Research progress on the correlation between corneal neovascularization and lymphangiogenesis (Review)

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Abstract. The cornea is a clear connective tissue membrane at the front of the outer layer of the eyeball wall. It plays a crucial role in the refractive system of the eyeball, making it essential to maintain its transparency. Neovascularization and lymphangiogenesis in the cornea significantly impact corneal transparency and immune privilege. The growth of corneal neovascularization (CNV) and corneal lymphangiogenesis (CL) vessels is interconnected yet independent. Currently, there is a substantial amount of clinical and experimental research on CNV and CL vessels. However, due to the relatively recent focus on CL vessel research compared with CNV research, most scholars tend to concentrate on CNV, with few articles offering a comprehensive comparison and discussion of the two processes. The present review emphasizes the similarities and differences between CNV and CL and summarizes recent research progress on their correlation in animal models, growth characteristics, cytokine effects and related diseases.

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1. Introduction

The cornea, located at the front of the eye, is not only part of the fibrous membrane of the eyeball but also a crucial component of the refractive system and a physical barrier of the eye. As a key refractive structure, the transparency of the cornea is essential for maintaining normal vision. However, corneal neovascularization (CNV) and corneal lymphangiogenesis (CL) can lead to an increase in corneal turbidity and damage normal vision. In addition, because these processes are integral to the immune response in the cornea, where lymphatic vessels serve as afferent arms for transporting antigen-presenting cells (APCs) and blood vessels function as efferent arms, facilitating the transport of immune effector molecules and generating an immune response, they disrupt the inherent immune privilege of the non-vascular cornea, ultimately reducing the survival time of transplanted corneas (1). Nevertheless, certain studies have indicated that CNV and lymphatic vessels also contribute to the defense against corneal inflammation by enhancing immune responses (2). Therefore, CNV and CL have dual functions in the cornea.

CL shares some similarities with CNV in terms of immune responses. For example, in animal models, CL often occurs concurrently with CNV. The cytokines and signaling pathways that promote or inhibit the development of CNV and/or CL also largely act synergistically in the occurrence and progression of the disease. However, there are differences in numerous aspects between the two groups, such as growth characteristics and occurrence time. The influence of cytokines on the two processes is also different, mainly in terms of cytokine preferences and dosage sensitivity (3).

However, due to the lack of research on CL, few studies have systematically described the relationship between them. Therefore, the present article reviews the research progress on the relationships among the establishment of animal models, growth characteristics, the influence of the cytokine pathway and the role of disease.

2. The different roles that the two play in the cornea

Under certain physical and chemical conditions, such as alkaline burn irritation, corneal infectious diseases, immune privileges are destroyed, neovascularization and lymphangiogenesis appear, and the inflammatory response is

intensified. CNV and CL play different roles in this inflammatory response. As an input arm of the immune system, corneal lymphatics produce transport APCs that transport foreign and soluble antigen molecules to local lymph nodes, where they activate immune effector cells such as T cells. On the other hand, CNV acts as an output arm of the immune response, transporting activated immune effector cells to the site of inflammation, thereby intensifying the immune response (4) and acting as anti-inflammatory agents, which may be related to ATP binding cassette subfamily B member 5 (5). In addition to specific immune-related effects, CNV can also play non-specific immune-related effects through components such as neutrophils and macrophages (6), and can promote corneal transparency by removing excess hyaluronic acid (HA) (7). It has been hypothesized that it may also help eliminate edema. In addition, it has been suggested that moderate CNV may promote wound healing by generating an inflammatory response to fight infection and by forming granulation tissue to promote tissue repair (8). However, other studies have revealed that CNV is associated with corneal edema, lipid deposition, and further decrease in corneal clarity (9). In certain studies, CL is also suspected to be related to the regression of corneal edema and play a role in the fluid homeostasis of the cornea (10). The specific role of these two needs to be further explored.

3. Relationship between CNV growth and lymphangiogenesis

CNV and CL often accompany each other, but they are regulated by different signaling systems. At the same time, their growth patterns are different. Their processes of appearance, growth and regression are very similar but concurrently have numerous distinct characteristics. They play different roles in the cornea, thus the newly formed lumen structure also has different functions to perform the corresponding functions. It is important to understand the relationship between the two to understand the occurrence and development of the corresponding diseases. The present study of CNV primarily employs CD31 staining for identification, given that CD31 is highly expressed on vascular endothelial cells (ECs) (11). By contrast, CL is primarily identified through staining with lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), as LYVE-1 is an analogue of CD44 and a receptor for HA, which is abundantly expressed in the lymphatic system (12).

The difference and relationship between the two processes. While CNV and CL often occur concurrently, they are similar and different at the same time.

The growth patterns of CNV. The existing research on angiogenesis is relatively thorough. Shi *et al* (13) described CNV using five models (suture induction, alkali burn, fungal infection and immunogen or tumor cell implantation) with three key steps in its formation: Sprouting, progressive and declination phases. Although the initial corneal edema and vasodilatation in these five models are consistent with the findings of Cogan (14), Shi *et al* (13) provided a more) detailed description of the pathogenesis of CNV. First, the basement membrane (BM) of the limbal capillary network is degraded by proteases released by ECs. Second, ECs migrate and invade the extracellular matrix (ECM), followed by proliferation. Finally, the lumen of new vessels forms, and the BM reshapes. An increasing number of studies suggests that CNV and CL are initiated by the budding of existing limbal vessels and lymphatics (3,15). Additionally, accumulating evidence indicates that corneal edema is not a prerequisite for the development of CNV (16,17). The processes involved in CNV vary depending on the trigger, but most steps are consistent. Therefore, the process of CNV can be summarized as follows:

i) When corneal injury or inflammatory irritation occurs, corneal edema, along with venules and capillary congestion, corneal edema can also stimulate cells, such as corneal epithelial cells, corneal ECs, vascular ECs and immune cells, to release angiogenic cytokines, including VEGF and basic fibroblast growth factor (bFGF). The upregulation of proangiogenic factors and the downregulation of angiogenesis inhibitory factors, such as soluble fms-like tyrosine kinase 1 (sFlt-1), metalloprotease 3, proteinase inhibitors, angiostatin, endostatin, platelet response protein, interleukin-1 receptor antagonist, pigment epithelial derived factor, vasoactive intestinal peptide and α-melanocyte-stimulating hormone, can occur under normal physiological conditions.

ii) Then, the combination of angiogenic factors and receptors activates the ECs of corneal perilimbal blood vessels. Corneal limbal ECs secrete matrix metalloproteinases to breakdown the ECM and vascular BM. Simultaneously, the combination of VEGF-A and VEGFR2 triggers vascular migration of ECs through the RAS-RAF-MAPK-ERK signaling cascade, the PLCγ-ERK 1/2 pathway, Ca^{2+} signaling, the PI3K-AKT pathway, small G protein, the SRC pathway, stress kinases and STATs. This enables ECs to migrate towards the cornea through chemotaxis (12,18).

iii) Some of these vascular ECs germinate and form vascular buds on the original blood vessels. VEGF-induced ECs exhibit a promigratory phenotype and undergo chemotaxis toward inflammatory sites. At those sites they form a structure that looks like a dendritic cell with a number of pseudopod-like structures to invade the corneal stroma. These cells are called tip cells and stalk cells. Tip cells are motile and invasive and can extend numerous filamentous pseudopodia that can respond to growth factors, the ECM, and other attractive or repulsive signals.

iv) A portion of ECs will undergo a transformation into a tip cell phenotype and initiate sprouting. Subsequently, the ECs (stalk cells) proliferate and contribute to the maintenance of the structural and functional integrity of neovascularization. The tips of the aforementioned pseudopod-like structures formed by ECs continue to secrete MMP and promote further growth of the blood vessels. However, new blood vessels are vulnerable due to the absence of pericyte support (19).

v) As neovascularization progresses, platelets release platelet-derived growth factor, which binds to receptors on pericytes, ultimately leading to their proliferation and migration. The chemotactic movement of pericytes to new blood vessels combines with them to stabilize new blood vessels (20).

vi) Other stimuli that can induce CNV include inflammation, hypoxia, limbal stem cell deficiency and denervation. Different stimuli stimulate different cells and cytokines to generate CNV. Inflammation triggers the release of cytokines such as VEGF, bFGF and BMP from corneal epithelial cells, corneal ECs, vascular ECs and immune cells, which promote

CNV. Hypoxia leads to the expression of VEGF in the corneal epithelium, ECs and endothelium of the limbal blood vessels. Limbal stem cell deficiency recruits macrophages, a significant source of VEGF, and causes secondary increases in TGF-β, both of which promote CNV. Finally, the deprivation of nerves results in a reduction in corneal angiogenesis inhibitors (21). Although the initial processes of these conditions vary, most of their downstream processes overlap with those caused by inflammation-induced vasculo-genesis. The common process is demonstrated in Fig. 1.

The growth patterns of CL. The formation process of new corneal lymphatic vessels differs from that of CNV. The currently recognized mechanism is that they sprout from the original blood vessels and veins. The process is as follows:

i) The original venous vascular ECs express high levels of VEGF-3 and LYVE-1, and a subset of bone marrow-derived macrophages undergo trans-differentiation. Subsequently, both cell types undergo the process of converting into lymphatic ECs.

ii) The original venous vascular ECs and a subset of bone marrow-derived macrophages start to express the transcription factor SOX18, leading to the upregulation of prospero-related homeobox 1 (Prox-1). This represents a pivotal step in the conversion of these cells into lymphatic ECs (22). Vascular ECs and bone marrow-derived macrophages initially differentiate and sprout towards lymphatic ECs (22).

iii) These two proteins express neuropilin-2 (NP-2), and the synergistic effect of NP-2 and VEGFR-3 renders cells sensitive to VEGF-3, which promotes the extension of lymphatic endothelial tip cells and the continuation of sprouting (23).

iv) Afterwards, the lymphatic vessels bifurcate from the vein. Lymphatic ECs express podophyllotoxin, which promotes platelet aggregation, leading to the separation of lymphangiogenesis vessels from veins.

v) VEGFR-3 activates downstream pathways such as the Akt and p42/p44MAPK pathways, protecting lymphatic ECs from apoptosis and further promoting EC migration (24,25).

These are the classic steps of CL. These steps are also demonstrated in Fig. 2. However, it is important to note that not all lymphangiogenesis originates from the CNV. In the case of dry eye disease (DED), there is a unique condition in which corneal neo-lymphatic vessels are generated, but CNV is not. Multiple studies have revealed that this may be related to the specific cytokine mechanism of dry stress in DED (26), particularly the secretion of IL-17 by Th-17 cells, which induces an increase in VEGF-D (27), and the downstream pathways that lead to an increase in VEGF-3 induced by SP (28). In addition to budding from veins, some studies have suggested that lymphatic vessels may originate from mesenchymal lymphatic stem cells (29), but this theory has not been described for CL and has not been confirmed in mammals. Therefore, it will not be discussed in the present review.

The process of generating CNV and CL are described in detail. There are several similarities between CNV and CL in terms of stimulating factors and activating cells, but they also have their own unique features. The details are presented in Table I.

Tubular morphology. CNV is a tubular structure surrounded by ECs, with a wall composed of a single layer of ECs surrounded by smooth muscle cells and peripheral cells and an incomplete BM on the outermost layer. CNV arises from the sprouting of veins behind existing capillaries, and the process of its formation can be explained by the tips and stalk cells. In addition, CL involves a tubular structure surrounded by a single layer of lymphatic ECs. The ECs of the lymphatic vessels are surrounded by an incomplete BM, while the lumen is protected from reflux by lymphatic flaps. The formation of CNV occurs by sprouting from existing veins (30-32) Their morphologies are shown in Fig. 3.

Both structures share several similarities in terms of luminal morphology. They are both surrounded by a single layer of ECs, have discontinuous BMs, and sprout from pre-existing veins. However, there are also numerous differences:

i) CL involves the presence of a lymphatic valve that assists the entry and exit of APCs, but it does not involve the presence of blood vessels;

ii) The wall involved in CNV contains pericytes and smooth muscle cells in addition to ECs. On the other hand, the wall of the corneal lymphatic vessel consists of a single layer of cells with a large lumen;

iii) CNV clearly involves red blood cells, while CL does not (30,31). However, in addition to the difference between CNV and the formation of new lymphatic vessels, there are also differences in the signaling pathways that induce new blood vessels and lymphatic vessels; (iv) Studies on CNV indicate that lymphatic vessels affected by VEGF-A/VEGFR-2 are structurally and functionally different from those affected by VEGFR-3 ligands. These vessels exhibit a relatively dilated, leaky, and poorly functional phenotype (26). In terms of new corneal lymphatic vessels, Cao *et al* (33) reported that the lymphatic vessels affected by bFGF and VEGF-C were larger than those affected by bFGF alone, which may be related to the synergistic effects of different cytokines.

Temporal regularity of angiogenesis. As early as 2002, Cursiefen *et al* (34,35) reported that although CL occurs simultaneously with CNV, CL degrades earlier than CNV. This result was obtained again in 2006. Although CNV and lymphangiogenesis generally occur together, subtle differences exist in the temporal regularity of angiogenesis in different models.

CNV coexists with corneal lymphatic vessels. In the case of bacterial keratitis, CNV resulted in significant proliferation on the seventh day of modelling, but CL increased more slowly on the fourteenth day than did CNV (2). In the case of alkaline burns, although CL vessels grow parallel to CNV vessels, CL vessels sprout approximately at the third day after neovascularization occurs, but at the same time point of detection, the density and length of CL vessels are smaller than those of neovascularization vessels, and there is still a certain lag compared with CNV vessels (36). In the corneal suture model, the situation of CL vessels and CNV is similar to that of alkali burns. However, the generation of CL vessels is greater than that of alkali burns, and even on the third day, the area of CL vessel proliferation is greater than that of CNV (36,37). Although CNV and CL have distinct characteristics in these three types of patients, they are still concomitant overall.

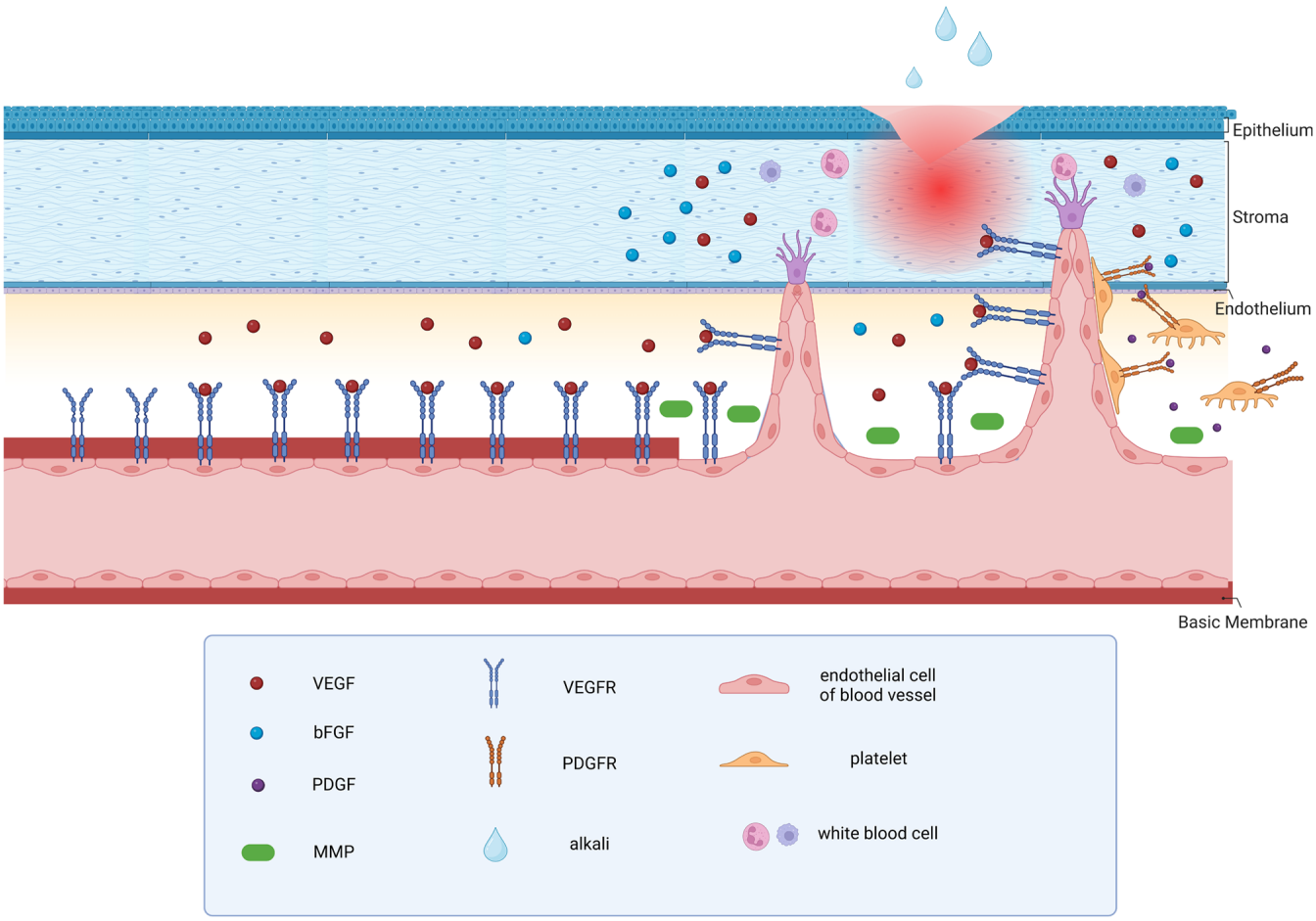

Figure 1. A schematic diagram of the corneal neovascularization process. bFGF, basic fibroblast growth factor; PDGF, platelet derived growth factor subunit B.

Only corneal lymphatics are present. In DED, only CL occurs, without CNV. This suggests that corneal neogenesis and lymphatic vessels grow in parallel or that the preference for blood vessels over lymphatic vessels is not universally applicable. In DED, this phenomenon has been proven to be related to specific cytokine pathways; for example, TH-17 cells secrete IL-17 directly, leading to an increase in VEGF-D, which promotes lymphatic vessel growth (27). SP promotes an increase in downstream VEGF-3 secretion in signaling pathways (28). There is currently no consensus on why CNV does not occur in DED. However, according to the experiments conducted by Chang *et al* (3), when the dose of bFGF was reduced to 12.5 ng, lymphatic vessel proliferation was significant, accompanied by only a small amount of neovascularization. The process of CNV is highly sensitive to dose, therefore it can be inferred that this phenomenon may be related to the concentration of cytokines that stimulate corneal growth. This is despite the fact that there are now cases where only CL are present. However, by reviewing the relevant articles, no case was found where only CNV occurs without CL. This idea is very novel and provides a direction for the future research.

4. Methods of animal modelling

In most cases, due to the coexistence of CNV and CL vessels, the modelling of CNV and CL vessels is universal. Currently, the most commonly used animal models are alkali burn and corneal suture models. In addition, there are specific models developed for different situations, such as the infectious keratitis model, which explores the generation of corneal blood vessels and lymphatic vessels during corneal infection; the low- and high-risk corneal transplantation model, which explores the impact of neovascularization and lymphatic vessels on the success rate of corneal transplantation; and the corneal stromal implantation bFGF model, which explores the effect of bFGF on CNV and lymphatic vessel generation.

Alkali burn model. The alkali burn model is a frequently employed model for studying chemical eye trauma in experiments. As early as 2010, Shi *et al* (36) confirmed the presence of blood vessels and lymphatic vessels in a corneal alkali burn model. The modelling method calculates the dosage according to the different animals, and then an appropriate dose of sodium hydroxide solution is used to directly drip into the eyes or soak the sodium hydroxide solution of this concentration on the upper filter paper and stick it on the animal cornea. After a period of time, the alkaline solution is discharged, or the filter paper is removed (35). Although this model is primarily used to investigate the treatment of corneal alkali burns, it is also utilized to examine the impact of various substances on blood vessels and lymphangiogenesis following an alkali burn. For example, Song *et al* (38) utilized this model to study the

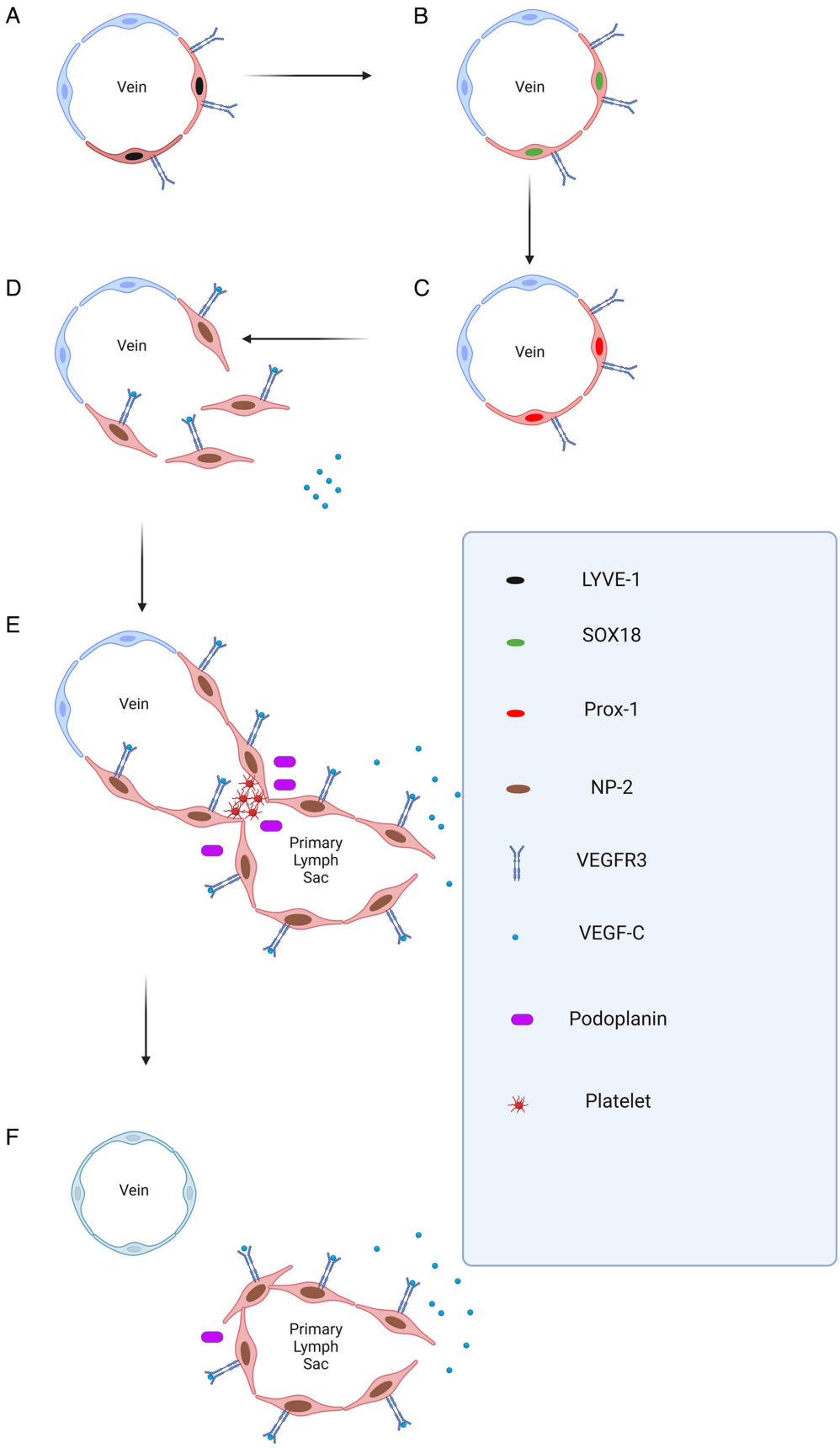

Figure 2. A schematic diagram of the corneal lymphangiogenesis process. (A) The original venous vascular ECs express high levels of LYVE-1 (30), and they begin to undergo conversion into lymphatic ECs. (B) The original venous vascular ECs started to express the transcription factor SOX18. (C) The expression of SOX18 leads to the upregulation of Prox-1. This represented a pivotal step in the conversion of these cells into lymphatic ECs. (D) These cells express NP-2, and the synergistic effect of NP-2 and VEGFR-3 renders cells sensitive to VEGF-3, which promotes the extension of lymphatic endothelial tip cells and the continuation of sprouting. (E) Lymphatic vessels bifurcate from the vein. Lymphatic ECs express podophyllotoxin, which promotes platelet aggregation, leading to the separation of lymphangiogenesis from the veins where the primary lymph sac is formed. (F) Newly formed lymph vessels. ECs, endothelial cells; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; NP-2, neuropilin-2; Prox-1, prospero homeobox 1.

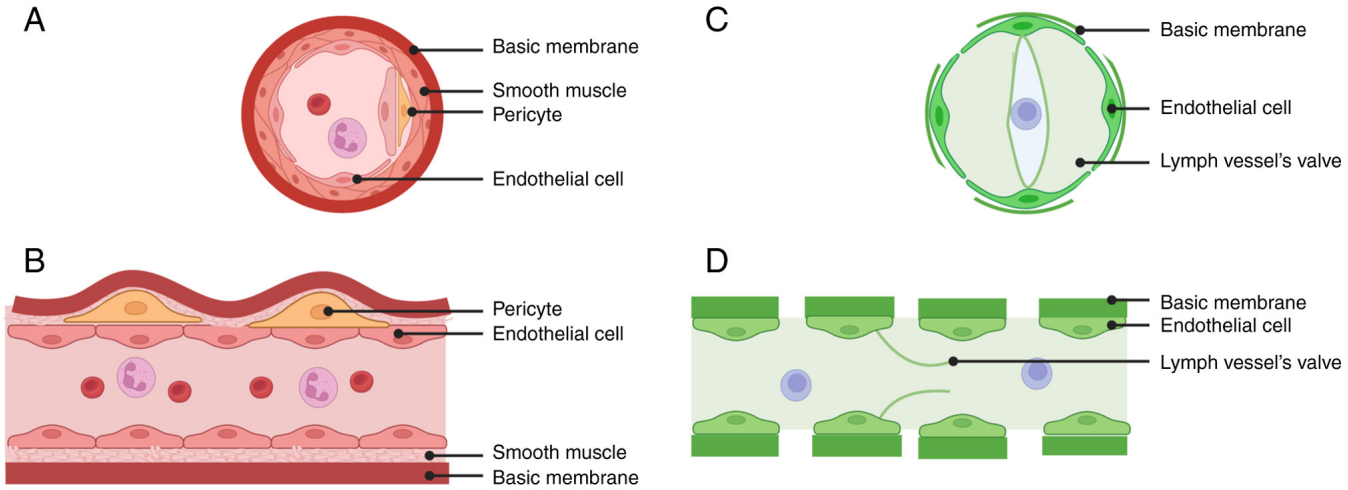

Figure 3. Morphology of CNV and CL. (A) Coronal plane of CNV. (B) Sagittal plane of CNV. (C) Coronal plane of CL. (D) Sagittal plane of CL. CNV, corneal neovascularization; CL, corneal lymphangiogenesis.

impact of leucine rich α-2-glycoprotein 1 (LRG-1) on CNV and lymphangiogenesis in the cornea (38). Compared with other methods, the model offers several advantages. First, the modelling method is simple and has minimal requirements for reagents and resources. Second, it is reproducible and can significantly reduce interference from factors other than variables. Third, it provides high controllability. Finally, the corneal alkali burn model can effectively simulate the condition of the cornea during alkali burns, which is very useful for studying the regulatory mechanism of corneal angiogenesis, the process of corneal inflammation and fibrosis, and the effects of related drugs. This method also has several limitations, such as its consistency being affected by equipment and drug specifications, the absorption of indoor air CO_2 by NaOH solution impacting the results, and other factors. Overall, this method has more advantages than disadvantages and remains a common technique for mold preparation (39).

Corneal suture model. Corneal suturing is another widely used method to study the relationship between the two parameters. It was first used to induce CNV and was later also used for studying CNV and the formation of new lymphatic vessels in the cornea (40). Its main molding method involves placing three stitches in the corneal stromal layer using a 10/0 nylon suture under a surgical microscope. The needle is then inserted into the center of the pupil from a distance slightly <2.0 mm from the corneal limbus (specific methods may vary for different animals). It is important to note that the specific method used may vary between animals. At present, this modelling method is mainly applied to study the roles of different cytokines in CNV and CL vessels. For example, Maier *et al* (41) used a corneal suture model to study the effect of TNF-α receptor deficiency on mouse CNV and lymphangiogenesis. This method is simple to use and prevents corneal damage caused by chemical reagents. The incidence of postoperative infection is low, but it can be influenced by the skill level of the operator and the size of the suture line and may induce protective eye rubbing in experimental animals, leading to model failure. Further research is needed to determine how to avoid these shortcomings.

Infectious keratitis model. Infection by various pathogens can lead to corneal inflammation, which in turn triggers the formation of new lymphatic vessels and blood vessels. At present, the most common model of bacterial keratitis is *Pseudomonas aeruginosa*-induced keratitis (42), while the most common model of viral keratitis is caused by the herpes simplex virus type 1 (HSV-1) (43). Currently, these two models are primarily used to investigate the impact of corneal blood vessels and corneal lymphatic vessels on the prognosis of patients with infectious keratitis. For example, Narimatsu *et al* (2) used *Pseudomonas aeruginosa*-induced infectious keratitis to study the impact of CL vessels on the prognosis of infectious keratitis. Moreover, these two models also have applications in studying the role of macrophages in neovascular and lymphatic vessels, as well as the factors that influence the timing of their formation (44). This method can effectively simulate the conditions of infectious keratitis. Because keratitis caused by different pathogens may require different treatment methods, the current approach is not reproducible and cannot establish a standard process. Therefore, further improvement is still needed.

The cytokine particle implantation model. In this novel modelling method, cytokine particles related to CNV and the development of CL vessels are implanted at the corneal edge to study the corresponding roles of these cytokines. The specific methods used are as follows: The sucralfate and cytokines are placed in a centrifuge tube and completely dissolved. A certain amount of hydron dissolved in anhydrous ethanol is added, and the mixture is dried on a nylon mesh to prepare particles containing cytokines. Cytokine particles are implanted into the corneal edge within 1 mm of the corneal stroma. At present, bFGF particles have been successfully implanted into the corneal margin in experimental research, and it has been verified that they can promote the production of blood vessels and lymphatic vessels (45).

The keratoplasty model. With the widespread use of keratoplasty, there is an increasing interest in studying the role of neovascularization and lymphatic vessels in this procedure.

Table I. Characteristics of CNV and CL.

Points of comparison	CNV	CL	(Refs.)
Stimulating factor	Infection, chemical burns, corneal sutures, immune response	Infection, chemical burns, corneal sutures, immune response	(2,36, 40,46)
Stimulating cytokines	Ang-1,2	VEGF-A, C, D, bFGF, Ang-1	(33,83, 84,112)
Activating cells	Vascular endothelial cells	Primary venous vascular endothelial cells, bone marrow-derived macrophages	(18,22)
Activation pathway	VEGF-A/VEGFR-1,2 VEGF-C/VEGFR-2	VEGF-C, D/VEGFR-3	(33)
Migratory route	Hydrolyses the basement membrane, invading the ECM and forming a lumen	The original venous endothelial cells and bone marrow-derived macrophages are transformed into lymphatic endothelial cells, and the blood vessels emit the anterior lymphatic tip. After the lumen sprouts and bifurcates, the new lymphatic vessels are separated from the veins to form lymphatic valves	(13,18-25)
Cells produced by the process	Vascular endothelial cell	Lymphatic endothelial cells	(30,31)
Key steps in development	Sprouting, progressive and declination phases	Germination, branching, proliferation, differentiation and remodeling processes	(13,18-25)
Luminal markers	Endoglin (membrane glycoprotein), neuropilin-1 (transmembrane receptor), collagen IV (ECM molecule)	Prox1 (transcription factor), podoplanin (transmembrane glycoprotein), LYVE1 (transmembrane receptor), VEGFR3 (receptor tyrosine kinase), CCL21 (expressed in secondary lymphoid organs)	(112)

CNV, corneal neovascularization; CL, corneal lymphangiogenesis; Ang, angiotensin; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; ECM, extracellular matrix; PROX1, Prospero-related homeobox-1; LYVE1, lymphatic endothelial hyaluronan receptor-1; CCL21, CC-chemokine ligand-21.

The use of animals to simulate human corneal transplantation is also one of the currently available methods for inducing CNV and lymphatic vessel formation. Its main modelling method is to simulate the situation of corneal transplantation. For example, the specific process of simulating penetrating keratoplasty involves replacing and removing the corneas of both the donor and recipient mice, suturing the corneas of the donor mice to the eyes of the recipient mice, using nylon thread sutures, injecting sterile air and performing antibacterial treatment, and finally removing the thread. Similar to other types of surgical methods, the process of human corneal transplantation can be reproduced in animals. The application of this method has focused primarily on studying the relationship between the two in the context of corneal transplantation (46).

Other. In addition to the aforementioned model, there are other models used to induce CNV and CL, such as the incision model and the limbal injury model. The incision model simulates corneal trauma by using a surgical blade after central cyclin labelling of the mouse cornea (47). The limbal injury model involves debridement after corneal injury to disrupt the integrity of the limbal epithelium to study its effect on corneal repair (48), although this approach is less commonly used.

5. Effects of different cytokine signaling pathways on CNV and lymphangiogenesis

Vascular endothelial growth factor (VEGF) family

Direct role of VEGF in CNV and lymphangiogenesis. VEGF and its associated receptor VEGFR are important cytokines that maintain the growth of corneal neovascular ECs and CL cells. The main members of the VEGF family include VEGF-A, VEGF-B, VEGF-C and VEGF-D, all of which are associated with the generation of CNV or CL vessels. They act by stimulating VEGFR1-3, in which VEGF-C plays a role in promoting the growth of corneal lymphatic vessels in newborns primarily by stimulating VEGFR-3, but it can also occasionally stimulate VEGFR-2. Moreover, VEGF-C can stimulate CNV by stimulating VEGFR-2. VEGF-D is generally similar to VEGF-C. The main function of VEGF-A is to stimulate neovascularization, and it can specifically stimulate VEGFR-1 and VEGFR-2, promoting corneal vascular growth (1).

In addition, VEGF-A activation of VEGFR-2 also plays a role in promoting lymphangiogenesis. VEGF-A stimulation of VEGFR-2 leads to the formation of lymphatic vessels that differ in morphology from those formed by VEGF-C. Compared with those induced by the VEGFR-3 ligand,

VEGF-A/VEGFR-2-induced vessels exhibit a dilated, leaky and poorly functional phenotype (49). However, in keratitis caused by HSV-1 infection, VEGF-A, which is driven by the immediate early gene product of HSV-1-infected cell polypeptide 4, also activates corneal lymphatic vessel growth promoted by VEGFR-2, which is the primary pathway for corneal lymphatic vessel growth (50,51).

Since VEGF itself is closely related to the formation of CNV and CL, drugs targeting VEGF and its receptors have been widely used in clinical ophthalmic diseases to reduce the formation of CNV and CL. Faricimab, as a novel specific antibody against VEGF-A, has been widely used in the treatment of macular degeneration, but there is no study on its clinical application in CNV (52). In addition to Faricimab, anti-VEGF-A drugs currently on the market also include aflibercept, Ranibizumab and Brolucizumab, which are mainly applied to retinal and choroidal diseases such as retinal vascular obstruction and macular degeneration (53-56). Except these drugs, there are numerous anti-VEGF drugs on the market, such as Ivonescimab for small cell lung cancer (57). However, these anti-VEGF drugs have not been used in the clinical trials' field of inhibiting CNV and CL.

Cytokines mediated by VEGF in CNV and CL. The effects of numerous inflammatory cells on CNV and CL can be attributed to the secretion of VEGF. For example, Cho *et al* (58) reported that mast cells can secrete VEGF-A to promote angiogenesis and can specifically secrete high levels of VEGF-D to promote lymphangiogenesis. The effects of other cytokines on CL and neovascularization also depend on this pathway. TNF-α can drive CL and CNV (59), primarily by activating the NF-κB signaling pathway, which stimulates macrophages to produce VEGF-C (60) or corneal dendritic cells and macrophages to express VEGF-C/VEGFR-3 (61,62). Interleukins such as IL-1β and IL-10 promote CNV by attracting neutrophils and macrophages and enhancing VEGF-A and VEGF-C expression in macrophages (63-65). IL-1β mainly attracts macrophages through IL-1β secretion and the IL-1RI receptor (66). The chemokine receptor CXCR-3 on macrophages recruits marrow-derived macrophages to sites of inflammation, which is closely associated with CNV. Its absence can increase VEGF levels and drive corneal angiogenesis (67). In 2019, Narimatsu *et al* (2) demonstrated that VEGF also plays an important role in bacterial keratitis caused by *Pseudomonas aeruginosa*. Specifically, bacterial lipopolysaccharide (LPS) stimulates F4/80-positive macrophages to promote VEGF-C release through the Toll-like receptor (TLR) 4 pathway, promoting the growth of new corneal lymphatic vessels and neovascularization (2). Song *et al* (38) reported that LRG-1, an emerging factor that promotes CNV and CL, is also related to VEGF. The authors hypothesized that LRG-1 induces VEGF secretion through TGF-β/Smad signaling or HIF-1α activation. However, the underlying mechanism still needs to be investigated (38,68,69).

Signaling pathways that function through VEGF. At present, Notch pathway is the signaling pathway that plays a role through VEGF. Current studies on Notch pathway have found that the Notch pathway and VEGF system interact; VEGF can stimulate the Notch pathway, and the Notch pathway stimulates VEGF-A expression through a feedback loop. In addition, Hu *et al* (70) reported that the bone morphogenetic protein 4 (BMP4/Smad pathway can enhance the Notch pathway through hairy and enhancer of split 1 (HES1) expression, and that overexpression of HES1 can inhibit CNV. Previously, BMP4 has been revealed to affect CNV by binding to BMPR-I/II, causing c-Src phosphorylation and activation of VEGFR-2 (71).

In addition to the Notch pathway, numerous of the signaling pathways that promote CNV and CL can ultimately be attributed to the influence of the VEGF family, therefore numerous therapeutic approaches also focus on blocking or inhibiting the signaling pathways acting through VEGF to reduce the production of CNV and CL. Shokirova *et al* (72) found that local proliferation of corneal vascular ECs during CNV could lead to upregulation of KOR expression. They also found that nalfurafine-activated KOR can inhibit VEGF production by inhibiting the g-αi/o pathway of adenylate cyclase. Liu *et al* (73) inhibited the IL-1/IL-1RI/ERK signaling pathway through AS-1 to reduce the production of pro-inflammatory cytokines, recruitment of neutrophils and macrophages, and thus reduce pro-angiogenic factors. The pathway of TLR2/NF-κB inducing macrophage production of VEGF-C and VEGF-D has also been confirmed by Song *et al* (74). This pathway can be inhibited by the secretion of TNF α-induced protein 6 (TSG-6) by mesenchymal stem cells (MSCs). In addition, it was found that TSG6 may indirectly reduce macrophage recruitment by regulating TLR/NF-κB signaling in laparoscopic endoscopic cooperative surgery. In addition to these studies, Bai *et al* (75) found that Wilms' tumor 1-associated protein could regulate N6-methyladenosine modification to influence C-C motif chemokine ligand 2, consequently promoting recruitment of macrophages to secrete VEGF to promote CNV and CL.

The use of inhibitory molecules acting on VEGF and its receptors to reduce CNV and CL is also a research direction. Zhang *et al* (76,77) and Le *et al* (78) focused on studying the VEGF receptor TrapR1R2, which can bind to VEGF to inactivate it. They found that the use of eye drops effectively inhibited CNV and CL and improved survival after transplantation. Salabarria *et al* (79) inhibited the effect of VEGF-C/D through VEGFC/DTrap and inhibited the growth of corneal lymphangiogenic vessels, but the results did not show an improvement in graft survival. In addition, synthetic L-783277 derivatives have also been reported to inhibit VEGFR-2 and VEGFR-3 (80). Cho *et al* (81,82) reported that both the β-blocker timolol and the insecticide resistant albendazole inhibited CNV and CL by blocking the VEGF-VEGFR pathway.

bFGF. The mechanism by which bFGF affects CNV and CL is complex. In general, the pathways that promote CL depend not only on VEGF-C and VEGF-D acting on VEGFR-3 (83,84) but also on dose-specific regulation of CNV and CL (85). When the dose of bFGF was reduced to 12.5 ng, lymphatic vessel hyperplasia became evident, and only a small amount of neovascularization was observed (3). According to the findings of Xie *et al* (45), the Notch/Dll4 pathway can activate the bFGF system, while the Notch pathway and the VEGF system simultaneously interact. VEGF can stimulate the Notch pathway, which can stimulate the expression of VEGF-A through a feedback loop (86,87). This finding demonstrated the intricate relationship between the VEGF family and bFGF. However, in

some cases, such as HSV-1 infection, bFGF can act as a master regulator of other proangiogenic and lymphangiogenic factors. This has profound effects on HSV-1-induced CNV and is associated with improved visual outcomes (44). Because bFGF is beneficial to the recovery of corneal injury, it has the potential to be applied to promote the recovery of corneal surface injury. However, bFGF-related drugs have not been approved by FDA (88). In China, although bFGF is also prohibited for injection, topical gel has been approved for clinical trials of injury healing, especially stomatology-related injuries (89). At present, there are no reported drugs that inhibit CNV and CL by inhibiting bFGF. In general, the current clinical application of bFGF is focused on promoting corneal injury recovery.

Neuropirim. Neuropilin-1 and neuropilin-2 are the coreceptors of VEGFR-2 and VEGFR-3, respectively. Currently, researchers are exploring their role in inhibiting CNV and CL. The class 3 semaphorin family is a soluble ligand of neuropilin, which can inhibit its ability to promote CNV and CL. Sema3F is a new member of this family and has the same function (32,67).

Calcitonin gene-related peptide (CGRP). CGRP is a 37-amino-acid neuropeptide that is extensively expressed in both the central and peripheral nervous systems. The trigeminal ganglion in the cornea has also been revealed to secrete CGRP, mainly by forming heterodimers with the calcitonin receptor and receptor activity-modifying proteins to send signals (90). Zhu *et al* (91) reported that although CGRP reduced the production of the inflammatory cytokines TNF-α and IL-1β in the cornea after $CD45^+$ leukocyte infiltration and mechanical injury *in vivo*, CGRP promoted VEC proliferation, migration and blood vessel formation *in vitro*. The effect of CGRP on lymphangiogenesis is similar to that on angiogenesis; CGRP increases the formation and branching of lymphatic vessels in the cornea. According to Majima *et al* (92) VEGF is the downstream signaling molecule of CGRP. Zhu *et al* (91) demonstrated that changes in VEGF-A expression correlate with changes in CGRP signaling *in vivo*. However, further research is needed to determine the exact relationship between these changes and their mechanism of action.

Chemokines. Chemokines play a crucial role in CL and CNV, and there is currently considerable research on them. For example, Li *et al* (93) recently found that the cell surface chemokine receptor CXCR-3 can attract marrow-derived macrophages to sites of inflammation, and its reduction leads to increased VEGF, promoting corneal angiogenesis. There is also a class of atypical chemokine receptors known as decoy receptors for chemokines. These receptors have the ability to neutralize chemokines and inhibit their effects (94). Chevigné *et al* (95)reported that ACKR2 can bind CC chemokines and CXCL10 to inhibit CNV and CL in an HSK mouse model. The mechanism may be related to its ability to indirectly limit corneal angiogenesis by clearing macrophages. Muramatsu *et al* (96) reported that SDF-1/CXCL12 and its receptor system are associated with corneal opacity and promote CNV and the formation of new lymphatic vessels, while DSCR-1 can inhibit this process.

Other cytokines. At present, studies on the cytokine pathway involved in CNV and CL have focused mainly on VEGF and bFGF. Most studies on other factors, such as BMP and TGF, primarily attribute their effects to VEGF. The remaining factors, such as integrins, HA and IL-17, are not currently available, therefore they will not be discussed again.

6. Diseases related to both

Corneal transplantation. There are typically no blood vessels present in the normal cornea. Due to its avascular nature, the cornea has immune privilege and a lower rejection rate. However, when the cornea is damaged, CNV and CL vessels are produced, forming the immune arms of the cornea. The CL vessels act as the afferent arms, transporting antigen substances and sensitizing lymph nodes, while CNV acts as the efferent arms, promoting activated immune cells to infiltrate the inflammatory site, which undermines the immune privilege of the cornea and reduces the success rate of corneal transplantation (1). According to the study by Zhang *et al* (77), CNV and CL are more prevalent in cases of corneal transplantation than in other models. This finding also highlights the significance of CNV and CL in the rejection of corneal transplantation.

At present, numerous researchers are also focusing on various methods to inhibit the generation and development of CNV and lymphangiogenesis vessels to improve the success rate of corneal transplantation. Reuer *et al* (67) utilized the combination of Sema3F and neuropirin to inhibit the activation of VEGFR and the production of CNV and lymphatic vessels, and especially after a period of treatment cessation, the graft survival rate was also very high, indicating that this treatment method has certain clinical application value. Ren *et al* (97) reported that myeloid-derived suppressor cells increase the expression of the antiangiogenic factor Tsp-1 and decrease the expression of the proangiogenic factors VEGF-A and VEGF-C through the iNOS pathway, inhibiting the generation of CNV and CL vessels. Ren *et al* (98) found that promoting the effect of myeloid suppressor cells through rapamycin can improve the success rate of corneal transplantation, while Wei *et al* (99) proposed that Cannabidiol can also enhance the effect of myeloid suppressor cells. In addition to myeloid suppressant cells, Kang *et al* (100) found that human umbilical cord MCSs and human adipose MSCs could also inhibit the formation of neovascular lymphatic vessels and corneal neovascularity to improve the survival rate of corneal transplantation, which was stronger than human adipose MSCs. Concurrently, Yu *et al* (101) found that insulin-like growth factor-1 modified mRNA can enhance the role of human adipose MSCs and promote corneal injury repair. Intravenous injection of cytokine-treated bone marrow cells can improve the survival rate of corneal transplant grafts (97). Zhu *et al* (102) also reported that isolated induced myeloid-derived suppressor cells could inhibit CNV through the iNOS pathway, improving the success rate of corneal transplantation. Shokirova *et al* (72) found that inhibiting adenylyl cyclase through Gαi/o using the κ opioid receptor agonist nanofuranin could suppress the production of VEGF and produce analgesic effects similar to those of opioid receptor agonists, but it does not have side effects caused by MOR activation, making it an ideal treatment for

inflammation after corneal transplantation. Zhu *et al* (103) proposed that membrane collagen could be crosslinked with riboflavin and UVA to combat corneal inflammation. Subsequently, Hou *et al* (104) further confirmed that this therapy can promote the regression of mature CNV and CL vessels after transplantation and can be used to improve the survival rate of corneal grafts. Zhang *et al* (76) proposed that local application of VEGFTrapR1R2 can further inhibit CNV and CL vessels by specifically inhibiting VEGF, thereby improving the survival rate of corneal transplantation (98). Unlike Zhang *et al* (76), the soluble form of VEGF-C/D used by Salabarria *et al* (79) is VEGFR-3. It can inhibit the formation of new lymphatic vessels and angiogenesis in the cornea after transplantation, but it cannot promote corneal graft survival. In addition to acting on VEGF itself and its upstream pathway, blocking its downstream pathway is also feasible at present. Dietrich-Ntoukas *et al* (105) found that CNV can be inhibited by blocking Integrin-α5β1 downstream of VEGF.

Infectious keratitis. Infectious keratitis is caused mainly by infections caused by bacteria, viruses and fungi. *Pseudomonas aeruginosa* is a common pathogen of bacterial keratitis and can cause keratitis in individuals who wear contact lenses for a long time. LPS from *Pseudomonas aeruginosa* can activate VEGF-C through the TLR4 pathway and subsequently induce lymphangiogenesis. The main pathway by which *Pseudomonas aeruginosa* promotes CL is also VEGF-C/VEGFR-3. However, research has shown that in bacterial keratitis, lymphatic vessels not only play a proinflammatory role but also, to some extent, reduce macrophage infiltration and alleviate corneal edema (2,47,63).

Herpetic keratitis is a type of infectious keratitis caused by HSV-1 (106). It is the primary cause of infectious corneal blindness in developed countries. Unlike bacterial keratitis caused by *Pseudomonas aeruginosa*, the lymphangiogenesis effect of HSV-1 does not rely on VEGF-3, VEGFR-3, or macrophage infiltration but mainly relies on VEGF-A and VEGFR-2. VEGF-A can also promote CNV. Studies have revealed that lymphangiogenesis promotes the progression of herpetic keratitis, resulting in visual damage by directing destructive inflammatory mediators to infected areas. Compared with the visual impairment caused by HSV-1-induced CNV, CL is more harmful (44,107). In addition to affecting VEGF, HSV-1-induced keratitis is also closely related to chemokines, especially CXCL-10, which is also related to CC chemokines but not as closely related to CXCL-10. Both factors affect the infiltration of leukocytes, which in turn affects the generation of CNV and CL vessels (108).

At present, in the treatment of infectious keratitis, researchers are exploring new treatments in addition to the traditional approach of using antibiotics for anti-inflammatory purposes and using glucocorticoids to inhibit CNV and CL. Zhu *et al* (103) proposed that corneal collagen can be cross-linked with riboflavin and UVA to treat infectious keratitis, which has the advantages of direct sterilization, reducing inflammatory cell infiltration, and inhibiting CNV and CL vessel formation. Shokirova *et al* (72) reported that κ opioid receptor agonists can also be used to combat infectious keratitis. This is due to their ability to inhibit transplanted CNV, promote lymphangiogenesis, and provide analgesic effects. For herpetic keratitis, the most commonly used method is to inhibit the generation of VEGF-A. Gurung *et al* (44) reported that regulating FGF-2 can indirectly inhibit the generation of VEGF-A, and this method can partially preserve the vision of HSV-1-infected mice, which has certain clinical value. However, inhibiting FGF-2 can also lead to nerve degeneration and has potential side effects. However, further research is needed to determine its effectiveness for clinical application. The CC chemokine ligand ACKR-2 is considered to be an important treatment for HSV-1 keratitis. It specifically binds to CC chemokines, reducing the number of free CC chemokines and promoting the resolution of inflammation. Recent findings have shown that it can also bind to CXCL-10, a factor involved in the pathogenesis of HSV-1 keratitis, further demonstrating that ACKR-2 can effectively inhibit HSV-1-induced keratitis (95,108).

DED. The order of formation of CNV and CL is different, but they often occur together in corneal diseases. However, in DED, only lymphangiogenesis occurs. Currently, the mechanism underlying the selective generation of corneal lymphatic vessels in DED is not fully understood. The results of the experiments conducted by Chang *et al* (3) may be related to differences in the sensitivity of corneal lymphatic vessels and CNV to cytokine dosage. Current treatment methods also mostly exert their effects through the side effects of anti-CNV and anti-CL (28).

Corneal alkali burn. Corneal alkali burn injury is a commonly used technique for establishing mouse CNV and CL models. It is detected in some laboratories and chemical plants and can cause accidental damage. Due to the lipophilicity of alkali, it often leads to more severe keratitis than acid burns (109). Li *et al* (93) reported that knockout of the CXCR-3 gene in an alkali burn model exacerbates CNV and CL in mice. This may be related to the gene's regulation of VEGF function. Song *et al* (38) reported that LRG1 also regulates the expression of VEGF and VEGFR, as well as CNV and CL, in alkaline burn models. Additionally, Oh *et al* (110) targeted DDR1 through microRNA-199a/b-5p and inhibited DDR1 expression, ultimately reducing lymphangiogenesis and neovascularization in alkaline burn models. However, the mechanism by which DDR1 promotes CNV and the formation of new corneal lymphatic vessels remains unclear. At present, for the treatment of CNV and CL after alkali burn, Li *et al* (111) proposed that shark cartilage-derived anti-angiogenic peptide could be used, because it could inhibit VEGF, MMP1 and other factors.

Others. In addition to the aforementioned diseases, CNV and CL also play significant roles in the development of other conditions, such as allergic keratitis and corneal edema, which are associated with angiogenesis and lymphangiogenesis. However, recent advancements in research on this topic have been limited, and the present topic will not be further discussed.

7. Summary

In recent years, with the progress of research on the mechanisms of corneal diseases, our understanding of the mechanisms of

CNV and CL, regulatory factors, and their relationship with diseases has become increasingly comprehensive. Relevant animal models can be created and treatment plans can be proposed based on these mechanisms. Currently, VEGF factor blockers are widely used in clinical practice to improve the success rate of corneal transplantation, and other therapies are in different stages of development.

However, due to the complex mechanisms related to CNV and CL, which involve numerous cells and cytokines, further research is required. Further understanding of the mechanisms by which various cytokines influence each other and the relationship between CNV and CL are needed to improve the treatment of related diseases.

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Authors' contributions

ZZ conceived and wrote the main manuscript. RZ prepared figures and helped write the manuscript. YH helped write the manuscript and provided valuable advice. XW and MY helped to write the manuscript. Data authentication is not applicable. All authors read and approved the final version of the manuscript.

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Competing interests

The authors declare that they have no competing interests.

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