagen (Gene Company, Shanghai, China). Takara Biotechnology (Dalian, China) supplied the polymerase chain reaction (PCR) kit.

Mice Specific pathogen-free male BALB/C mice (aged 6-8 weeks) were obtained from the Shanghai SLAC Laboratory Animal Co. (Shanghai, China). Animals were kept in our animal facility under specific pathogen-free conditions and fed regular lab chow and water ad libitum. All animal experiments were performed in accordance with the Association for the Use of Animals in Ophthalmic and Vision Research and complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals of Chinese Medical Academy and the Soochow University Animal Care Committee.

Alkali –induced corneal injury model and CCR3 – antagonist treatment Eighty male BALB/c mice were divided into 8 groups. Each group contained 10 mice. Corneal injury was induced by placing a 2mm filter disk saturated with 1mol/L NaOH on the left eye of the mouse for 40 seconds as previously described[15]. CCR3-antagonist was immediately dissolved in 2g/L sodium hyaluronate (Sigma-Aldrich, St. Louis, MO) before the topical application. Four group's alkali-treated eyes received 5μL of CCR3-atagonist dissolved in 2g/L sodium hyaluronate at the concentration of 125μg/mL, 250μg/mL and 500μg/mL, or 5μL of 2g/L sodium hyaluronate as vehicle three times per day for 7 days immediately after alkali injury, respectively. Another 4 group's alkali-treated eyes received 5μL of CCR3-atagonist dissolved in 2g/L sodium hyaluronate at the concentration of 125μg/mL, 250μg/mL and 500μg/mL, or 5μL of 2g/L sodium hyaluronate as vehicle three times per day for 2 weeks from day 7 after alkali injury respectively. At the indicated time after alkali injury, mice were sacrificed and the corneas were removed and fixed in acetone for 20 minutes for whole mount CD31 staining. In other experiments for target gene detection or F4/80 immuohistochemical staining, 66 BALB/c mice were divided into 2 groups. Each group contained 33 mice. After corneal injury, eyes received 5μL of CCR3-atagonist (500μg/mL) or vehicle three times a day. For target gene detection, at the indicated time intervals, 5 mice in each group were sacrificed. For F4/80 staining, at the indicated time intervals, 9 mice in each group were sacrificed. The eyes were snap-frozen in OCT compound for histological analysis, or the corneas were removed and placed immediately into RNALate (Qiagen, Shanghai, China), and kept at -86℃ until total RNA extraction was performed. Each experiment step was repeated at least three times.

Biomicroscopic examination Under anesthesia, photographs of corneas were obtained using a digital camera (Nikon, Tokyo, Japan) linked to the slit lamp from Haag streit (BQ 900, Swiss made), on day 14.

Table 1 Specific set of primers and conditions of PCR

Primers	Nucleotide sequence(5'→3') sense/anti-sense	Annealing Temperature(℃)	PCR Cycles
CCR3	5'-TGTGTTTGCCCTTCGAGCCCG-3' 5'-CCCTCTGGATAGCGAGGACTGC-3'	59	40
MCP-1	5'-TGCTGTTCACAGTTGCCGGCT-3' 5'-TTGGGACACCTGCTGCTGGT-3'	58	37
MCP-3	5'-GCCACGCTTCTGTGCCTGCT-3' 5'-ACAGCTTCCCAGGGACACCG-3'	58	37
GAPDH	5'-ACCACAGTCCATGCCATCAC-3' 5'-TCCACCACCCTGTTGCTGTA-3'	58	25

Detection of corneal neovascularization The areas of corneal whole mount were stained with CD31, and blood vessels in the corneas were measured according to previous reports [8]. In brief, corneas were separated from the entire eyes with ophthalmic operating scissors under ophthalmic operating microscope and were fixed in acetone for 20 minutes. And then corneas were washed with phosphate-buffered saline (PBS), and incubated with 10% donkey serum for 1 hour. Thereafter, the corneas were stained with rat anti-mouse CD31 (1:150; BD Pharmingen) at 4℃ overnight, washed, and then incubated with Alexa Fluor 488 donkey anti-rat IgG (1/100) for 1 hour at room temperature in the dark and detected by microscope. Digital pictures of the flat mounts were taken. Then, the area covered by CD31 was measured morphometrically on these flat mounts using NIH Image software (National Institutes of Health, Bethesda, MD). The total corneal area was outlined using the innermost vessels of the limbal arcade as the border. The total area of neovascularization was then normalized to the total corneal area, and the percentage of the corneal covered by vessels was calculated. The relative neovascular area was compared between experiment group and control group.

Semi-quantitative reverse transcription-polymerase chain reaction Total RNAs were extracted from the corneas with the use of the RNeasy Mini Kit (Qiagen), and the resultant RNA preparations were further treated with ribonuclease-free deoxyribonuc (Life Technologies, Inc, Gaithersburg, MD) to remove genomic DNA. 2μg of total RNA was reverse transcribed at 42℃ for 1 hour in 20μL of reaction mixture containing mouse moloney leukemia virus reverse transcriptase and hexanucleotide random primers (Qiagen). Serially, 2-fold diluted complementary DNA (cDNA) was amplified for GAPDH (Table 1) to estimate the amount of transcribed cDNA. Then, equal amounts of cDNA products were amplified for the target genes using the primers under the following conditions: denaturation at 94℃ for 2 minutes, followed by optimal cycles of 20 seconds at 94℃, 30 seconds at 58℃-59℃, 30 seconds at 72℃, and a final 8-minute extension step at 72℃ (Table 1). The amplified polymerase chain reaction products were fractionated on a 25g/L agarose gel and visualized by ethidium bromide staining. The band intensities were measured and the ratios to GAPDH were determined with the aid of National Institutes of Health Image Analysis software.

Immunohistochemical analysis The eight micrometer-thick fixed cryosections were subjected to immuohisto-chemical staining. Endogenous peroxidases were quenched in 0.3% (vol/vol) hydrogen peroxide for 30 minutes. After washing with PBS, the slides were incubated with blocking reagent for 1 hour. Thereafter, the sections were incubated with rat anti-mouse F4/80 (1:120) overnight at 4℃. The slides were further incubated with biotin-conjugated anti-rat Ig antibody as the second antibodies. The immune complexes were detected by using an ABC kit and a DAB Substrate Kit from Vector Laboratories according to the manufacturer's instructions. Slides were then counterstained with hematoxylin and mounted. The numbers of positive cells were counted on 5 randomly chosen fields of corneal sections in each animal at 400-fold magnification by an examiner without any prior knowledge of the experimental procedures. The numbers of positive cells per square micrometer were calculated as described previously[9].

Statistical Analysis The means and standard error of the mean were calculated on all parameters determined in the study. Data were analyzed statistically using 2-tailed Student's t test with statistic software SPSS 13.0. A value of $P<0.05$ was accepted as statistically significant.

RESULTS

Dynamic intracorneal expression of CCR3 after alkali injury Published data indicates that CCR3 participated in the development of CNV in AMD, and CCR3 blockage was effective to reduce CNV [3]. We speculate that CCR3 may be also expressed in the corneal tissue after alkali injury, and CCR3 blockage may inhibit the development of CRNV. In order to confirm our assumption, we first examined the intracorneal mRNA expression of CCR3 by RT-PCR. We found CCR3 was time kinetic expressed in corneal tissues at the time point of day 0, day 2, day 4, day 7, and day 14 after alkali injury we determined (Figure 1). This result suggested the possible involvement of CCR3 signal in alkali injury induced CRNV.

Effect of CCR3-antagonist on alkali-induced corneal neovascularization To observe the effect of CCR3 signal on CRNV, the alkali-injured corneas were locally

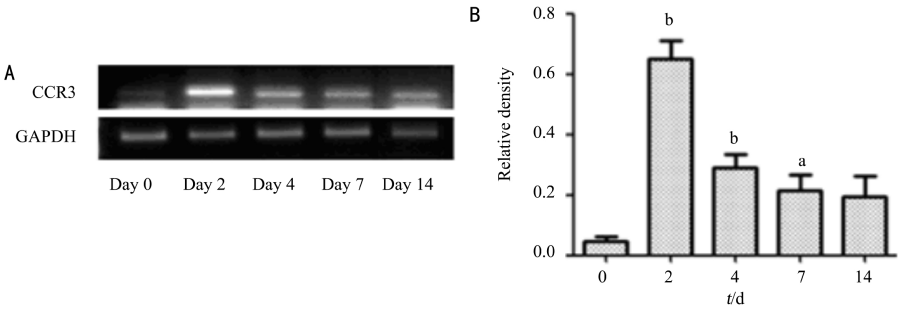

Figure 1 Expression of CCR3 in corneas after alkali injury A: Semi-quantitative RT-PCR to assess mRNA expression of CCR3. Corneas were harvested at the indicated time points, and five corneas at each time point were pooled to extract total RNAs. RT-PCR was performed using the obtained total RNAs; B: The ratios of CCR3 to GAPDH were determined. All values represent mean±SEM of the three to five independent measurements. $^{a}P<0.05$, $^{b}P<0.01$ *vs* day 0.

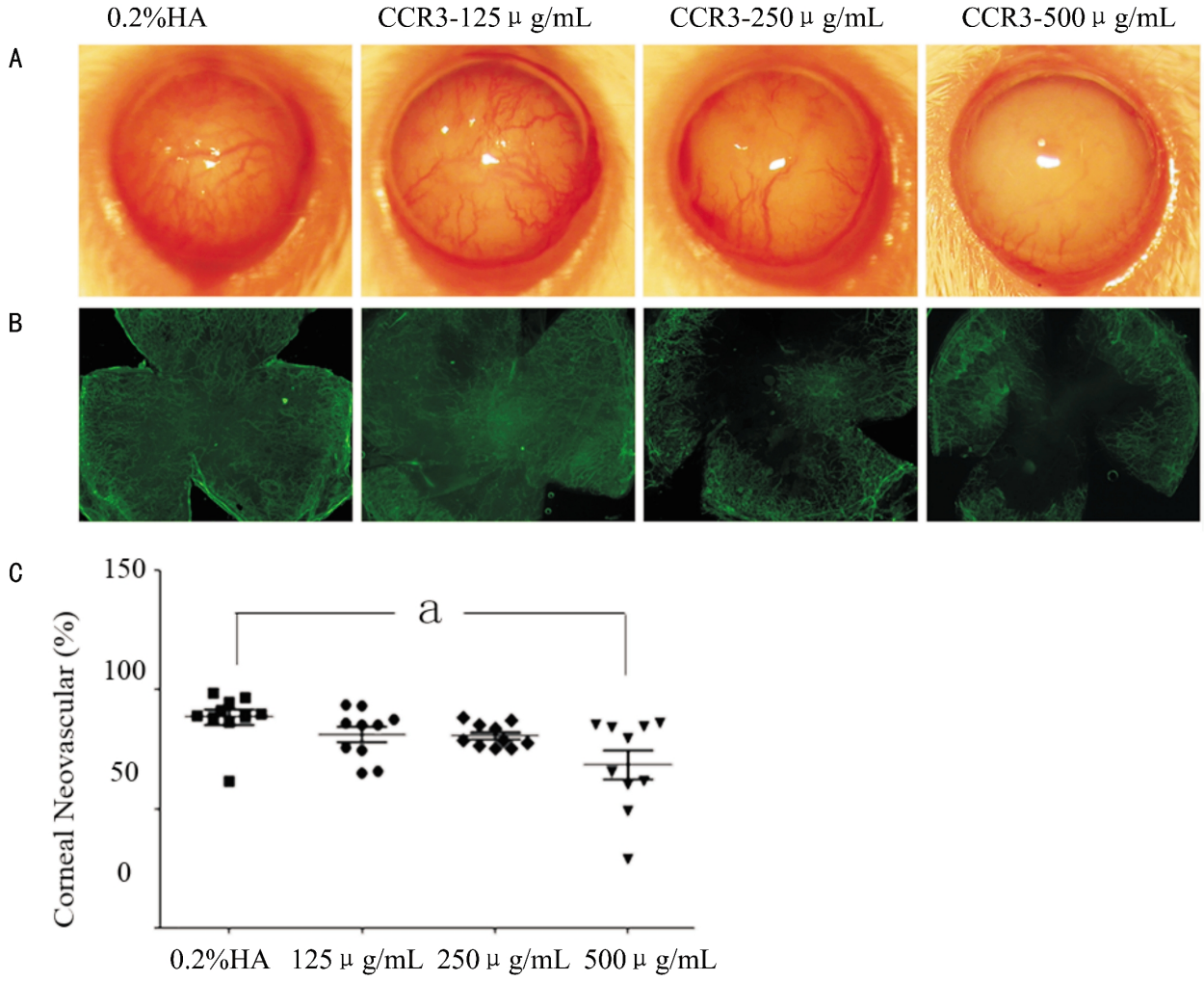

Figure 2 Alkali-induced CRNV after CCR3 antagonist treatment in early phase A: Macroscopic appearance of different concentration of CCR3-antagonist (125μg/mL, 250μg/mL, 500μg/mL in 2g/L HA) treated and 2g/L HA treated mice eyes 2 weeks after alkali injury in early phase; B: Corneal neovascularization was assessed by CD31 corneal whole mount staining. The percentages of the corneal covered by vessels which were normalized to the total corneas were calculated; C: The relative neovascular areas were compared between treated groups and control group, the 500μg/mL CCR3-antagonist treated mice exhibited significantly decreased CRNV area compared to the control (n=10; t=2.715, $^{a}P<0.05$ *vs* control group).

administrated with CCR3-antagonist to inhibit the effect of CCR3 immediately for 1 week or from 7 days after alkali injury for 2 weeks, the areas of CRNV were compared between CCR3-antagonist treated and control mice by fluorescence immunohistochemical analysis. Macroscopic observation indicated that CRNV was inhibited by CCR3-antagonist at the concentration of 500μg/mL ($P<0.05$), but not the 125μg/mL and 250μg/mL administrated at early stage (Figure 2A). Similar results were obtained from CD31 corneal whole mount staining (Figures 2B and 2C). We did not found obvious alkali-induced CRNV changes when CCR3-antagonist was administrated in the late phase

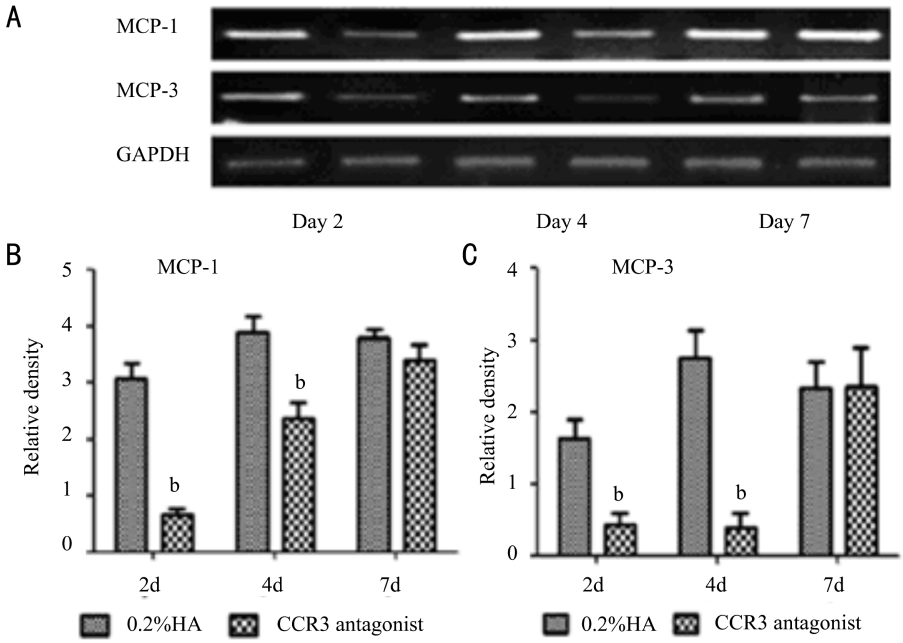

Figure 3 Intracorneal MCP-1 and MCP-3 mRNA expression A: Representative RT-PCR results from three independent experiments of MCP-1 and MCP-3 mRNA expression of 500μg/mL CCR3-antagonist treated and control mice on day 2, day 4 and day 7 after alkali injury; B: Ratios of MCP-1 to GAPDH of CCR3-antagonist treated and control mice determined by RT-PCR at the indicated time intervals ($^bP<0.01$ *vs* control group); C: The relative level of MCP-3 mRNA expression at the indicated time between treated group and control. ($^bP<0.01$ *vs* control group).

(data not shown).

Molecular mechanism of CCR3-antagonist on alkali-induced CRNV We next focused on the molecular mechanism of 500μg/mL CCR3-antagonist treatment in the early phase on alkali-induced CRNV. Among the angiogenic factors VEGF, MCP-1 and MCP-3 we detected, we observed that 500μg/mL CCR3-antagonist treated mice exhibited decreased intracorneal MCP-1 and MCP-3 mRNA expression in the early phase (day 2, day 4) after alkali injury compared with control group (Figure 3). Moreover, we did not detect significant difference of CCR3 mRNA expression between CCR3-antagonist treated and control mice (data not shown). The result revealed that topical administration of CCR3-antagonist efficiently inhibited the MCP-1 and MCP-3, but not CCR3 expression in the corneas after alkali injury.

Impaired alkali-induced intracorneal macrophage recruitment after 500μg/mL CCR3-antagonist treatment We previously observed that F4/80-positive macrophages infiltrated the injured corneas, reaching their peak levels in 2-4 days after the injury in mice [13]. In our study, we found that the mRNA expression of MCP-1 and MCP-3 were decreased in the experiment group at day 2 and day 4, which promoted the chemotaxis of macrophage to the injured corneas. We supposed that the infiltration of macrophages into the corneas might decrease after treated with CCR3-antagonist compared to control group at day 2 and day 4. Immunohistochemical analysis against macrophages

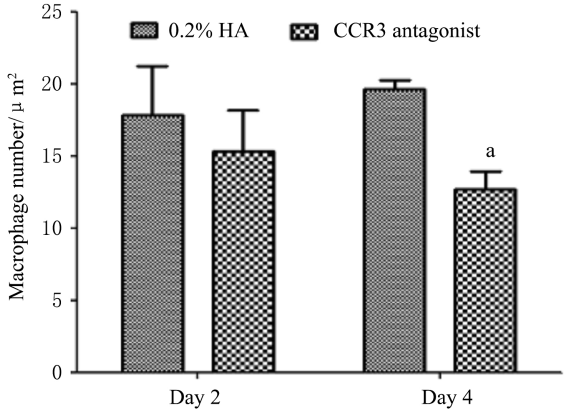

Figure 4 Macrophage intracorneal infiltrations after alkali injury The numbers of infiltrated F4/80-positive macrophages of 500μg/mL CCR3-antagonist treated mice and control mice at day 2 and day 4 were determined as described in materials and methods and the mean and SEM are shown there. ($n=9$, $^aP<0.05$ *vs* untreated mice).

further demonstrated that CCR3-antagonist treatment significantly inhibited F4/80-positive macrophages infiltration into the cornea at day 4 (Figure 4).

DISCUSSION

Corneal avascularity is maintained by means of the balance between pro-angiogenic and anti-angiogenic factor expression. Corneal injury enhanced the expression of various growth factors, cytokines and chemokines, which contribute to cornea tissue repair in a coordinated manner. Corneal injuries lead to break the balance between

angiogenic and anti-angiogenic factor expression, and finally cause CRNV. We previously found, in alkali-induced CRNV, many inflammatory factors such as VEGF, MCP-1 produced, and cells such as neutrophil, macrophage infiltrated into the injured corneas. They peaked at 2 days to 4 days after alkali injury. CRNV was evident at 7 days and peaked at 14 days after injury, and declined gradually there after[1,9,10,12,13].

CCR3, a G-protein-coupled receptor, is well known for its role in promoting eosinophil and mast cell trafficking, and its involvement in the development atopic asthma, atopic dermatitis, and allergic rhinitis[14-16]. Recent research reported that CCR3 and its ligands eotaxin-1, -2 and -3 were specifically expressed in CNV endothelial cells in human with AMD and CNV was suppressed by CCR3 blockade, which was due to direct inhibition of endothelial cell proliferation[3].Moreover,several independent studies suggested that CCR3 might have some interaction with Notch pathway, VEGFR2 and prostaglandin E_2, which were responsible for CNV in AMD or corneal neovascularization induced by suture injury[5,8,17].

These observations prompted us to investigate the roles of the CCR3 signal in alkali injury-induced CRNV by comparing the molecular and cellular pathological changes between CCR3-antagonist treated and control group. We found that CCR3 was time kinetic expressed in the corneal tissue at day 0, day 2, day 4, day 7 and day 14 after alkali injury. Thus, we speculate that CCR3 was involved in the pathological process of alkali-induced CRNV. We used a small molecule CCR3 selectivity antagonist SB 328437 to intervene the effect of CCR3 signal on the development of CRNV induced by alkali injury. We found that CRNV was inhibited by CCR3-antagonist at the concentration of 500μg/mL when local administrated at early stage. However, in current study, we did not find difference between CCR3-antagonist treated groups and control group when local administrated in the late phase. Although further work about CCR3 signal on vascular endothelial cells during alkali induced CRNV need to be done, we speculate that CCR3 signal involved in the development of CRNV through the inflammatory molecules and cells in the early phase but not the vascular endothelial cells. We next further focused on the molecular and cellular changes in the early phase after CCR3-antagonist intervention.

Our RT-PCR results showed that in alkali-induced CRNV, the expressions of MCP-1 and MCP-3, which were previously reported to promote chemotaxis of macrophage, in CCR3-antagonist treated groups were inhibited at day 2 and day 4. Consistently, we observed the number of macrophage infiltration into cornea was reduced in the CCR3-antagonist treated group at day 4. Our results indicate there is relationship between CCR3 signal and macrophage infiltration associated factors such as MCP-1 and MCP-3, although it is still need to be further investigation. Firstly, consistent with our observation, Mori *et al*[18] found that the highly potent selective CCR3-antagonist could decrease the concentrations of MCP-1 and MCP-3 in bronchoalveolar lavage fluid in the allergic airway inflammation models measured by ELISA. We speculate that the CCR3-antagonist might inhibit the engagement of CCR3 ligand to their receptors on MCP-1 and MCP-3-releasing cells, such as T cell. Secondly, Ogilvie *et al*[19] reported that eotaxin-3 was a natural antagonist for CCR2 and exerted a repulsive effect on human monocytes. Published data suggest that CCR2-expressing macrophages may be proangiogenic [10]. It is reasonable that the CCR3-antagonist inhibited the engagement of eotaxin-3 and CCR3, on the contrary increased the chance of the combination of eotaxin-3 and CCR2, and reduced macrophage infiltration. Finally, Wang *et al*[17] found that CCR3 and VEGF work synergistically in triggering downstream signaling pathways, CCR3-antagonist inhibited the effect of VEGF by means of decreasing the phosphorylation of VEGFR2, which promoted the migration of choroidal endothelial cells. Thus, we believe CCR3-antagonist may also decrease the phosphorylation of VEGFR2 and consequently inhibit the development of CRNV.

Collectively, our observation suggests that the CCR3 signal play crucial roles in the development of alkali-induced CRNV. However, the development of CRNV was extraordinary complex and the effects of CCR3 signal on CRNV have not completely understood. More investigative works need to be done to clarify the role of CCR3 and its ligands in CRNV, which will provide experimental basis for clinical treatment of CRNV.

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