CXCL12/CXCR4 axis regulates neovascularization and lymphangiogenesis in sutured corneas in mice

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Abstract. The present study aimed to determine the plausible functional role of chemokine (C-X-C motif) ligand 12 (CXCL12)/chemokine (C-X-C motif) receptor 4 (CXCR4) in inflammatory corneal hemangiogenesis and lymphangiogenesis in vivo. Corneal hemangiogenesis and lymphangiogenesis were induced by placing an 11-0 nylon suture in an intrastromal position. The expression levels of the vascular endothelial growth factor (VEGF) family, CXCL12 and CXCR4 in the corneas were investigated in the corneas using reverse transcription-quantitative polymerase chain reaction and immunohistochemistry. Corneal hemangiogenic and lymphangiogenic responses were assessed by immunofluorescence using specific antibodies against cluster of differentiation 31 and lymphatic vessel endothelial hyaluronan receptor-1. Subconjunctival injection of AMD3100 to the sutured corneas was also performed. CXCL12/CXCR4 mRNA and protein expression levels increased markedly in suture-induced corneal neovascularization (CNV) and decreased with AMD3100 treatment. Hemangiogenesis and lymphangiogenesis were captured in images using immunofluorescence and were shown to be markedly increased with suture placement and reduced with AMD3100 treatment. VEGF-A/VEGFR-1 and VEGF-C/VEGFR-3 mRNA expression levels were upregulated in the suture placement and control groups, whereas the expression levels of all the factors were downregulated in the AMD3100 treatment group. The results from the present study demonstrated that CXCL12/CXCR4 interactions regulate hemangiogenesis and lymphangiogenesis in suture-induced CNV. AMD3100 may be a novel therapeutic target for the prevention of blindness.

Introduction

The maintenance of corneal transparency is required for optimal vision, thus, the cornea must preserve avascularity and alypathicity (1). It is widely recognized that new blood and lymphatic vessels in the cornea are strong risk factors for corneal transparency. Corneal neovascularization (CNV), also referred to as angiogenesis or hemangiogenesis (HA), is the formation of ectopic corneal vasculature from preexisting vasculature (2). CNV is promoted by a wide variety of proangiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-6, platelet-derived growth factor (PDGF), hepatocyte growth factor (3-7). In addition, recent studies have demonstrated that anti-angiogenic factors, such as angiostatin and endostatin, inhibit CNV (3).

Lymphangiogenesis (LG) is the formation of novel lymphatic vessels from pre-existing vasculature. In contrast to angiogenesis, research on the lymphatic system has been slowed by a lack of molecular markers to identify the vessels. The recent discovery of several lymphatic endothelial molecular markers, including VEGF receptor-3 (VEGFR-3), lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), and Prox-1, has enabled further research and progress in the field (8-10).

Signaling via VEGF-C/D and VEGFR3 is a central pathway for LG (11-12). VEGF-C+ mice are viable but suffer from lymphatic deficiency and subsequent lymphedema (13). Similarly, inhibition of VEGFR3 signaling during the formation of lymphatic vessels induces lymphatic regression and lymphedema in mouse embryos and neonates (14). VEGF-A is widely used for angiogenesis research as it promotes several processes of angiogenesis, including proteolytic activities (dissolution of the membrane of the original vessel), endothelial cell proliferation, migration, and capillary tube formation. Notably, the requirement of VEGF in corneal angiogenesis was demonstrated by the inhibition of NV following stromal implantation of an anti-VEGF blocking antibody in a rat model (15).

Chemokine (C-X-C motif) ligand 12 (CXCL12) is a member of the CXC subfamily of chemokine peptides, also known as stromal cell-derived factor-1 (SDF-1). However, unlike other chemokines that interact with multiple G-protein coupled receptors, CXCL12 mediates its effects via its only known receptor, chemokine (C-X-C motif) receptor 4 (CXCR4). CXCL12 is highly conserved with 99% homology between humans and mice, allowing it to exert effects across species barriers (16).
The CXCL12/CXCR4 axis is essential for multiple biological processes, including development, hematopoiesis, organogenesis, and vascularization (17-20). Furthermore, CXCR4 is highly expressed in a variety of cancers (21). The interaction between CXCL12 and CXCR4 is important in tumorigenesis (22) and metastasis (21,22,23). AMD3100, a well-known antagonist of CXCR4, reversibly inhibits interaction of CXCL12 and CXCR4 (24). Previous studies have identified the CXCL12/CXCR4 axis is associated with NV (25-27). However, the association between the CXCL12/CXCR4 axis and LG remains to be elucidated.

The role of the CXCL12 (SDF-1)/CXCR4 axis in mediating LG in suture-induced CNV remains to be elucidated. To investigate the roles of CXCR4 signaling, the process of suture-induced CNV was analyzed using mice with suture placement or CXCR4 antagonist (AMD3100)-treated mice in comparison with control-treated mice. The present study also investigated whether VEGF signaling was involved in the molecular signaling pathway resulting in corneal LG due to placement of a suture.

Materials and methods

Animals and anesthesia. All animal protocols were approved by the local animal care committee of The First Affiliated Hospital of Harbin Medical University (Harbin, China), and were in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. A total of 25 male C57BL/6 mice (age, 6-8 weeks) were purchased from the Changchun Animal Center (Changchun, China). Mice were housed in three groups (9 mice in control group, and 8 mice in AMD3100 and suture placement groups each) under a 12 h light/dark cycle at moderate temperature and humidity with free access to food and water. Animals were anesthetized with a mixture of ketamine and xylazine (Zhejiang Jiuxu Pharmaceutical Co., Ltd.; Jinhua, China) at 120 mg/kg body weight and 20 mg/kg body weight, respectively. At the end of the experiment, the mice were sacrificed by CO₂ inhalation.

Suture-induced CNV. The mouse model of suture-induced inflammatory CNV was used as previously described (28). The central cornea was marked with a 2-mm diameter trephine and three 11-0 nylon sutures (Lingqiao, Ningbo, China) were placed in the intrastromal position. The outer point of suture placement was selected to be near the limbus, and the inner point was selected to be near the corneal center equidistant from the limbus. Sutures were removed after 7 days. Mice were randomly selected to receive subconjunctival injection of the CXCR4 antagonist, AMD3100 (1 g/l; Abcam, Cambridge, UK) or balanced salt solution once a day from day 1 of the suture-induced model.

Measure of suture-induced CNV. CNV was examined using a slit lamp every other day following the corneal suture. Measurements of NV were made using a slit lamp by an ophthalmologist. Vessel growth onto the cornea was recorded in millimeters on day 7. CNV was quantified by calculating the wedge-shaped area of vessel growth with the formula: 

\[ A = C/12 \times 3.1416[r^2 - (r - l)^2], \]

where A is the area, C is the time (h), l is the radius from the center to the border of vessel growth, and r is the radius of the cornea (29).

Immunohistochemistry. Corneas were cut and fixed in 10% neutral buffered formalin (Beijing Yili Fine Chemicals Co., Ltd.; Beijing, China) for 24 h. Paraffin-embedded tissue sections (4 μm) were deparaffinized, rehydrated, and treated with 0.3% hydrogen peroxide in methanol (Beyotime Institute of Biotechnology, Inc., Haimen, China) for 30 min, to eliminate endogenous peroxidase activity. The tissue sections were incubated for 60 min at room temperature with rabbit anti-mouse CXCR4 polyclonal antibody (1:200 dilution; cat. no. ab2074; Abcam). Following three washes of 3 min with phosphate-buffered saline (PBS; Beyotime Institute of Biotechnology, Inc.), a 3,3'-diaminobenzidine detection kit (PV9000; ZSGB-BIO, Beijing, China) was used for CXCR4 staining, according to the manufacturer's protocols. Images were captured with the Leica DM4000B biological microscope equipped with a Leica DFC 550 digital camera and Leica Application Suite version 4.2.0 software (Leica Microsystems GmbH, Wetzlar, Germany).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocols. Total RNA (400 ng) was reverse transcribed using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed using SYBR® Premix Ex Taq™ II (Takara Biotechnology Co., Ltd.) in a LightCycler 480 Real-time PCR system (Roche Diagnostics, Basel, Switzerland). The thermocycling conditions were as follows: Initial denaturation step of 95°C for 30 sec; 40 cycles of 95°C for 5 sec and 60°C for 30 sec; followed by

| Gene          | Sequence (5'-3') |
|---------------|------------------|
| GAPDH         | F: GTATTGGGCGCCTGGTACC |
|               | R: CGCTCCTGGAAGTAGTGATGG |
| VEGF-A        | F: ACACGGTGTTGGAGGAAGAG |
|               | R: CAACTGCTCCTGGGACAGAA |
| VEGF-C        | F: CTACAGATGTGCGGGTTGCT |
|               | R: GATTGCGAAAATCTAGTGTG |
| VEGFR-1       | F: CTGGACTGAGAACCAAGCCCAAG |
|               | R: GTCGAGATTCACTGTCTGAC |
| VEGFR-3       | F: CTCTGATCCATTGGAGATCTG |
|               | R: CTTGGCTAGATGATAGCCTG |
| CXCR4         | F: AGCATGACGCAAGTACC |
|               | R: GATGATGATGGACGCTTACAC |
| CXCL12        | F: GAGAGCCACATCGCCAGAG |
|               | R: TTTGGGCTTAATGCACACTTG |

F, forward; R, reverse; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CXCR4, chemokine (C-X-C motif) receptor 4; CXCL12, chemokine (C-X-C motif) ligand 12.
an additional denaturation step of 95°C for 5 sec and 60°C for 60 sec, as a subsequent melt curve analysis to check amplification specificity. The assays were conducted three times, and were performed in triplicate. Results were derived from the 2-ΔΔCq method (30) and glyceraldehyde-3-phosphate dehydrogenase served as an internal control for normalization. Primers used in the RT-qPCR are presented in Table I.

**Western blot analysis.** The corneas were harvested and lysed in ice-cold radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology, Inc.) with the addition of protease inhibitors. Following separation by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 120 V for 2 h, proteins were transferred onto nitrocellulose membranes (Pall Life Science, Port Washington, NY, USA). The membranes were incubated in blocking solution of 2% bovine serum albumin (BSA; Beyotime Institute of Biotechnology, Inc.) in Tris-buffered saline with Tween-20 for 1 h at room temperature, then incubated with rabbit anti-mouse CXCR4 polyclonal antibody and rabbit anti-mouse CXCL12 polyclonal antibody (cat. no. ab25117; Abcam) at 1:1,000 dilution overnight at 4°C. Each step was followed by extensive washing. β-actin was used as loading control (mouse anti-mouse β-actin monoclonal antibody; cat. no. A00702-100; 1:1,000 dilution; GenScript, Nanjing, China). The membranes were then incubated with horseradish peroxidase-conjugated rabbit anti-mouse polyclonal immunoglobulin (IgG) (1:5,000 dilution; cat. no. A9044; Sigma-Aldrich, St. Louis, MO, USA) or goat anti-rabbit polyclonal IgG secondary antibody (cat. no. A0545; 1:5,000 dilution Sigma-Aldrich) for 1 h at room temperature and developed using an enhanced chemiluminescence system (GE Healthcare, Little Chalfont, UK). Images were captured on X-ray film.

**Corneal immunofluorescence microscopy assays and quantification.** The immunofluorescence experiments were performed as previously described (31). The excised corneas from the CNV assay were rinsed in PBS and fixed in acetone (Beyotime Institute of Biotechnology, Inc.) for 30 min. Following washing and blocking with 2% BSA in PBS for 2 h, the corneas were stained overnight at 4°C, with a rabbit anti-mouse LYVE-1 antibody (cat. no. ab14917; 1:500 dilution; Abcam) and a rat anti-mouse cluster of differentiation (CD)31 antibody (1:100; cat. no. 550274; BD Pharmingen, San Diego, CA, USA). On day 2, the tissue was washed three times in PBS and stored at 4°C in the dark. LYVE-1 antibody was detected with an Alexa Fluor® 647-conjugated goat anti-rabbit IgG antibody (1:200; cat. no. A-21244; Invitrogen; Thermo Fisher Scientific, Inc.) and the CD31 antibody was detected with Alexa Fluor 488®-conjugated goat anti-rat IgG polyclonal antibody (1:200; cat. no. A-11006 Invitrogen; Thermo Fisher Scientific, Inc.).

The stained whole mounts were analyzed with a fluorescence microscope (EVOS f1; Thermo Fisher Scientific, Inc.). Each whole mount picture was quantified using Image J software version 1.24o (National Institutes of Health, Bethesda, MD, USA) analysis software as described previously (32). The mean vascularized area of the suture placement was defined as being 100%; vascularized areas were then relative to this value (vessel ratio).

**Statistical analysis.** Statistical analysis was performed by the Student's t-test using SPSS version 13.0 software (Armonk, NY, USA). Results were expressed as the mean ± standard error of the mean and P<0.05 was considered to indicate a statistically significant difference. Graphs were drawn using GraphPad Prism, version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

**Results**

Expression levels of CXCL12 and CXCR4 in a mouse model of suture-induced inflammatory CNV. The present study
analyzed CXCL12 and CXCR4 mRNA and protein expression levels in normal and vascularized corneas by RT-qPCR, immunohistochemistry and western blot analysis. The immunohistochemical staining demonstrated CXCR4 was only detected in epithelial cells in normal corneas (Fig. 1A). Immunohistochemical staining showed a high expression of CXCR4 in corneas on day 7 following suture placement in the control group, and a weak expression of CXCR4 in AMD3100 subconjunctival injection (Fig. 1B-D). CXCL12 and CXCR4 mRNA was detected at low levels in normal eyes, but was significantly increased following suture placement and decreased with treatment with AMD3100 (P<0.01; Fig. 2A and B). CXCL12 and CXCR4 mRNA expression levels presented no significant change between the suture placement and control groups (P>0.05; Fig. 2A and B). Furthermore, the western blot assay also indicated a marked upregulation of CXCL12 and CXCR4 expression levels in the suture placement and control groups, and the downregulation of CXCL12 and CXCR4 expression levels with AMD3100 treatment (Fig. 2C). The increased mRNA and protein expression levels of CXCL12 and CXCR4 resulted in further investigation into whether CXCL12/CXCR4 interactions are involved in suture-induced CNV. Slit lamp examinations demonstrated that untreated corneas were avascular, however, suture placement markedly increased the vascular areas in corneas and the vascular areas decreased following AMD3100 treatment (P<0.01; Fig. 3). Slit lamp

Figure 2. CXCL12/CXCR4 expression levels in normal and treated corneas. (A and B) Quantitative polymerase chain reaction analysis of CXCR4/CXCL12 mRNA was performed in corneas following suture placement and AMD3100 treatment. (C) Western blot assays demonstrated the protein expression levels of corneal CXCL12/CXCR4 under different conditions. The images are representative of three independent experiments. β-actin served as a loading control. **P<0.01. NS, not significant; CXCL12, chemokine (C-X-C motif) ligand 12; CXCR4, chemokine (C-X-C motif) receptor 4.

Figure 3. CNV was examined by slit lamp on day 7. (A) Suture-induced CNV (B) Suture-induced CNV with subconjunctival injection of balanced salt solution. (C) Suture-induced CNV with subconjunctival injection of AMD3100. CNV was measured and data are expressed as the mean ± standard deviation (n=5). (D) The AMD3100-treated group demonstrated decreased CNV area. **P<0.01 vs. the suture-induced CNV and balanced salt solution subconjunctival injection groups. CNV, corneal neovascularization.
examination was consistent with results from the analysis of mRNA and protein expression levels.

**CXCL12/CXCR4 axis regulates CNV.** CXCL12 is one of the major chemokines associated with CXCR4, thus, the present study hypothesized that the CXCL12/CXCR4 axis regulates NV and LG. To confirm the bioactivity of CXCL12/CXCR4 on NV, a mice model of suture-induced CNV was established in vivo. The effect of CNV was evaluated by comparing the CNV area between mice from the suture placement and AMD3100 treatment groups on day 7 (Fig. 3). The results demonstrated that the size of the areas of CNV indicate no significant difference between the control and the suture placement groups. The area following AMD3100 treatment was smaller than that in the suture placement and control groups, suggesting CXCL12/CXCR4 axis regulates CNV (0.54±0.11, 2.34±0.24 and 2.65±0.32, respectively; Fig. 3A-C). Corneal suture placement induced a robust CNV response that emerged from day 3 and reached suture placement sites at day 7 following suture injury (31). The densities of platelet endothelial cell adhesion molecule-1 (CD31)-positive blood vessels were detected by immunofluorescence on day 7 (Fig. 4A-C). Quantitative immunofluorescence and morphometric analyses demonstrated that the number of blood vessels in mice treated with AMD3100 was significantly decreased (P<0.01), whereas the number of blood vessels in the suture placement group showed no changes compared with balanced salt solution group (P=0.46; Fig. 4J). These results suggest that the CXCL12/CXCR4 axis is associated with CNV.

**CXCL12/CXCR4 axis regulates LG.** Lymphatic vessels express multiple specific markers, including LYVE-1, Prox-1, and VEGFR-3 (8-10). LYVE-1 is specifically expressed on newly formed lymphatic vessels but not on blood vessels in the mouse corneal model (33). Thus, the present study used LYVE-1 as a specific maker for detection of lymphatic vessels in the present study. On day 7, the densities of LYVE-1 positive lymphatic vessels were detected by immunofluorescence. The lymphatic vessels of suture placement had vascular ‘tree-like’ structures (Fig. 4D-F). Quantitative immunofluorescence and morphometric analyses demonstrated that corneal LG was induced by suture placement and the vessel ratio of lymphatic vessels in mice treated with AMD3100 were significantly decreased (P<0.01; Fig. 4K) compared with the suture placement and control groups. The present study supports that CXCL12/CXCR4 regulates corneal LG.

**CXCL12/CXCR4 pathway is dependent on the VEGF-A/VEGFR-1 pathway in the regulation of CNV.** VEGF-A/VEGFR-1 is considered to induce angiogenesis. To investigate whether VEGF-A/VEGFR-1 was associated with CXCL12/CXCR4, RT-qPCR for VEGF-A/VEGFR-1 was performed. VEGF-A/VEGFR-1 mRNA expression levels were upregulated in the suture-induced CNV and control groups (Fig. 5), whereas VEGF-A/VEGFR-1 expression levels in the corneas treated with AMD3100 were downregulated. The results are consistent with the results of CXCL12/CXCR4. Thus, these results demonstrate that the CXCL12/CXCR4 pathway is dependent on the VEGF-A/VEGFR-1 pathway in regulating CNV.
CXCL12/CXCR4 pathway is dependent of the VEGF-C/VEGFR-3 pathway in the regulation of LG. The abovementioned results demonstrated that CXCL12/CXCR4 mediates LG. However, whether CXCL12/CXCR4 mediation of LG is associated with VEGF-C/VEGFR-3 remains to be elucidated. To further investigate the roles of VEGF-C and VEGFR-3 in suture-induced CNV, RT-qPCR was used to examine mRNA expression levels. VEGF-C and VEGFR-3 expression levels were significantly downregulated in mice treated with AMD3100 (P<0.05), whereas the expression levels of the two factors indicated no significant difference between the suture-induced and control groups (Fig. 5). Thus, these results demonstrate that the CXCL12/CXCR4 signaling pathway is dependent on the VEGF-C/VEGFR-3 pathway.

Discussion

To the best of our knowledge, the present study is the first to demonstrate that CXCL12/CXCR4 regulates LG in vivo in a corneal suture-induced mouse model. Giacomini et al (34) quantified and compared HA and LG following alkali burn-and suture-induced CNV. They demonstrated that LG was more marked in the suture-induced model as compared with the alkali burn model, and the two types induced similar HA. Thus, the present study used a suture-induced CNV mouse model.

In the current study, there are low levels of expression of CXCL12/CXCR4 in normal corneal epithelium. Bourcier et al (35) detected CXCL12/CXCR4 mRNA expression levels in normal human corneas by in situ hybridization with similar results to those in the present study. CXCL12/CXCR4 expression levels in suture-induced CNV was investigated by immunohistochemical analysis, RT-qPCR and western blotting. The present study observed SDF-1 and CXCR4 mRNA overexpression on day 7 in suture-induced CNV, consistent with a previous study (36). These findings suggest that suture-induced inflammation increases CXCL12/CXCR4 expression levels in the cornea, and that CXCL12/CXCR4 is crucial to the induction of inflammatory corneal HA and LG. AMD3100 is a specific antagonist of CXCR4, with no cross reaction with other chemokines. According to the immunofluorescence results, corneal HA and LG were markedly reduced in eyes receiving subconjunctival injections of AMD3100 compared with the suture placement group. Subconjunctival injection of AMD3100 was an effective method to decrease the expression of CXCL12/CXCR4. These results suggest that CXCL12/CXCR4 was involved in CNV and, particularly, LG.

Corneal avascularity is maintained by the balance between angiogenic and anti-angiogenic factor expression (37,38). Corneal wounding enhances predominantly the expression of angiogenic factors, including VEGF and bFGF (25,39), and skewed the balance toward angiogenesis, thus resulting in CNV. The crucial role of VEGF-A in the pathophysiology of CNV has been extensively previously reported (15,40,41). CXCL12 and its receptor CXCR4 promote glioma stem cell-initiated glioma growth and angiogenesis by stimulating VEGF-A production (25). Antoniou et al (42) demonstrated VEGF-A and SDF-1
Inhibition of lymphangiogenesis with Novel aspects of corneal angiogenic and

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Education Department (no. 12521262) and the Scientific Natural Science Foundation of China (no. 81470618), The present study was supported by grants from the National corneal suture-induced mouse model. 

strate evidence that CXCL12 inhibit LG. Notably, the present study is the first to demon

ated in corneas treated with AMD3100. The results are consistent with the results from investigation of CXCL12/CXCR4, thus, the current study hypothesizes CXCL12/CXCR4 may enhance CNV by increasing VEGFA/VEGFR-1.

Among the known lymphangiogenic factors, VEGF-C, which exerts its functions via VEGFR-3, is the most potent and specific growth factor acting directly on lymphatic vessels. Although bFGF, angiopoietin-1/2, insulin-like growth factor-1/2, hepatocyte growth factor, and PDGF-BB have been demonstrated to be prolymphangiogenesis factors, the VEGF-C/VEGFR-3 signaling pathway is common for a number of lymphangiogenic factors. bFGF has also been reported to upregulate VEGF-C expression in endothelial cells, and its lymphangiogenic property is mediated by VEGF-C (43).

The present study hypothesized that CXCL12/CXCR4 activates a signaling pathway dependent on that triggered by the VEGF-C/VEGFR-3 axis. The present study has demonstrated that VEGF-C and VEGFR-3 expression were significantly upregulated in the suture placement and control groups, whereas the expression levels of the two factors were downregulated in mice treated with AMD3100. Although it is possible that CXCL12 may also have indirect effects in promoting LG via inducing other factors, the VEGF-C/VEGFR-3 pathway and CXCL12/CXCR4 signaling pathways are involved in LG, and the CXCL12/CXCR4 pathway may depend on VEGF-C/VEGFR-3 pathway in regulating LG. The present study suggests that the CXCL12/CXCR4 axis is a potent positive-regulator of LG.

In conclusion, the results from the present study demonstrate CXCL12/ CXCR4 regulates HA and LG following corneal suture placement, and provides a novel strategy to inhibit LG. Notably, the present study is the first to demonstrate evidence that CXCL12/CXCR4 modulates LG in a corneal suture-induced mouse model.

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