Original Article

An Association between Corneal Inflammation and Corneal Lymphangiogenesis after Keratoplasty

Weihua Li[1], Wencong Wang[1], Shiqi Ling[2,*]

1 State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Centre, Sun Yat-sen University, Guangzhou 510060, China,

2 Department of Ophthalmology, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou 510630, China

Abstract

Purpose: To examine the relationship between corneal inflammation and corneal lymphangiogenesis after keratoplasty.

Methods: Rat corneal lymphangiogenesis was examined by lymphatic vessel endothelial receptor (LYVE-1) immunohistochemistry and whole mount immunofluorescence at 1, 3, 7, 10, and 14 days after corneal transplantation. Corneal inflammation was evaluated by inflammation index (IF) grading and NF-κB immunohistochemistry at the same time points. The association between lymphatic vessel counting (LVC) and the IF scores was then examined.

Results: LYVE-1 positive lymphatic vessels occurred in the corneal stroma on day 3, developed throughout days 7 and 10, and peaked in number at day 14 after keratoplasty. Corneal inflammation was strong on day 3, and then resolved gradually, but increased again from days 7 to 14 after the transplantation. LVC was strongly and positively correlated with IF after keratoplasty (r=0.41; P<0.05). However, changes in IF scores and LVC were not parallel.

Conclusion: A close, but not parallel, relationship was found between corneal lymphangiogenesis and corneal inflammation after corneal transplantation. (***Eye Science 2014; 29:78-84***)

Keywords: lymphatic vessel; inflammation; corneal transplantation

DOI: 10.3969/j.issn.1000-4432.2014.02.004

Funding: This study was supported by China Natural Science Foundation (81070711); Guangdong Provincial Science and Technology Projects (2012B031800456); Doctoral Program of the Ministry of Education (20110171120104)and the Young Teachers Training Program of Sun Yat-sen University (11ykpy42).

* **Corresponding author**: Shiqi Ling, E-mail: lingshiqi123@163.com

Introduction

Our previous studies demonstrated the occurrence of corneal lymphangiogenesis in normal and high-risk cornea beds after keratoplasty[1,2]. This implies that the corneal graft has a chance of connecting to both the blood and lymphatic systems. The blood vessels provide a route of entry for immune effector cells (e.g., CD4 + alloreactive T lymphocytes, memory T lymphocytes). By contrast, corneal lymphangiogenesis enables the exit of antigenic material, antigen-presenting cells, *etc.*, from the graft to the regional lymph node, which can induce alloimmunization and subsequent graft rejection[3,4]. Recent studies indicate that corneal lymphatic vessels play a more important role than blood vessels in allograft failure; consequently, corneal lymphangiogenesis is receiving increasing attention[5].

Current evidence from transplanted human and animal corneas indicates a parallel relationship between corneal lymphatic and blood vessels, suggesting that lymphatic vessels are more likely to be present in strongly vascularized corneas than in mildly vascularized corneas after keratoplasty[6,7]. In addition to blood vessels, inflammation is an important factor that induces corneal lymphangiogenesis, as confirmed in recent experimental and clinical trials[8].

The possible association of the degree of corneal inflammation with the degree of corneal lymphangiogenesis in transplanted corneas also warrants further investigation for two reasons. First, inflammation appears to be the common denominator in the postnatal events that overlap with lymphatic vessel growth. Second, the state of corneal inflammation is one of

the most important factors in allograft immune responses[8]. However, to our knowledge, little published information exists regarding the relationship between corneal lymphatic vessel numbers and inflammation after corneal transplantation.

The aim of this study was to examine the association between corneal lymphangiogenesis and corneal inflammation after keratoplasty. The findings reported here may broaden our understanding of the mechanisms that underpin corneal lymphangiogenesis and corneal transplant rejection.

Materials and methods

Animals

Fifty-six of 62 Lewis rats($RT1^l$) were used as recipients, while the remaining 6 served as controls. Twenty-eight Fischer 344 rats ($RT1^{lv1}$) were used as donors for corneal transplantation. All rats were male and aged 1-2 months, with weights ranging from 150 to 200 μg. They were provided by the animal center of Sun Yat-sen University, China. The experimental conditions used in the study conformed to good laboratory practices (National Research Council USA 1996) and all animals involved in the study were handled in accordance with the ARVO statement for the use of Animals in Ophthalmic and Vision Research. Prior to treatment, we confirmed that all rats were free from ocular disease.

Orthotopic corneal transplantation

The corneal transplantation procedure was adapted from the technique described previously by Williams et al[9]. In brief, rats were anesthetized with chloral hydrate (300 mg/kg) through the abdominal cavity, and 1% atropine was used 20 minutes before the operation. Donor corneas (3 mm in diameter) were removed and placed in Optisol solution. The recipient's right cornea was marked with a 2.75 mm trephine and excised under the operating microscope, and then the donor graft was sutured into the recipient's bed with six interrupted sutures (10-0 nylon). Equilibrium liquid was injected to maintain the anterior chamber and 2000 μg gentamicin was injected into the subconjunctiva. The sutures were removed 7 days after the transplantation.

At 1, 3, 7, 10, and 14 days after penetrating corneal transplantation, corneal lymphangiogenesis was examined by LYVE-1 immunohistochemistry and whole-mount immunofluorescence. The state of corneal inflammation was evaluated by inflammatory index (IF) scoring, histopathological examination, and NF-κB immunohistochemistry at the same intervals. For each time point after the transplantation, six corneas (the right cornea in Lewis rats, including both parts of the donor and recipient) were used for NF-κB and LYVE-1 immunohistochemistry, two for whole mount immunofluorescence, and two for histopathology. Six recipient right eyes were used for IF examination, and six right corneas from normal Lewis rats were used as controls for immunohistochemistry (two corneas), and immunofluorescence (two corneas), and histopathology (two corneas).

Immunohistochemistry

The corneas were fixed in 10% neutral formalin for 24 h, embedded in paraffin, serially sectioned at 4-μm thickness, and rehydrated with graded ethanol-water mixtures, followed by washing with distilled water. Endogenous peroxidase activity was blocked by incubation with 30 ml/L hydrogen peroxidase for 20 min. Tissue sections were then autoclaved at 121℃ in 10 mmol/L citrate buffer (pH 6.0) for 10 min for antigen retrieval and cooled at room temperature for 30 min. The cornea sections were then incubated with goat anti-rat LYVE-1 monoclonal antibody (Boshide Bio-Project Co., Wuhan, China) and goat anti-rat NF-κB P65 monoclonal antibody (Boshide Bio-Project Co.) for 3 h, followed by biotin-labeled rabbit anti-goat immunoglobulin as the secondary antibody. Streptavidin biotin complex (SABC)-peroxidase was used as the immune check system. The slides were visualized for peroxidase activity with diaminobenzidine (DAB) and counterstained with hematoxylin. For NF-κB stains, the brown-staining cells were NF-KB (+) cells and for LYVE-1 stains, the brown-staining vessels were LYVE-1 (+) lymphatic vessels. Lymphatic vessel counting(LVC) was performed as previously described. Briefly, LVC of corneas was evaluated independently by two observers without prior knowledge of the experimental details and the counts were performed in duplicate. Each cornea sample was excised into 20 slices. The LVC number was calculated by summing all LYVE-1(+) lymphatic vessels in the 20 slices and dividing

by 20.

Histopathological examination

The rats were deeply anesthetized and serial photographs of their corneas were taken. The eyeballs were then removed and fixed in 10% neutral buffered formalin for 24 h. The corneal specimens were embedded in paraffin, cross-sectioned, and stained with hematoxylin and eosin.

Immunofluorescence of whole-mount rat corneas

The method was modified from one described previously[10,11]. Briefly, 10 ml lectin-FITC from Lycopersicon esculentum (Sigma, St. Louis, Missouri, USA) (20 μg/ml) was injected intracardially and allowed to perfuse for 2 min. The cornea was dissected and fixed in acetone at -20°C for 15 min and then incubated in phycoerythrin-labeled anti-rat PECAM1 (CD31-PECAM) (BD Biosciences, San Diego, California, USA) (1:50) in TNB blocking buffer (TSA Biotin System, NEN Life Science, Boston, Massachusetts, USA) overnight at 4℃. Corneas were digitally photographed using a confocal fluo rescence microscope (ZEISS, LSM 510 META, Carl Zeiss, Jena, Germany).

Inflammatory index (IF)

The IF was evaluated using a slit lamp and serial photographs were taken of the cornea. The IF was scored as previously described[12], based on the following parameters: ciliary hyperemia (0:absent; 1: present but less than 1 mm; 2: present between 1 and 2 mm; 3: present and more than 2 mm); central corneal edema (0: absent; 1+: present with visible iris details; 2: present without visible iris details; 3: present without visible pupil); and peripheral corneal edema (0: absent; 1: present with visible iris details; 2: present without visible iris details; 3: present with no visible iris). The final IF result was obtained by summing the scores obtained for the different parameters and dividing by 9.

Statistical analysis

Pearson's analysis (SPSS 10.0 statistical software) was used to analyze the correlation between corneal lymphangiogenesis and the IF scores. Values are presented as mean±SD. Differences were accepted as significant at $P<0.05$.

Results

The development of corneal lymphangiogenesis after keratoplasty

We visualized and distinguished corneal lymphatic and blood vessels by injecting i.v. FITC-conjugated lectin to color the blood vessels green, and then coloring all vessels red by incubating the whole cornea with PE-conjugated anti-CD31. Merging of the green and red fluorescent images yielded blood vessels that appeared yellow and lymphatic vessels that appeared red. This method showed that corneal lymphangiogenesis occurred in parallel with corneal hemangiogenesis, and extended gradually from the limbus to the graft after keratoplasty (Figure 1). We then used LYVE-1, one of the most powerful markers of lymphatic structure and function, to further examine the tissue sections for lymphatic vessel development after keratoplasty. LYVE-1 positivity was not observed in control rat corneas, whereas LYVE-1 positive lymphatic vessels occurred on day 3, developed throughout days 7 and 10, and peaked in number at

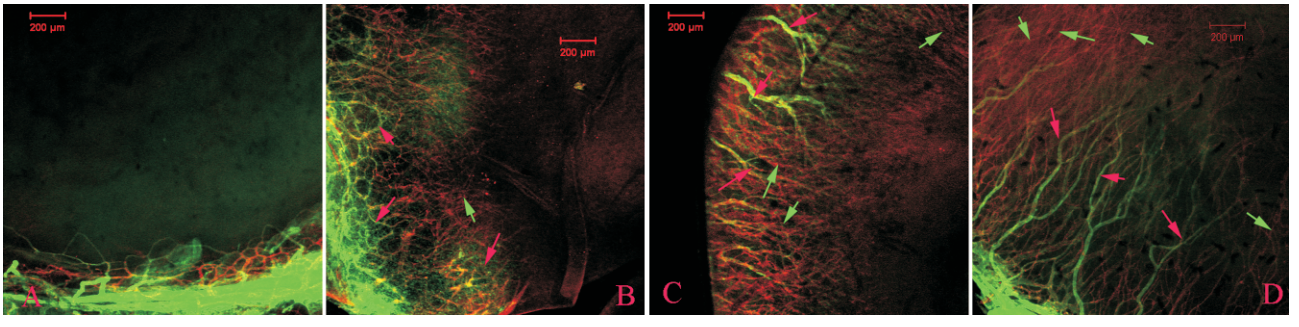

Figure 1 Whole-mount immunofluorescence of a rat cornea after keratoplasty. Lymphatic and blood vessels were present only in the normal rat limbus (A). Some new blood and lymphatic vessels appear in the cornea 3 days after keratoplasty (B), then extend forward gradually 7 days after keratoplasty (C), corneal lymphangiogenesis and hemangiogenesis both peak 14 days after keratoplasty (D).(Green arrows: lymphatic vessels; red arrows: blood vessels. Magnification ×100)

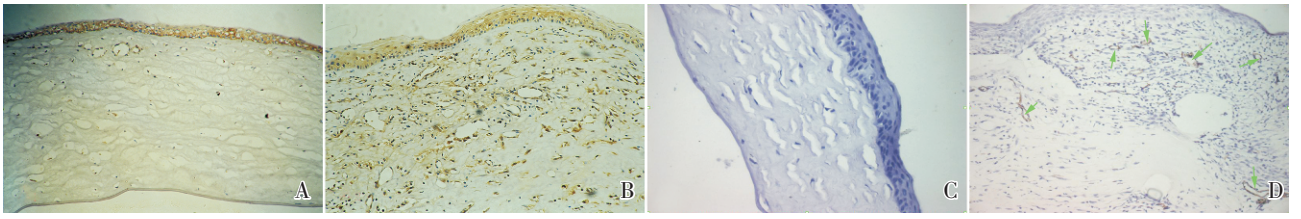

Figure 2 Immunohistochemistry of the rat cornea after keratoplasty. NF-κB (A) or LYVE-1 (C) proteins were not present in the normal rat corneal stoma, but the expression of NF-κB in the stroma was dramatically increased 2 weeks after keratoplasty (B), and some LYVE-1-positive lymphatic vessels merged into larger vessels at the same time (D). (Magnification ×200)

day 14 after corneal transplantation (Figure 2).

The development of corneal inflammation after keratoplasty

We evaluated the state of corneal inflammation by first examining the histopathology of the grafts after corneal transplantation. We found a large number of inflammatory cells invading the stroma at day 3 and day 14 after keratoplasty (Figure 3). Immunohisto chemistry of the grafts revealed that NF-κB was not expressed in the normal corneal stroma but was dramatically upregulated 14 days after keratoplasty (Figure 2). Slit lamp examination of the grafts of six recipients after corneal transplantation revealed inflammation so intense that it clouded the pupil on day 3, and the IF scores also increased dramatically at this time point. This corneal inflammation resolved gradually and was weakest (with the lowest IF scoring) at day 7 after keratoplasty. Corneal inflammation increased again after this time point, and the IF scores were highest on day 14 after keratoplasty (Figures 3 and 4).

The relationship between corneal lymphangiogenesis and corneal inflammation after keratoplasty

We investigated the association between corneal lymphangiogenesis and corneal inflammation by first evaluating the relationship between LVC and IF after keratoplasty. The LVC was strongly and positively correlated with IF (r=0.41; P<0.05). The LVC and

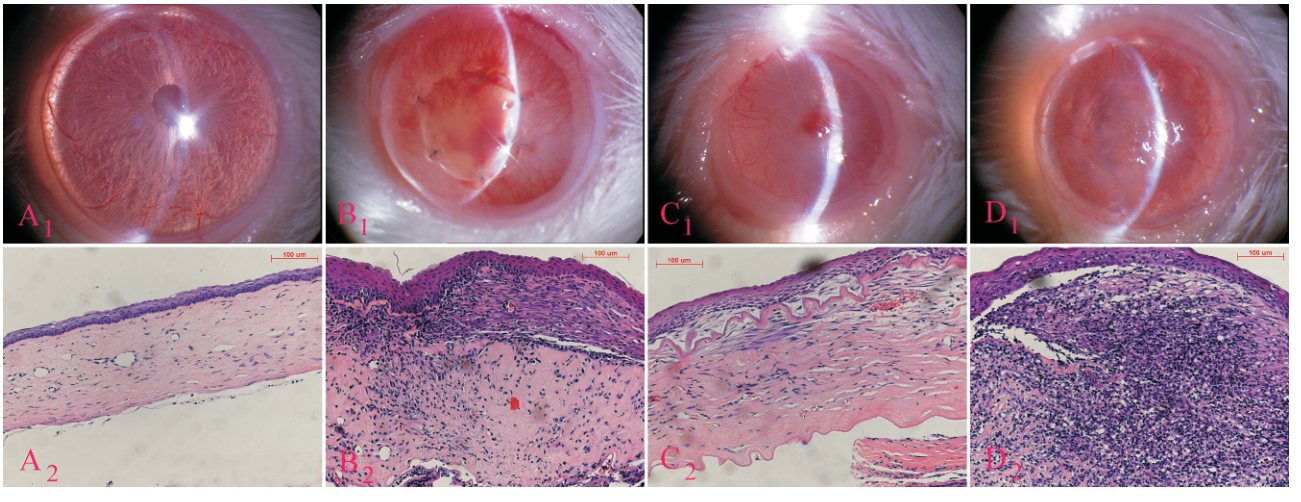

Figure 3 Observation under a slit lamp microscope and histopathological examination after corneal transplantation. The normal rat cornea was transparent with no invading inflammatory cells in the stroma (A). On day 3 after the transplantation, corneal inflammation was so intense it obscured the pupil. The corneal hypothallus was obviously thickened, with a large number of infiltrating inflammatory cells (B). Corneal edema and inflammation decreased gradually; the grafts became transparent and the pupils could be visualized. Few inflammatory cells invaded into the stroma 7 days after the transplantation (C). Inflammatory cells and blood vessels increased dramatically after 7 days. A great many inflammatory cells appeared in the stroma together with many new blood vessels, giving a hairbrush-like appearance from the limbus to the central region, filling the whole cornea, suggesting that corneal inflammation peaked at that time point (D). (Footnote 1: photograph of burned rat cornea; Footnote 2: Histopathological examination; Magnification: (A1, B1, C1, D1) ×25; (A2, B2, C2, D2) ×200)

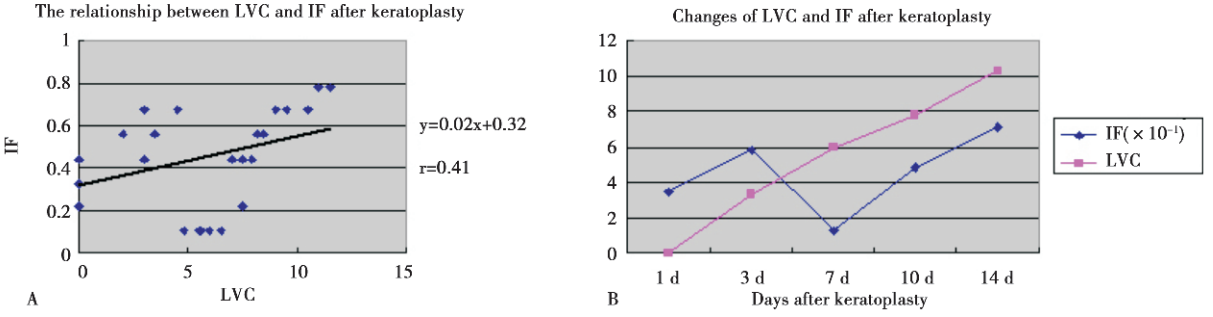

Figure 4 The relationship between LVC and IF after keratoplasty. A significantly positive correlation was evident between LVC and IF after the transplantation (r=0.41 P<0.05) (A). However, the changes in corneal lymphangiogenesis and corneal inflammation did not occur in parallel (B).

IF scores were both maximal at day 14 after keratoplasty. We then plotted the data for further analysis of the association between IF and LVC. A close relationship was generally evident between corneal lymphangiogenesis and inflammation, but the changes in LVC and IF did not occur in parallel (Figure 4).

Discussion

This study indicates that corneal lymphangiogenesis correlates closely with corneal inflammation after keratoplasty. The chance of lymph vessels being present in transplanted rat corneas was significantly higher in strongly inflammatory corneas than in mildly inflammatory corneas.

Corneal lymphangiogenesis plays a crucial role in allograft rejection, but oculists cannot easily identify the state of corneal lymphangiogenesis. This inability to visualize the features of corneal lymphatics prevents the oculist from assessing the effect of drugs targeting lymphangiogenesis after keratoplasty (if available) or choosing a better time for regrafting. In the present study, we considered the possibility that corneal inflammation, which can be easily assessed by a slit lamp, might be useful as an indirect assessment of the state of corneal lymphangiogenesis. We first tested this possibility by using LYVE-1 to label lymphatic vessels in tissue sections.

Compared with angiogenesis, lymphangiogenesis is poorly understood, partly because of the lack of specific lymphatic endothelium markers. This situation has improved since the identification of LYVE-1[13], a hyaluronan receptor related to CD44 that is expressed on lymph vessel endothelial cells in both normal and neoplastic tissues and on both the luminal and abluminal surfaces of the lymphatic endothelial cells. It is a powerful marker of lymphatic structure and function[14,15]. We used LYVE-1 to conduct lymphatic vessel counting (LVC) and plotted the data to obtain a time course for corneal lymphangiogenesis in transplanted corneas.

We also used NF-κB immunohistochemistry to eva luate the extent of corneal inflammation. During the course of corneal transplantation, a number of cytokines and growth factors are up-regulated in corneal cells and contribute to tissue inflammation. A majority of the inflammatory cytokines use the NF-κB pathway for signaling following ligand binding to cell surface receptors. Consequently, NF-κB plays a critical role in allograft rejection, and blocking the NF-κB pathway is beneficial in promoting graft survival[16,17].

We also determined IF scores and plotted these data to show the development of corneal inflammation after keratoplasty. Comparison of the IF and LVC plots indicated that corneal lymphangiogenesis occurred on day 3, developed throughout days 7 and 10, and reached a maximum at day 14 after corneal transplantation. Corneal inflammation was intense on day 3, resolved gradually on day 7, and increased again, with the highest IF scores appearing on day 14 after keratoplasty. Although a significant relationship was evident between corneal inflammation and lymphangiogenesis, the development of corneal lymphatic vessels did not occur in parallel with

corneal inflammation in transplanted rat corneas.

We previously found that transplantation of rat corneas under similar conditions led to allograft failure at day 14 after transplantation. Therefore, we examined the corneal lymphangiogenesis and the development of corneal inflammation from days 1 to 14 after keratoplasty[18]. In another similar experiment, Cursiefen et al[19]. examined the time course of hemangiogenesis and lymphangiogenesis after corneal suturing. They left the sutures in place for 14 days and found a progression of hemangiogenesis and lymphangiogenesis that reached a peak on day 14. After 14 days, the lymphatic vessels started to regress[19]. In our study, corneal lymphatic vessels appeared after the suturing, developed gradually, and also reached a maximal number by day 14. The time course of lymphangiogenesis seen in the present study agreed in general with the data reported by Cursiefen's group. However, we removed the sutures 7 days after the transplantation and did not see any regression in corneal lymphangiogenesis as was observed by Cursiefen et al. In contrast, we observed a steady development that peaked at 14 days after the transplantation. We observed high IF scores on day 3, but then the IF scores declined gradually, followed by an increase on day 7, and a peak at day 14 after keratoplasty. The strong corneal inflammation on day 3 might be a consequence of injury due to sutures and the operation, while the increasing IF scores from day 7 to 14 might be induced by allograft rejection. This could explain why corneal lymphangiogenesis continued to develop until day 14 although the sutures had been removed 7 days after the transplantation.

In summary, our study examined the development of corneal inflammation and corneal lymphangiogenesis and revealed a close, but not parallel, relationship between them in transplanted corneas. Strategies for corneal anti-inflammatory therapy should be investigated to reduce corneal lymphangiogenesis and improve the prognosis of corneal transplantation.

Acknowledgments

This study was supported by China Natural Science Foundation (81070711); Guangdong Provincial Science and Technology Projects(2012B031800456); Doctoral Program of the Ministry of Education (20110171120104)and the Young Teachers Training Program of Sun Yat-sen University (11ykpy42). We thank Dr. Chaoyang Li and Dr. Wenhui Kuang for their invaluable technical support.

Reference

1 Ling S,Qi C,Li W,et al.Crucial role of corneal lymphangiogenesis for allograft rejection in alkali-burned cornea bed.Clin Experiment Ophthalmol, 2009,37(9):874–883.

2 Ling S,Lin H,Xiang D,et al.Clinical and experimental research of corneal lymphangiogenesis after keratoplasty. Ophthalmologica, 2008,222(5):308–316.

3 Maruyama K,Ii M,Cursiefen C,et al.Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages.J Clin Invest,2005, 115: 2363–2372.

4 Cursiefen C,Chen L,Dana MR,et al.Corneal lymphangiogenesis:evidence,mechanisms,and implications for corneal transplant immunology (review).Cornea,2003, 22:273–281.

5 Dietrich T,Bock F,Yuen D,et al.Cutting edge:lymphatic vessels,not blood vessels,primarily mediate immune rejections after transplantation.J Immunol,2010,184 (2): 535–539.

6 Cursiefen C,Chen L,Dana MR,et al.Corneal lymphangiogenesis:evidence, mechanisms,and implications for corneal transplant immunology.Cornea,2003,22:273 – 281.Review.

7 Cursiefen C,Schlotzer-Schrehardt U,Kuchle M,et al. Lymphatic vessels in vascularized human corneas:immunohistochemical investigation using LYVE-1 and podoplanin.Invest Ophthalmol Vis Sci,2002,43:2127 – 2135.

8 Mouta C,Heroult M.Inflammatory triggers of lymphangiogenesis(review). Lymphat Res Biol,2003,1:201–218.

9 Williams KA, Coster DJ. Penetrating corneal transplantation in the inbred rat:a new model. Invest Ophthalmol Vis Cci 1985;26:23–30.

10 Chang L,Kaipainen A,Folkman J.Lymphangiogenesis new mechanisms.Ann N Y Acad Sci,2002;979:111–119. Review.

11 Chang LK,Garcia-cardena G,Farnebo F,et al.Dosedependent response of FGF-2 for lymphangiogenesis.Proc Natl Acad Sci USA,2004,101:11658-11663.

12 Zhang Z,Ma JX,Gao G,et al.Plasminogen kringle 5 inhibits alkali-burn-induced corneal neovascularization.Invest Ophthalmol Vis Sci, 2005,46(11):4062–4071.

13 Bono P, Wasenius VM, Heikkila P, et al.High LYVE-1-positive lymphatic vessel numbers are associated with poor outcome in breast cancer.Clin Cancer Res, 2004, 10: 7144-7149.

14 Banerji S, Ni J, Wang S, Clasper S, et al.LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan.J Cell Biol, 1999, 144: 789-801.

15 Jackson DG.Biology of the lymphatic marker LYVE-1 and applications in research into lymphatic trafficking and lymphangiogenesis (review).APMIS, 2004, 112: 526-538.

16 Ueki S, Yamashita K, Aoyagi T, et al.Control of allograft rejection by applying a novel nuclear factor-kappaB inhibitor, dehydroxymethylepoxyquinomicin. Transplantation, 2006, 82: 1720-1727.

17 Wei JF, Zheng SS.NF-kappa B in allograft rejection.Hepatobiliary Pancreat Dis Int, 2003, 2: 180-183.Review.

18 Ling S, Hu Y.Ipsilateral lymphadenectomy to inhibit corneal allograft rejection in rats.J Huazhong Univ Sci Technolog Med Sci, 2005, 25(1): 88-90.

19 Cursiefen C, Maruyama K, Jackson DG, et al.Time course of angiogenesis and lymphangiogenesis after brief corneal inflammation. Cornea, 2006, 25: 443-447.