• Basic Research •

Blockade of angiopoietin-2/Tie2 signaling pathway specifically promotes inflammation-induced angiogenesis in mouse cornea

Zhi-Xin Yan[1], Yi Luo[1], Ning-Fei Liu[2]

[1]Department of Plastic & Burn Surgery, Affiliated Hospital of Jiangsu University, Zhenjiang 212001, Jiangsu Province, China
[2]Lymphology Center of Department of Plastic & Reconstructive Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200011, China
Correspondence to: Ning-Fei Liu. Lymphology Center of Department of Plastic & Reconstructive Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhi Zao Ju Road, Shanghai 200011, China. liuningfei@126.com
Received: 2017-01-24 Accepted: 2017-06-01

Abstract

• AIM: To investigate angiopoietin-2 (Ang-2)/Tie2 signaling pathway involving in inflammatory angiogenesis.

• METHODS: Three interrupted 11-0 nylon sutures were placed into the corneal stroma of BALB/c mice (6wk old) to induce inflammatory neovascularization. Expression of Ang-2 and Tie2 protein on neovascularization were examined by immunofluorescence. The dynamic expression of Ang-2 mRNA on neovascularization was examined by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Finally, the mouse model of suture-induced corneal neovascularization was used to assess the role of Ang-2/Tie2 signaling pathway in inflammatory angiogenesis by systemic application of L1-10, an Ang-2 specific inhibitor. Mouse corneal hemangiogenesis were evaluated by whole mount immunofluorescence.

• RESULTS: Both Ang-2 and Tie2 were expressed on newly generated blood vessels in inflammatory cornea. Ang-2 expression was gradually upregulated around 2wk following injury, which was concurrent with an increased number of blood vessels. Blockade of Ang-2/Tie2 signaling pathway obviously promoted angiogenesis in inflammatory cornea.

• CONCLUSION: Ang-2/Tie2 signaling pathway seems to play an important role during angiogenesis in inflammatory cornea. This may open new therapeutic applications in pathological processes such as corneal graft survival, wound healing and carcinogenesis.

• KEYWORDS: angiogenesis; angiopoietin-2; Tie2; inflammation

DOI:10.18240/ijo.2017.08.01

Citation: Yan ZX, Luo Y, Liu NF. Blockade of angiopoietin-2/Tie2 signaling pathway specifically promotes inflammation-induced angiogenesis in mouse cornea. *Int J Ophthalmol* 2017;10(8):1187-1194

INTRODUCTION

Angiogenesis, such as the formation of new blood vessel from preexisting blood vessels, plays a key role in multiple physiological and pathological processes. Three of these processes have gained extensive attention in the past decades years: the involvement of blood vessels in wound healing[1], proliferation of tumor cells depending on nutrition supply *via* blood vessels[2], and the role of blood vessels in (corneal) graft survival[3]. The outgrowth of blood vessels is initially induced by the vascular endothelial growth factor (VEGF) and its receptor (VEGFR). While more evidence indicates that VEGF signaling often keeps a critical rate-limiting step in pathological angiogenesis[4], there is consensus that angiogenesis is very complex and coordinated processes, needing the sequential activation of a series of signaling [5-7]. Members of VEGFs and angiopoietins are thought to function in a complementary way during angiogenesis.

Angiopoietin-2 (Ang-2), the second member of the angiopoietin family, is a key molecule for blood vessel formation, which is known as a secreted protein ligand of the receptor tyrosine kinase Tie2[8-9]. Ang-2 gene was reported to be expressed in the endothelium and the associated cells of the arterial vessels, inner stripe of the renal outer medulla[8,10-12], and ocular blood vessels[13] during embryonic and postnatal development. Ang-2 is speculated to destabilize blood vessels as a natural Tie2 antagonist[8,14-15]. It is thought to cause blood vessel regression in the absence of VEGF-A, whereas promote hemangiogenesis in the presence of VEGF-A[14-15]. However, biochemical researches on Ang-2 have reached disputed results[8-10,16-17]. Ang-2 blocks Ang-1-induced Tie2 activation in endothelial cells (ECs) but triggers Tie2 phosphorylation when Tie2 is genetically introduced into NIH3T3 fibroblast cells[8]. While other researches elucidate that high level of Ang-2 stimulation activates Tie2 in vascular ECs[16] and triggers vascular tube formation[18-19], revealing the complexity of Ang2

in angiogenesis. Inflammation is the body's physiological reaction to injury or inflammation, but it may also develop during and involve in multiple pathological processes. Angiogenesis can be induced by inflammation during various pathological processes, including grafting, carcinogenesis and wound healing. However, there are limitations and concerns associated with the function of Ang-2 in the progress of inflammatory angiogenesis, and underlying mechanisms of Ang-2/Tie2 signaling pathway on inflammatory angiogenesis *in vivo* remain also uncharacterized.

Here, the murine model of combined inflammatory hemangiogenesis and lymphangiogenesis in the normally avascular cornea was used to investigate the contributions of Ang-2/Tie2 signaling pathway to the progression of inflammatory angiogenesis.

MATERIALS AND METHODS

Animals The mouse models of sutured cornea were used to assess inflammatory neovascularization[20]. BALB/c mice (female, aged 6-8wk), weighing 20-25 g, were purchased from the Animal Care Centre of Pudong Shanghai and the Experimental Animal Centre of Jiangsu University, China. All animals participated in the research were managed according to the Jiangsu University and Shanghai Jiao Tong University School of Medicine Administration Office of Laboratory Animals Guidelines for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research[20]. The study follows to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Prior to experiment, all mice were confirmed to be free from corneal diseases by using slit lamp microscope and other disorders.

Mouse Suture-induced Corneal Model of Inflammation We used microsurgical techniques to establish the mouse suture-induced corneal model of inflammation as formerly described[20-22]. Before surgical procedures, the intramuscular injection of ketamine (100 mg/kg) plus xylazine (10 mg/kg) were used to anesthetize mice which were euthanized at experimental end points with a lethal dose of CO_2 asphyxiation. Three 11-0 nylon sutures (Jinhua, China) were laid intrastromally, with two stromal incursions extending over 120° of corneal circumference each. The outer point of suture placement was selected near the limbus, and the inner suture point was selected near the corneal centre equidistant from limbus for obtaining standardized angiogenic responses. Sutures were kept in place until the end of experiment[23].

Immunofluorescence Staining protocols of cryosections were standardized as previously described[20,24-26]. Shortly, indirect immunofluorescence was used to localize Ang-2 and Tie2 in blood vessels in the pathologically vascularized mouse corneas and the normal nonvascularized mouse corneas at the limbus. For these experiments, murine eyes were cryopreserved in optimal cutting temperature embedding medium, and 5 to 7 μm cryosections were harvested. Sections were dried (15min, 37℃) and fixed in acetone for 15min on slides. After being rinsed with phosphate-buffered saline (PBS) (3×5min), specimens were permeabilized with 0.2% Triton X-100 in PBS for 5min and incubated with PBS containing 2% bovine serum albumin (BSA) at room temperature for 1h. Specimens were incubated with the mixed primary antibody's fluid in PBS containing 2% BSA followed overnight at 4℃. To localize Ang-2 expression on blood vessels, the mixed primary antibody's fluid of rabbit anti-mouse Ang-2 antibody (Abcam, United Kingdom, 1:500) and rat anti-mouse CD31 Biotin antibody (BD Biosciences pharmingen, USA, 1:400) in PBS containing 2% BSA was used. To localize Tie2 expression on blood vessels, the mixed primary antibody's fluid of rabbit anti-mouse Tie2 antibody (Abcam, 1:500) and rat anti-mouse CD31 Biotin antibody (BD Biosciences pharmingen, 1:400) in PBS containing 2% BSA was used. On the second day, the antibodies were rinsed with PBS (5×5min) and blocked with 2% BSA in PBS for 1h at room temperature, and then specimens were incubated with the mixed fluid of Alexa Fluor®555 donkey anti-rabbit antibody (Invitrogen, USA, 1:1000) and Streptavidin-Dylight™488 (Biolegend, CA, 1:200) in PBS containing 2% BSA for 1h at 37℃ in the dark. Then, the antibodies were rinsed with PBS (3×15min, on a shaker) in the dark, and specimens were incubated with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, 1:500) in PBS for 5min at 37℃ in the dark. All incubations of staining were carried out in a humid chamber. After a last rinsing step (3×5min PBS), sections were covered using fluorescent mounting medium (DAKO Corporation, Denmark) and stored at 4℃ in the dark humid chamber. Fluorescence microscopy and photography was taken with a confocal laser scanning microscope (Zeiss Confocal LSM 710 microscope, Germany), and digital pictures were done with Zen 2010 Light Edition (Carl Zeiss, Germany).

RNA Isolation and Purification Prior to suture and 1, 7, 11 and 14d after suture, the eyes of mice were removed under anaesthesia. To each time point, all RNA was extracted from the 6 corneas with Trizol® Reagent (Invitrogen). RNA was prepared following according to the manufacturer's protocol. The RNA pellets were washed with 75% ethanol, centrifuged and dried. Pellets were dissolved in DEPC-treated water. The concentration and purity of RNA were determined by measuring optical density at 260 nm and 280 nm using a Beckman Coulter DU 800 UV/Vis spectrophotometer (Beckman Coulter, USA).

Real-time Reverse Transcriptase-polymerase Chain Reaction Analysis Complementary DNA synthesis was carried out by using a 20 μL reaction system. cDNA was synthesized from

2 μg of total RNA with ThermoScript reverse transcriptase (Invitrogen) following manufacturer's protocol. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out with gene-specific primers using a Stratagene Mx3000P qPCR System (Agilent Technologies Inc. USA). The sequences of primers for RT-PCR were as follows: β-actin:(sense: 5'-CTGTCCCTGTATGCCTCTG-3', antisense: 5'-TGTCACGCACGATTTCC-3'); Ang-2:(sense: 5'-TCTTCCTCCAGCCCCTACAT-3', antisense:5'-TCTCCACCATCTCCTTCTTCATC-3').

All reactions were carried out in triplicate. The melting curve analyses were carried out to guarantee the specificity of the quantitative RT-PCR reactions. The data analysis was carried out with the $2^{-\triangle\triangle Ct}$ method depicted previously[27], while β-actin was acted as reference gene. Values are presented as means±SEM. $P<0.05$ were regarded as significant. In the figure, the alphabet a is used to represent a significant difference between groups ($P<0.01$). Graph was drawn using origin7.5 (Originlab Inc., USA).

Neovascularization Assay of Suture-induced Inflammatory Cornea The mouse models of sutured cornea were randomly divided into two groups. The treatment group ($n=7$) received L1-10 (4 mg/kg), an Ang-2 specific inhibitor (Amgen Inc., USA), which was dissolved in PBS and injected subcutaneously every other day beginning with 1d before surgery. The control mice ($n=7$) received a same amount of PBS solution. Mice were sacrificed on 14d, morphological determination of corneal neovascularization was examined by whole mount immunofluorescence staining.

Whole Mount Preparations and Immunofluorescence Staining Preparation was done as previously depicted[20,24]. Briefly, mice were sacrificed under anaesthesia, the sutured eyes were taken out and the corneas were anatomized from the eyes in the rear of the corneal limbus. Corneas were washed 3×5min with PBS at room temperature. Fixation was done with acetone for 30min. After three more washing steps with PBS and blocking by 2% BSA containing 0.3% Triton X-100 in PBS for 2h at room temperature, corneas were stained overnight at 4℃ by rabbit anti-mouse LYVE-1 antibody (Abcam 1:500) plus 2% BSA in PBS. On 2d, after washing 5×5min with PBS, the antibody was blocked by 2% BSA in PBS for 2h. The secondary antibody Alexa Fluor®488 rat anti-mouse CD31 (Biolegend, USA), diluted 1:50 by PBS containing 2% BSA, was added to incubate with corneas overnight at 4℃ in the dark. On the third day, after washed 5×5min by PBS, 2% BSA in PBS was used to block the antibody for 2h. The third antibody, Alexa Fluor®555 donkey anti-rabbit antibody (Invitrogen), diluted 1:1000 by 2% BSA in PBS, was used to incubate with corneas for 45min at room temperature in the dark. As a last step, antibody was washed 3×15min with PBS. Corneas were moved to microscope slides, covered with DAKO fluorescent mounting medium and stored at 4℃ in the dark. Fluorescence microscopy and photography was taken with a confocal laser scanning microscope (Zeiss Confocal LSM 710 microscope, Germany), and digital pictures were done with Zen 2010 Light Edition (Carl Zeiss, Germany).

Dynamic Functional and Statistical Analysis and Graph Quantitative analysis of neovascularization was carried out in a standardized procedure by using Image-pro plus 6.0 (soft imaging system, USA) software *via* threshold analysis. In order to measure, we used rectangles of a standardized size (1.1 mm^2), aligning along the limbus as previously depicted[21]. The corneal area suffused with newborn vessels (hemvascularized or lymphvascularized area) was calculated in each rectangle. The ratio of vessel area was decided by the vascularized area of the treatment group in correlation to that of the control group. The vascularized areas of the control groups were regarded as being 100%. Analysis of differences between two samples was achieved by using a standard two-tailed Student's *t*-test (SPSS 17.0 statistical software, USA). Values are expressed as mean±SEM. *P* value of being less than 0.05 was regarded significant. In the figure, the alphabet 'a' is used to represent a significant difference between 2 groups ($P<0.01$). Origin 7.5 (Originlab Inc., USA) was used to draw graphs.

RESULTS

Ang-2 Expression on Physiological Blood Vessels Immunofluorescence staining was used to investigate whether Ang-2 was expressed on physiological blood vessels at the limbus (border between vascularized conjunctiva and nonvascularized cornea) in normal murine eyes. We found that blood vessels were only found in limbus (Figures 1A and 2A), and a low-level expression of Ang-2 was co-localized on the quiescent CD31-positive blood vessels at the limbal arcade and adjacent physiological vascularized conjunctiva in normal cornea (Figure 1D). While, some inside epithelial cells near matrix was found also to express Ang-2 (Figure 1B).

Ang-2 Expression on Pathological Blood Vessels Induced by Inflammation In this study, we used immunofluorescence staining to localize Ang-2 expression in pathologically vascularized murine corneas and observed that angiogenesis was obviously induced by inflammation (Figures 1E and 2E), and that the expression of Ang-2 was simultaneously intensely raised in the sutured cornea (Figure 1F). We also found that the newly generated blood vessels strongly expressed Ang-2 in the sutured cornea (Figure 1H).

Tie2 Expression on Physiological Blood Vessels Immunofluorescence staining was used to investigate whether Tie2 was expressed on physiological blood vessels at the limbus in normal murine eyes. We found that blood vessels were only found in limbus (Figures 1A and 2A), and a low-level expression of Tie2 co-localized on the quiescent CD31-positive

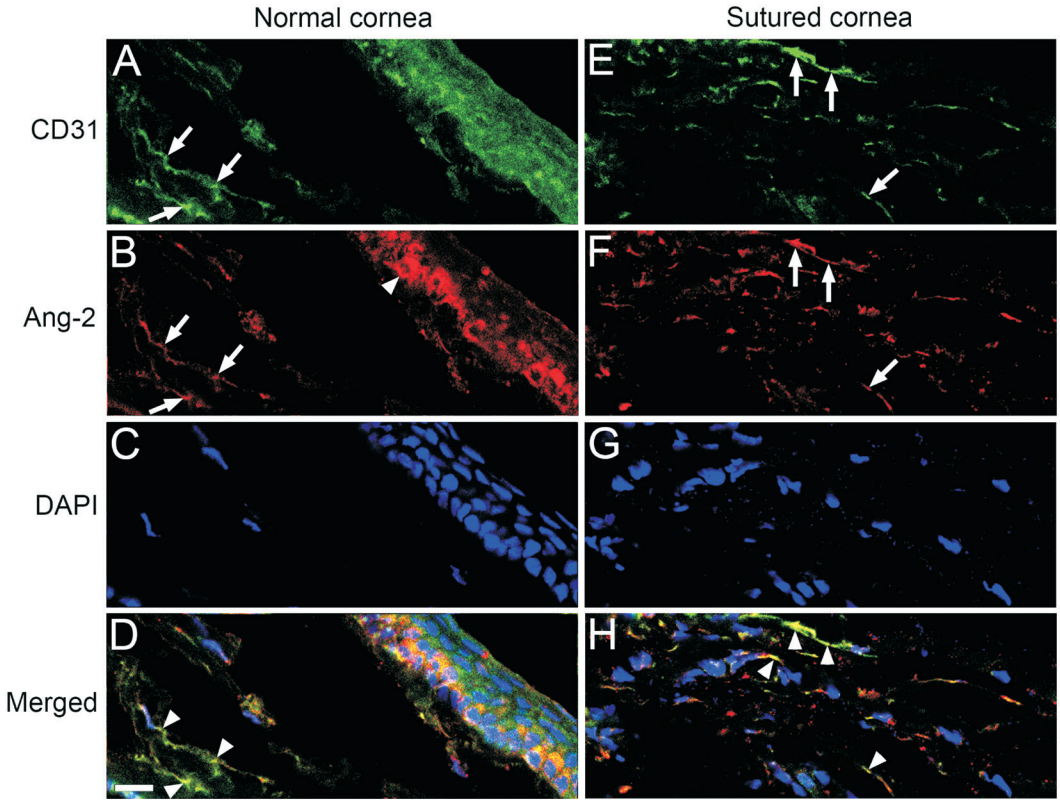

Figure 1 Representative images of Ang-2 expressed on the CD31-positive blood vessels in the cornea stained by immunofluorescence (A, B, C, D): Representative images of the immunofluorescence-stained limbus (border between vascularized conjunctiva and nonvascularized cornea) of normal murine eyes. A: Stained blood vessels at limbus (CD31, green, while arrows); B: Stained Ang-2 at limbus (red, white arrows) and on some epithelial cells (red, white arrowhead); C: Stained nuclei [4', 6-diamidino-2-phenylindole (DAPI), blue]; D: Merged images of A, B and C (arrowheads: Co-location of Ang-2 on quiescent blood vascular endothelial cells). E, F, G, H: Representative images of the cornea 10d after suture stained by immunofluorescence; E: Stained blood vessels (CD31, green, white arrows). F: Stained Ang-2 (red, white arrows); G: Stained nuclei (DAPI, blue); H: Merged images of E, F and G (arrowheads: Ang-2 is co-localized with CD31-positive blood vessels). Scale bar (D, bottom, left)=20 μm.

blood vessels at the limbal arcade and adjacent physiological vascularized conjunctiva in normal cornea (Figure 2D). While, some inside epithelial cells near matrix was found also to express Tie2 (Figure 2B).

Tie2 Expression on Pathological Blood Vessels Induced by Inflammation In this study, we used immunofluorescence staining to localize Tie2 expression in pathologically vascularized murine corneas and observed that angiogenesis was obviously induced by inflammation (Figures 1E and 2E), and that the expression of Tie2 was simultaneously intensely raised in the sutured cornea (Figure 2F). We also found that the newly generated blood vessels strongly expressed Tie2 in the sutured cornea (Figure 2H).

Expression of Ang-2 mRNA in Sutured Corneas We used the murine model of suture-induced corneal neovascularization to further explore the expression of Ang-2 mRNA in inflammatory angiogenesis. Quantitative RT-PCR analysis was used to estimate the expression level of Ang-2 mRNA. Our results show that normal corneas expressed a low-level Ang-2 mRNA, and that the expression of Ang-2 mRNA was triggered by inflammation in the sutured corneas (Figure 3). Accompanying inflammatory angiogenesis in inflamed corneas and the raised quantity and density of inflammatory blood vessels during this time (Figure 4D), the expression of Ang-2 mRNA gradually raised lasting at least 14d.

L1-10, a Specific Inhibitor of Ang-2, Increases Hemangiogenesis In our study, whole mount staining indicated that blood and lymphatic vessels were physiologically existing at the limbus (Figure 4A and 4B), and normal mouse corneas were lack of blood and lymphatic vessels (Figure 4A and 4B), and that corneal newborn blood and lymphatic vessels were interspersed through the stroma after suture about 2wk (Figure 4D and 4E). L1-10, an Ang-2 specific inhibitor, was used to judge the role of Ang-2 in inflammatory angiogenesis in murine models of sutured cornea. L1-10 was injected into mice subcutaneously every other day starting at one day before surgery. The experiment group received L1-10 (4 mg/kg), dissolved in PBS, while control group received the same total of PBS solution. Mice were given the treatment for 14d after suture. After 14d, the treatment group (n=7) showed significantly increased blood vessel growth with raised vascular density but slimmer in diameter (P<0.01) (Figure 4J)

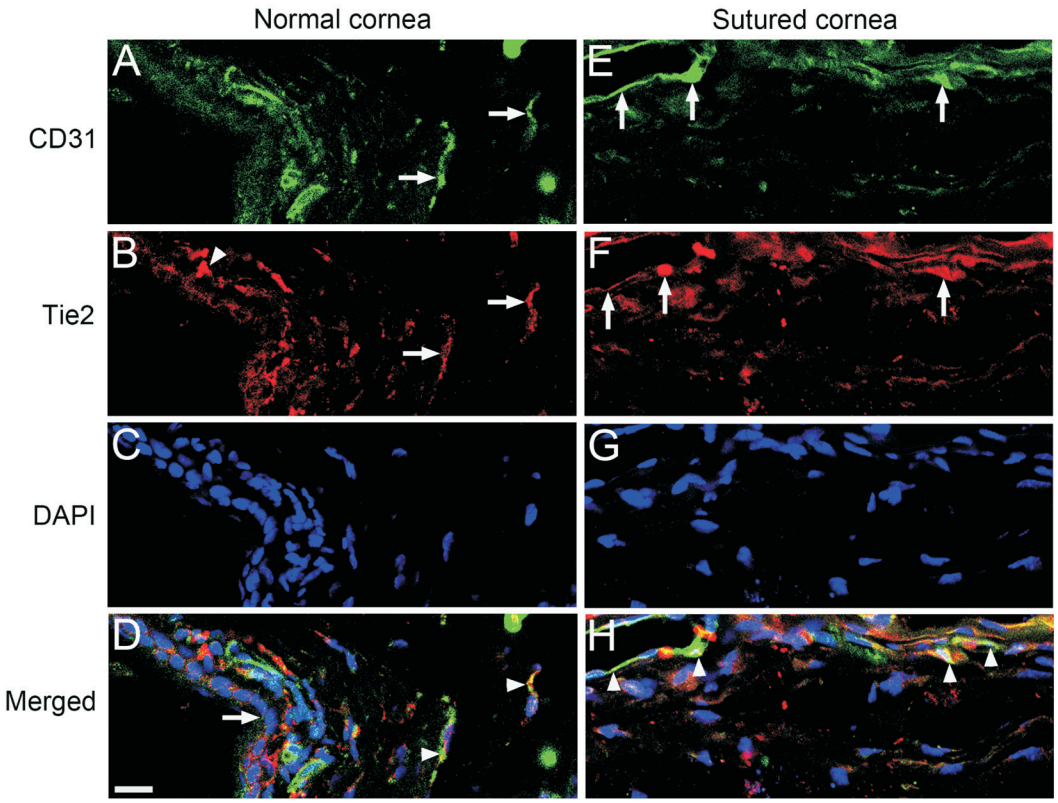

Figure 2 Representative images of Tie2 expressed on the CD31-positive blood vessels in the cornea stained by immunofluorescence A, B, C, D: Representative images of the limbus of normal murine eyes stained by immunofluorescence. A: Stained blood vessels at limbus (CD31, green, while arrows); B: Stained Tie2 at limbus (red, white arrows) and on some epithelial cells (red, white arrowhead); C: Stained nuclei (DAPI, blue); D: Merged images of A, B and C (arrowheads: co-location of Ang-2 on quiescent blood vascular endothelial cells, arrows: limbus). E, F, G, H: Representative images of the cornea 7d after suture stained by immunofluorescence; E: Stained blood vessels (CD31, green, white arrows); F: Stained Tie2 (red, white arrows); G: Stained nuclei (DAPI, blue); H: Merged images of E, F and G (arrowheads: Tie2 is co-localized with CD31-positive activated blood vessels). Scale bar (D, bottom, left)=20 μm.

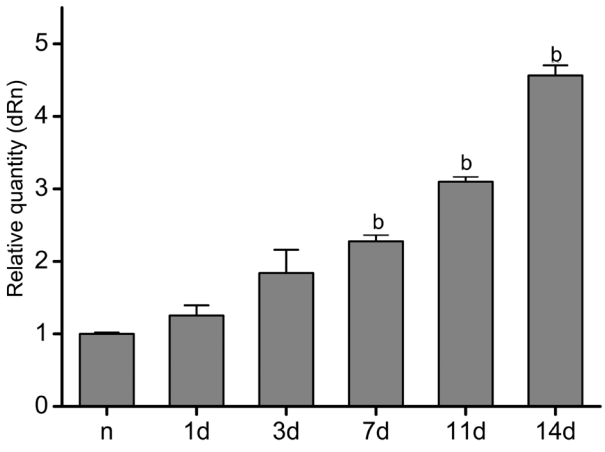

Figure 3 Expression of Ang-2 mRNA in the inflamed murine corneas Untreated corneas expressed a low-level Ang-2 mRNA. Expression of Ang-2 mRNA was increased gradually in inflamed murine corneas after suture lasting about 2wk, as proved by qRT-PCR analysis. n represents group of untreated corneas; 1, 3, 7, 11, and 14d represent groups of corneas 1, 3, 7, 11, and 14d after suture, respectively. Data are presented as the mean±SEM. $^{b}P<0.01$ *vs* group of normal corneas.

and reduced lymphatic growth ($P<0.01$) (Figure 4K) compared with the control group ($n=7$). Therefore, inhibition of the Ang-2/Tie2 signalling pathway by L1-10 efficiently promoted inflammatory angiogenesis but obviously blocked inflammatory lymphangiogenesis, indicating that Ang-2/Tie2 system is an important signaling pathway involving in regulating inflammatory angiogenesis.

DISCUSSION

Until now, the function of Ang-2/Tie2 signaling pathway in inflammatory angiogenesis remains largely undefined. In this study, we determine that both Ang-2 and Tie2 are merely weakly expressed on quiescent blood vessel endothelium, however, inflammation induces obvious expression of Ang-2 and Tie2 on newly generated blood vessels. Upregulation of the expression of Ang-2 and Tie2 occurred simultaneously with inflammatory angiogenesis. The expression of Ang-2 mRNA was also strongly induced by inflammation in sutured corneas, which was gradually upregulated for around 2wk following injury, while the number and density of inflammatory blood vessels also were raised during this time. These observations clearly demonstrate that Ang-2 expression is consistent, and strictly controlled and dynamic, and it is induced and obviously increased by exogenous stimuli which induce the pathological process of angiogenesis, such as inflammation

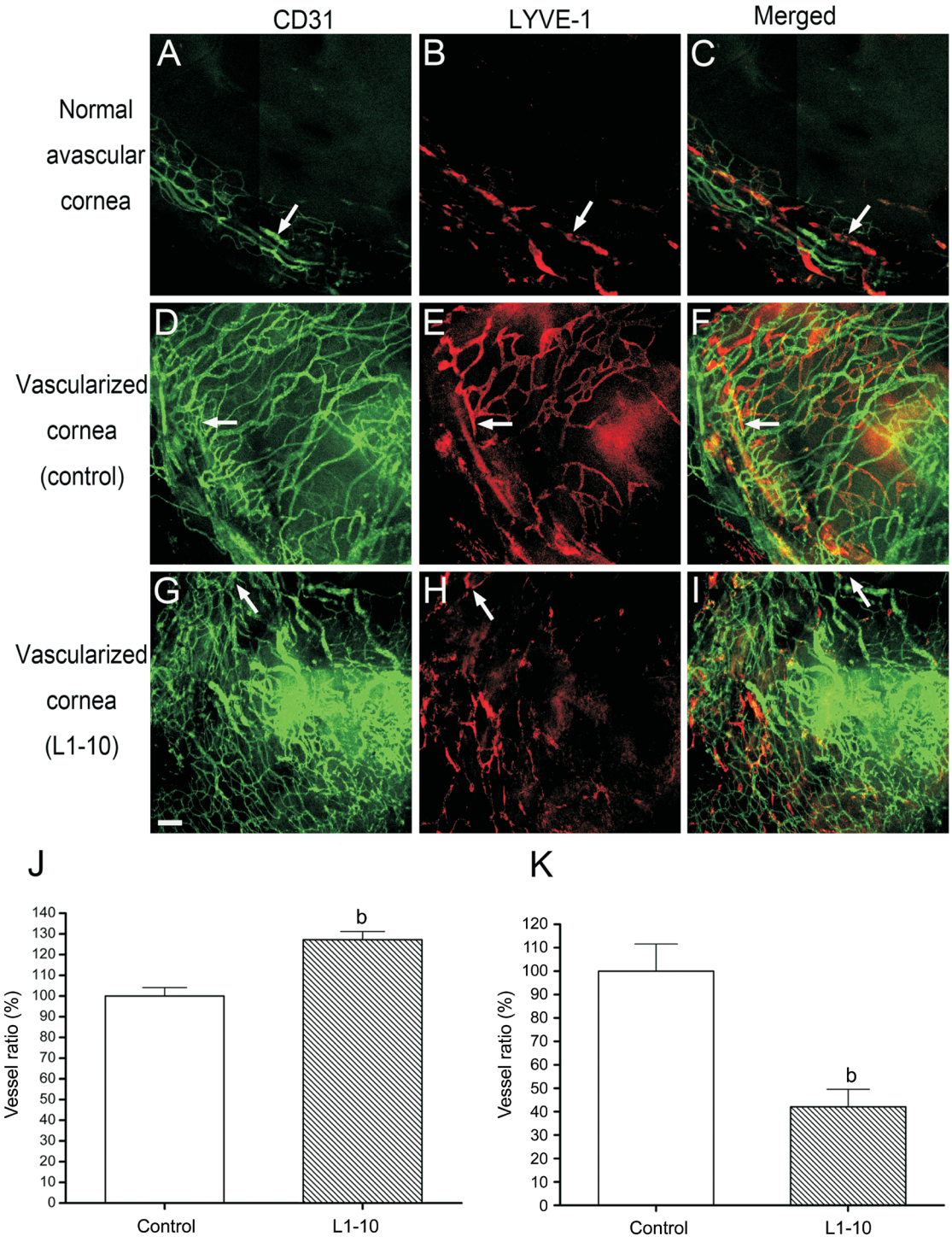

Figure 4 Systemic application of L1-10, an Ang-2 specific inhibitor, for 14d in the mouse models of suture-induced corneal neovascularization L1-10 obviously promoted hemangiogenesis in the inflamed murine corneas, but decreased lymphangiogenesis in the inflamed murine corneas compared with the control group 14d after suture. A-I: Representative segments of corneal whole mounts (green: blood vessels; red: lymphatic vessels). Arrows: Limbus. A, D, G: $CD31^+$ blood vessels; B, E, H: $LYVE\text{-}1^+$ lymphatic vessels; C, F, I: Merge of A and B, D and E, and G and H, respectively. Raising of hemangiogenesis (J) ($P<0.01$, $n=7$) and inhibition of lymphangiogenesis (K) ($P<0.01$, $n=7$) 2wk after treatment with L1-10 in a suture-induced neovascularization assay. Vessel area ratio: Area covered by blood/lymphatic vessels (%) in correlation to the control (set to 100%). Data are expressed as the mean±SEM. $^bP<0.01$ comparing the treatment group with the control group. Scale bar (G, bottom, left)=100 μm.

and hypoxia. For all we know, this is the first time to report the expression of both Ang-2 and Tie2 on inflammatory blood vessels, suggesting that Ang-2/Tie2 signaling pathway may play an important role in inflammatory angiogenesis.

Previous study shows Ang-2 plays an intricate role in regulation of vascular remodeling that leads to either vessel sprouting or regression, depending on its context[8]. To enucleate the direct function of Ang-2/Tie2 signaling pathway in inflammatory

angiogenesis, we treated the murine models of suture-induced inflammatory corneal neovascularization with the FC-fusion protein L1-10, which blocks Ang-2 binding to its receptor Tie2 and inhibits the proliferation of endothelial cells[28]. Current studies have proved that L1-10 is a specific and strong depressor of Ang-2[29-30]. In the current study, we showed that blockade of the Ang-2/Tie2 signaling pathway by using L1-10 obviously promoted inflammatory angiogenesis. These data suggest that Ang-2/Tie2 signaling pathway is a crucial system which involves in inflammatory angiogenesis. Because blood vessels show the identical morphological and functional characteristics in the eye as they do in other tissues[25], we can guess that the blockade of Ang-2 might affect angiogenesis in other organs, but the accurate timing and the molecular mechanisms might be other than in the cornea because of the different microenvironment.

In transplantation, the blood vessels and lymphatic vessels have received tremendous attention because of theirs association with graft survival and rejection[3]. A great amount of animal and clinical studies have showed that graft survival depends on both arms of the so-called immune reflex arc. This arc comprises the lymphatic vessels as the afferent arm and the blood vessels as the efferent arm. Through the lymphatic vessels antigens and dendritic cells can get to local lymph nodes and trigger an immune response[30], while via the blood vessels oxygen and nutrients can reach local tissue. Therefore, specifically promoting hemangiogenesis could provide the graft with more nutrients and benefit wound healing, and at the same time blockade of lymphangiogenesis could result in inhibition of the induction of an immune response. So it is important to distinguish ways to specifically upregulate hemangiogenesis to promote wound healing and graft survival. Previous studies show both inflammatory angiogenesis and inflammatory lymphangiogenesis can be blocked by inhibiting VEGF-A/VEGFR-2 system[21], and blocking the VEGF-C/-D/VEGFR-3 signaling pathway inhibits the outgrowth of inflammatory lymphatic vessels in contrast to hemangiogenesis[23]. However, there are limitations and concerns associated with anti-Ang-2/Tie2 signaling pathway until now. Previous studies also show Ang-2 stimulates pathologic angiogenesis, and inhibition of Ang-2 promotes neovascular regression[31-33]. Here, we show for the first time that the different role of Ang-2/Tie2 system in inflammatory angiogenesis. So, we speculate that Ang-2/Tie2 system is intricately involved in angiogenesis, and Ang-2/Tie2 system acts as accelerator or inhibitor, which is dependent on tissue microenvironment. Blocking Ang-2/Tie2 signaling pathway may found a new treatment option to promote graft survival by boosting inflammatory hemangiogenesis, which is also important for wound healing.

In general, inflammatory corneal angiogenesis seems to rely on Ang-2/Tie2 signaling pathway. By inhibiting this pathway, the ingrowths of blood vessels can be obviously promoted in inflammatory cornea, which provide new field for therapeutic exploration. This research effort is uncovering new, important molecular regulator of inflammatory angiogenesis. Illustrating the role of Ang-2/Tie2 signaling pathway in inflammatory angiogenesis might broaden our understanding of numerous pathological processes, and the discovering that Ang-2/Tie2 signalling pathway is participated in the regulation of inflammatory angiogenesis suggests that blockade of Ang-2/Tie2 system may have important therapeutic applications in some pathological processes such as (corneal) graft survival, wound healing and carcinogenesis.

ACKNOWLEDGEMENTS

Foundations: Supported by National Natural Science Foundation of China (No.81641174; No.30772262).

Conflicts of Interest: Yan ZX, None; **Luo Y**, None; **Liu NF**, None.

REFERENCES

1 Johnson KE, Wilgus TA. Vascular Endothelial Growth Factor and Angiogenesis in the Regulation of Cutaneous Wound Repair. *Adv Wound Care* (*New Rochelle*) 2014;3(10):647-661.

2 Paduch R. The role of lymphangiogenesis and angiogenesis in tumor metastasis. *Cell Oncol* (*Dordr*) 2016;39(5):397-410.

3 Fasciani R, Mosca L, Giannico MI, Ambrogio SA, Balestrazzi E. Subconjunctival and/or intrastromal bevacizumab injections as preconditioning therapy to promote corneal graft survival. *Int Ophthalmol* 2015;35(2):221-227.

4 Kumar VB, Binu S, Soumya SJ, K H, Sudhakaran PR. Regulation of vascular endothelial growth factor by metabolic context of the cell. *Glycoconj J* 2014;31(6-7):427-434.

5 Yamazaki Y, Morita T. Molecular and functional diversity of vascular endothelial growth factors. *Mol Divers* 2006;10(4):515-527.

6 Khan KA, Bicknell R. Anti-angiogenic alternatives to VEGF blockade. *Clin Exp Metastasis* 2016;33(2):197-210.

7 Kumar M, Dhatwalia SK, Dhawan DK. Role of angiogenic factors of herbal origin in regulation of molecular pathways that control tumor angiogenesis. *Tumour Biol* 2016;37(11):14341-14354.

8 Wang S, Park JK, Duh EJ. Novel targets against retinal angiogenesis in diabetic retinopathy. *Curr Diab Rep* 2012;12(4):355-363.

9 Isidori AM, Venneri MA, Fiore D. Angiopoietin-1 and Angiopoietin-2 in metabolic disorders: therapeutic strategies to restore the highs and lows of angiogenesis in diabetes. *J Endocrinol Invest* 2016;39(11):1235-1246.

10 Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D, Suri C, Campochiaro PA, Wiegand SJ, Yancopoulos GD. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. *Dev Cell* 2002;3(3):411-423.

11 Shimoda H. Immunohistochemical demonstration of Angiopoietin-2 in lymphatic vascular development. *Histochem Cell Biol* 2009;131(2):231-238.

12 Yuan HT, Suri C, Landon DN, Yancopoulos GD, Woolf AS. Angiopoietin-2 is a site-specific factor in differentiation of mouse renal vasculature. *J Am Soc Nephrol* 2000;11(6):1055-1066.

13 Hackett SF, Wiegand S, Yancopoulos G, Campochiaro PA. Angiopoietin-2 plays an important role in retinal angiogenesis. *J Cell Physiol* 2002; 192(2):182-187.

14 Visconti RP, Richardson CD, Sato TN. Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). *Proc Natl Acad Sci U S A* 2002;99(12):8219-8224.

15 Eklund L, Olsen BR. Tie receptors and their angiopoietin ligands are context-dependent regulators of vascular remodeling. *Exp Cell Res* 2006;312(5):630-641.

16 Kim I, Kim JH, Moon SO, Kwak HJ, Kim NG, Koh GY. Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. *Oncogene* 2000;19(39):4549-4552.

17 Bogdanovic E, Nguyen VP, Dumont DJ. Activation of Tie2 by angiopoietin-1 and angiopoietin-2 results in their release and receptor internalization. *J Cell Sci* 2006;119(Pt 17):3551-3560.

18 Mochizuki Y, Nakamura T, Kanetake H, Kanda S. Angiopoietin 2 stimulates migration and tube-like structure formation of murine brain capillary endothelial cells through c-Fes and c-Fyn. *J Cell Sci* 2002;115(Pt 1): 175-183.

19 Chen JX, Chen Y, DeBusk L, Lin W, Lin PC. Dual functional roles of Tie-2/angiopoietin in TNF-alpha-mediated angiogenesis. *Am J Physiol Heart Circ Physiol* 2004;287(1):H187-H195.

20 Yan ZX, Jiang ZH, Liu NF. Angiopoietin-2 promotes inflammatory lymphangiogenesis and its effect can be blocked by the specific inhibitor L1-10. *Am J Physiol Heart Circ Physiol* 2012;302(1):H215-H223.

21 Bock F, Onderka J, Dietrich T, Bachmann B, Kruse FE, Paschke M, Zahn G, Cursiefen C. Bevacizumab as a potent inhibitor of inflammatory corneal angiogenesis and lymphangiogenesis. *Invest Ophthalmol Vis Sci* 2007;48(6):2545-2552.

22 Cursiefen C, Maruyama K, Jackson DG, Streilein JW, Kruse FE. Time course of angiogenesis and lymphangiogenesis after brief corneal inflammation. *Cornea* 2006;25(4):443-447.

23 Bock F, Onderka J, Dietrich T, Bachmann B, Pytowski B, Cursiefen C. Blockade of VEGFR3-signalling specifically inhibits lymphangiogenesis in inflammatory corneal neovascularisation. *Graefes Arch Clin Exp Ophthalmol* 2008;246(1):115-119.

24 Cursiefen C, Chen L, Borges LP, Jackson D, Cao J, Radziejewski C, D'Amore PA, Dana MR, Wiegand SJ, Streilein JW. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest* 2004;113(7): 1040-1050.

25 Cursiefen C, Schlötzer-Schrehardt U, Küchle M, Sorokin L, Breiteneder-Geleff S, Alitalo K, Jackson D. Lymphatic vessels in vascularized human corneas: immunohistochemical investigation using LYVE-1 and podoplanin. *Invest Ophthalmol Vis Sci* 2002;43(7): 2127-2135.

26 Dietrich T, Onderka J, Bock F, Kruse FE, Vossmeyer D, Stragies R, Zahn G, Cursiefen C. Inhibition of inflammatory lymphangiogenesis by integrin α5 blockade. *Am J Pathol* 2007;171(1):361-372.

27 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25(4):402-408.

28 Oliner J, Min H, Leal J, *et al*. Suppression of angiogenesis and tumor growth by selective inhibition of angiopoietin-2. *Cancer Cell* 2004;6(5):507-516.

29 Tressel SL, Kim H, Ni CW, Chang K, Velasquez-Castano JC, Taylor WR, Yoon YS, Jo H. Angiopoietin-2 stimulates blood flow recovery after femoral artery occlusion by inducing inflammation and arteriogenesis. *Arterioscler Thromb Vasc Biol* 2008;28(11):1989-1995.

30 Fang SC, Zhang HT, Hu HD, Wang CY, Zhang YM. Effect of Endostar combined with angiopoietin-2 inhibitor on malignant pleural effusion in mice. *Med Oncol* 2015;32(1):410.

31 Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature* 2011;473(7347):298-307.

32 Rennel ES, Regula JT, Harper SJ, Thomas M, Klein C, Bates DO. A human neutralizing antibody specific to Ang-2 inhibits ocular angiogenesis. *Microcirculation* 2011;18(7):598-607.

33 Palmer GM, Tiran Z, Zhou Z, Capozzi ME, Park W, Coletta C, Pyriochou A, Kliger Y, Levy O, Borukhov I, Dewhirst MW, Rotman G, Penn JS, Papapetropoulos A. A novel angiopoietin-derived peptide displays anti-angiogenic activity and inhibits tumour-induced and retinal neovascularization. *Br J Pharmacol* 2012;165(6):1891-1903.