Three kinds of corneal host cells contribute differently to corneal neovascularization

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Background: Corneal neovascularization (angiogenesis and lymphangiogenesis) compromises corneal transparency and transplant survival, however, the molecular mechanisms of corneal host epithelial and stromal cells in neovascularization have not yet been fully elucidated. Furthermore, the contribution and mechanism of corneal host endothelial cells involved in neovascularization are largely unexplored.

Methods: Liquid chromatography-mass spectrometry, immunoblotting, and ELISA were used to screen and identify potential neovascularization-related factors in human full-thickness vascularized corneal tissues. Lipopolysaccharide was used to induce inflammation in three kinds of corneal host cells in vitro, including corneal epithelial, stromal, and endothelial cells. Fungus was used to establish an animal model of corneal neovascularization in vivo. Tube formation and spheroid sprouting assays were used to evaluate the contribution of three kinds of corneal host cells to the degree of neovascularization under various stimuli. Matrix metalloproteinase (MMP)-2, alpha-crystallin A chain (CRYAA), galectin-8, Bcl-2, neuropilin-2, MMP-9 plasmids, and recombinant human fibronectin were used to identify the key proteins of corneal host cells involved in corneal inflammatory neovascularization.

Findings: All three kinds of corneal host cells influenced corneal neovascularization to varying degrees. MMP-9 in human corneal epithelial cells, MMP-2, and CRYAA in human corneal stromal cells, and MMP-2 and galectin-8 in human corneal endothelial cells are potential key proteins that participate in corneal inflammatory neovascularization.

Interpretation: Our data indicated that both the effects of key proteins and corneal host cells involved should be considered for the treatment of corneal inflammatory neovascularization.

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1. Introduction

The cornea roughly consists of the epithelium, stroma, and endothelial tissue layers and is devoid of blood and lymphatic vessels. However, certain pathological conditions, particularly inflammation, can lead to corneal neovascularization, which decreases the transparency of the cornea and increases the corneal graft failure rate [1]. In clinical terms, corneal neovascularization can be divided into three categories according to the level of invasion: (1) superficial vascular pannus, in which blood and lymphatic vessels invade just below the corneal epithelium, is often seen in ocular surface diseases; (2) stromal neovascularization, in which vessels mostly invade the corneal stromal layer, is always observed in interstitial keratitis; (3) deep neovascularization overlying Descemet’s membrane, which is observed in luetic interstitial keratitis [2]. Corneal neovascularization is a sight-threatening condition that currently affects about 1.4 million individuals in the United States each year [3,4]. Despite this, the molecular mechanism of corneal neovascularization remains unclear.

In terms of the molecular mechanisms, it has been reported that the occurrence of ocular neovascularization relies on the balance between corneal neovascularization and anti-neovascularization-related factors [5,6]. In particular, vascular endothelial growth factor-A (VEGF-A) [7], VEGF-C [8], matrix metalloproteases (MMP)-2 [9], MMP-9 [9], galectin-8 [10,11], and neuropilin-2 [12,13] are regarded as neovascularization-related factors, whereas the tissue inhibitors of metalloproteinase (TIMP)-2 [9] and TIMP-1 [9] are reported to be anti-neovascularization factors. In addition, Bcl-2, Bax, and CD31 are also associated with neovascularization [14–16].

In terms of the cellular mechanisms, leukocytes such as macrophages [4], dendritic cells, neutrophils, and mast cells [17,18] have been reported to mediate corneal neovascularization through a...
paracrine mechanism via VEGF-A and VEGF-C. However, the therapeutic efficacy of steroids and anti-inflammatory drugs targeting leukocytes has not been satisfactory, and corneal neovascularization was still observed in animal models of leukopenia [19–21], indicating that, except leukocytes, corneal host cells, including corneal epithelial, stromal, and endothelial cells, could participate in corneal neovascularization. Under normal conditions, corneal epithelial [22–25] and stromal [21,23,25,26] cells have been reported to either promote or inhibit neovascularization. Moreover, under inflammation conditions, corneal epithelial cells have been reported to promote neovascularization through a paracrine mechanism via VEGF-A [5,27] and epithelial membrane protein-2 [28], whereas corneal stromal cells have been reported to mediate neovascularization through a paracrine mechanism via VEGF-A [5,27], transforming growth factor β [29–32], and MMP-13 [33]. However, the key proteins produced by corneal host epithelial and stromal cells leading to neovascularization have not yet been determined, such that the mechanism of corneal endothelial cells involved in neovascularization remains largely unexplored. In the present study, we investigated the contributions of corneal epithelial, stromal, and endothelial cells to corneal neovascularization, and identified the role of their key proteins in neovascularization.

2. Materials and methods

2.1. Human corneas

Corneas were collected from patients who had undergone penetrating keratoplasty of corneal transplantation. Patients with a previous history of topical, subconjunctival, or intraocular application of anti-VEGF agents and steroids were excluded. In total, 54 corneal tissues (20 females and 34 males) were collected for this study from patients who had undergone penetrating keratoplasty for various corneal diseases between 2012 and 2014 from the Eye Hospital of the First Affiliated Hospital of Harbin Medical University. The average age of patients was 52.35 ± 15.95 years, ranging from 18 to 85 years. In addition, 9 normal corneal tissues (4 females and 5 males) from cadaver donors were obtained from the Eye Bank of Heilongjiang Province (Harbin, China). The average age of the cadaver donors was 41.4 ± 11.2 years, ranging from 23 to 69 years. Corneas were classified into 3 groups: vascularized group (33 corneas), non-vascularized group (21 corneas), and normal control group (9 corneas). The details are shown in Table 1. All experiments were conducted in accordance with the Declaration of Helsinki Principles and were performed with the approval of the Internal Review Board of Harbin Medical University. We obtained informed consents from all patients prior to mortality or their family members following mortality for the inclusion of autopsy specimens.

2.2. Preparation of corneal extracts and determination of protein concentration

After their collection, corneas were frozen and stored at −80 °C. Human corneal tissues were ground and then lysed with cold RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) at 4 °C overnight. Thereafter, the supernatants were retained as the corneal extracts following centrifugation at 15000 × g for 20 min at 4 °C. The concentrations of corneal extracts were quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) [34]. Finally, the protein concentrations of all corneal extracts were unified to 0.35 μg/μl. The samples were stored at −20 °C until use.

2.3. Cell culture and collection of cell lysate and supernatant

Human umbilical vein endothelial cell line (HUVEC) (ATCC Cat# CRL-1730, RRID: CVCL_2995), human lymphatic endothelial cell line (HLEC), human corneal epithelial cell line (HCEp) (RRID: CVCL_0541), and human corneal endothelial cell line (HCEC) (DSMZ Cat# ACC-646, RRID: CVCL_2064) were obtained for this study. Primary human corneal stromal cells (HCSCs) were isolated from 6 corneoscleral rings retrieved from the Eye Bank of Heilongjiang Province as previously described [35]. The cell culture methods and the collection of cell lysates and supernatants were carried out according to a previous report [36].

2.4. Liquid chromatography–mass spectrometry (LC-MS) analysis

Two vascularized corneal tissues and one normal corneal tissue were used for LC-MS analysis. LC-MS analysis was carried out as previously reported [37].
2.5. Immunoblotting

Immunoblotting was performed as described previously [36]. The antibodies used in this study include rabbit polyclonal antibodies (pAbs) against MMP-2 (1:200, Santa Cruz Biotechnology Cat# sc-10736, RRID: AB_2250826) and neuropilin-2 (1:200, Santa Cruz Biotechnology Cat# sc-5542, RRID: AB_650370), mouse monoclonal antibodies (mAbs) against collagen-type XII alpha 1 (COL12A1) (1:200), Santa Cruz Biotechnology Cat# sc-168181, RRID: AB_635295), rabbit pAb against alpha-crystallin A chain (CRYAA) (1:2000, Abioscience Cat# ab116815), rabbit pAbs against Bax (1:1000, Assay Biotech Cat# B0773, RRID: AB_10685085), MMP-9 (1:1000, Assay Biotech Cat# C0275, RRID: AB_10685675), TIMP-1 (1:1000, Assay Biotech Cat# C0347, RRID: AB_10684440), CD31 (1:1000, Assay Biotech Cat# R12–2074), TFN-α (1:1000, Assay Biotech Cat# C30026), and p-38 (1:1000, Assay Biotech Cat# B0798, RRID: AB_10864722), rabbit pAbs against TIMP-2 (1:1000, Abcam Cat# ab180630), collagen-type VIII alpha 2 (COL8A2) (1:1000, Abcam Cat# ab112110, RRID: AB_10864383), and VEGF-A (1:1000, Abcam Cat# ab9570, RRID: AB_10685085), rabbit pAbs against TIMP-2 (1:1000, Abcam Cat# ab9570, RRID: AB_10864383), and VEGF-A (1:1000, Abcam Cat# ab9570, RRID: AB_308723), rabbit pAbs against fibronecin (1:1000, Proteintech Group Cat# 15613–1–AP, RRID: AB_2105691), IL-6 (1:1000, Proteintech Group Cat# 21865–1–AP, RRID: AB_11142677), and p21 (1:1000, Proteintech Group Cat# 10355–1–AP, RRID: AB_2077682), rabbit mAb against NF-κB p65 (1:60000, Cell Signalling Technology Cat# 8242, RRID: AB_10859369), rabbit pAb against p-NF-κB p65 (1:1000, Cell Signalling Technology Cat# 3033, RRID: AB_331284), rabbit pAb against p-p38 (1:1000, Cell Signalling Technology Cat# 4511, RRID: AB_2139682), rabbit pAb against galectin-8 (1:1000, Novus Cat# NB1–66520, RRID: AB_11006568), rabbit pAb against laminin α5 (1:2000, Elabscience Cat# E-AB-31903), and mouse mAb against beta-actin (1:2000, ZSBG-Bio Cat# TA-09, RRID: AB_2636897).

2.6. ELISA

The target protein levels in corneal extracts were measured using commercially available ELISA kits in accordance with the manufacturer’s protocols. The ELISA kits used in this study include MMP-2 (ml058669; Mlbio, Shanghai, China), TIMP-2 (E-EL-H1453c; Elabscience, Houston, USA), galectin-8 (CSB-EL012894H;CUSABIO, Wuhan, China), VEGF-C (CSB-EO4579H; CUSABIO, Wuhan, China), VEG-F-A (CSB-EI1718H; CUSABIO, Wuhan, China), COL5A2 (ml204018; Mlbio, Shanghai, China), Bcl-2 (E-EL-H0114c; Elabscience, Houston, USA), MMP-9 (CSB-EO8006H; CUSABIO, Wuhan, China), COL12A1 (ml241608; Mlbio, Shanghai, China), fibronectin (ml023813; Mlbio, Shanghai, China), CD31 (E-EL-H1640c; Elabscience, Houston, USA), Bax (E-EL-H0562c; Elabscience, Houston, USA), neuropilin-2 (CSB-EL016092HU; CUSABIO, Wuhan, China), and total laminin (E-EL-H0128c; Elabscience, Houston, USA). The antibodies used in the total laminin ELISA kit were pAbs against all types of laminin α, laminin β, and laminin γ subunits.

2.7. Lipopolysaccharide (LPS) administration

An in vitro model of LPS-induced inflammation was developed to investigate the pathogenesis of neovascularization-related factors targeting on HCEPs, HCSCs, and HCECs respectively. Firstly, HCEPs, HCSCs, and HCECs with a density of 1.0 × 10^5 cells/ml were seeded in 6-well plates for 24 h. Then, the cells were treated with LPS (L4516-1MG; Sigma, St. Louis, MO, USA) at 5 μg/ml for 48 h. The medium was then replaced with fresh medium. After further incubating for 24 h, the cell lysates and supernatants were collected. In addition, the collected supernatants were used to stimulate HLECs and HUVECs. For this experiment, HLECs and HUVECs with a density of 1.5 × 10^5 cells/ml were seeded in 6-well plates for 24 h. The supernatants of HCEPs/HCSCs/HCECs treated with LPS were then added to HLECs and HUVECs. After culturing for a further 48 h, the cell lysates were harvested.

2.8. Plasmid transfection

The plasmids containing full length of MMP-2, CRYAA, galectin-8, Bcl-2, neuropilin-2, or MMP-9, and blank vector[pCDNA3.1(+) were obtained from Bsgens Company (Harbin, China). Transfections were conducted as previously reported [38].

2.9. Treatment of HCEPs, HCSCs, and HCECs with recombinant human fibronectin (rhFN)

HCEPs, HCSCs, and HCECs with a density of 1.0 × 10^5 cells/ml were seeded in 6-well plates for 24 h. Then, the cells were treated with rhFN (APA037Huo1, Cloud-Clone Corp. Houston, USA) at 5 μg/ml for 48 h. The medium was then replaced with fresh medium. After further incubating for 24 h, the cell lysates and supernatants were collected.

2.10. Tube formation assay

The supernatants of HCEPs, HCSCs, and HCECs treated with LPS were collected as discussed in Section 2.7. Following the manufacturer’s instructions, 30 μl of Matrigel (Proteintech, Chicago, USA) thawed on ice was placed into the wells of a 96-well plate, and the plate was incubated at 37 °C for 30 min in a 5% CO₂ incubator. Then, 15 μl of HLECs and HUVECs at a density of 1.0 × 10^5 cells/ml mixed with 200 μl of supernatants of LPS treated HCEPs, HCSCs, or HCECs were added onto the gel. After four hours, five randomly-selected fields were photographed in each well, and the quantification of total tube length was performed using Image-J software (version 1.47, NIH). All experiments were performed in triplicate.

2.11. Spheroid sprouting assay

Four thousands of HUVECs in 100 μl culture medium containing 1.2% methylcellulose (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) were generated to form spheroids in 200 μl round-bottom non-adherent tubes. The spheroids were then harvested and embedded in collagen type 1 (2 mg/ml, pH 7.4) (Corning, Bedford, USA) for 30 min to obtain matrix polymerization. Then, 200 μl of the supernatants of LPS-treated (collected in Section 2.7), plasmid transfected (collected in Section 2.8), or rhFN-treated (collected in Section 2.9) HCEPs, HCSCs, or HCECs were added onto the gel. After four hours, five randomly-selected fields were photographed in each well, and the quantification of total tubule length was performed using Image-J software (version 1.47, NIH). All experiments were performed in triplicate.

2.12. Rat corneal neovascularization model

Six female Sprague-Dawley rats (3 for the fungus group and 3 for the normal control group) weighing 80–100 g were housed in the Harbin Medical University Experimental Animal Resources Center in accordance with the guidelines of the National Institutes of Health and operated upon using a research protocol approved by the Institutional Animal Care and Use Committee. The rats were placed under general anaesthesia with 3% isoflurane in an O2/room air mixture at a ratio of 1:1. As an additional topical anaesthesia, 0.4% benoxinate hydrochloride (Novesin, Ciba Vision, Hettlingen, Switzerland) was applied to the corneal surface. The rat model of fungus-induced corneal neovascularization was prepared as described below. Briefly, a 33-gauge Hamilton syringe (Hamilton Co., Reno, NV, USA) was used to inject 1.0 × 10^5 PFU of fungus (Candida albicans, ATCC 10231) in a volume of 10 μl into the corneal stroma. After injection, the eyes were treated with one drop of atropine and erythromycin ophthalmic ointment. At days 2 and 7, after anaesthesia, rat cornea photographs were taken with a digital CoolPix 995 camera (Nikon, Japan). At day 7, the rat cornneas...
were harvested. After removing the corneal epithelium and endothelium from the stroma, the rat corneal stromal layer was obtained. The rat corneal stromal layer extracts were prepared and their protein concentration was determined according to the procedure in Section 2.2 [39].

2.13. Statistical analysis

Student’s t-test, one-way ANOVA, and Pearson’s correlation using SPSS 18.0 software were used for statistical analysis. Values are shown as the mean value ± standard deviation (SD), while \( p < .05 \) was considered to indicate a significant difference.

3. Results

3.1. Neovascularization-related factors revealed by LC-MS

In this study, LC-MS was used to screen for potential neovascularization-related factors with two vascularized corneal tissues and one normal corneal tissue. By LC-MS, vascularized corneal tissues were found to have significantly higher expression levels of MMP-2, fibronectin, COL8A2, and CRYAA, and lower expression levels of COL12A1 and laminin α5, compared to the control (Table 2).

3.2. Neovascularization-related factors in corneal tissues identified by immunoblotting and ELISA

To further confirm the neovascularization-related factors, 33 vascularized corneal tissues, 21 non-vascularized corneal tissues, and 9 normal corneal tissues were employed to analyse the expression of neovascularization-related factors identified by LC-MS in this study and those reported previously, including VEGF-A [7], VEGF-C [8], MMP-9 [9], TIMP-2 [9], TIMP-1 [9], galectin-8 [10,11], neuropilin-2 [12,13], Bcl-2 [14], Bax [15], and CD31 [16].

By immunoblotting of corneal extracts, fibronectin, COL8A2, CRYAA, COL12A1, laminin α5, galectin-8, neuropilin-2, Bcl-2, MMP-9, TIMP-2, CD31, and VEG-C were detectable (Fig. 1a), whereas MMP-2, TIMP-1, VEGF-A, and Bax were not detectable (data not shown).

By ELISA and statistical analysis of the corneal extracts (Fig. 1b), the expression of MMP-2 \(( p = .01)\), TIMP-2 \(( p = .001)\), galectin-8 \(( p = .009)\), VEGF-C \(( p = .040)\), and VEGF-A \(( p = .025)\) in vascularized corneal tissues was significantly higher compared to that in non-vascularized corneal tissues. The expression of COL8A2 in vascularized corneal extracts tended to be higher than that in non-vascularized corneal extracts \(( p = .094)\). The expression of Bcl-2 in vascularized corneal tissues was significantly higher than that in normal control corneal tissues \(( p = .007)\). In contrast, the expression of MMP-9 in vascularized corneal tissues was significantly lower than that in normal control corneal tissues \(( p = .014)\). Although laminin α5 was not detectable in any of the corneal extracts by ELISA (data not shown), total laminin was detectable. The expression of total laminin, COL12A1, fibronectin, CD31, Bax, and neuropilin-2 in vascularized corneal tissues showed no differences compared to that in non-vascularized corneal tissues or normal control corneal tissues.

3.3. Correlation of neovascularization-related factors in vascularized corneal tissues

Based on the ELISA results, the possible correlations of various factors in vascularized corneal tissues were analysed. The levels of Bax were positively correlated with the levels of VEGF-C \(( R = .447, p = .010)\) (Fig. 2a), VEGF-A \(( R = .499, p = .004)\) (Fig. 2b), CD31 \(( R = .570, p = .0007)\) (Fig. 2c), and MMP-9 \(( R = .520, p = .002)\) (Fig. 2d), but negatively correlated with the levels of Bcl-2 \(( R = - .392, p = .027)\) (Fig. 2e). The levels of VEGF-A were positively correlated with the levels of CD31 \(( R = .431, p = .014)\) (Fig. 2f), MMP-9 \(( R = .364, p = .041)\) (Fig. 2g), and TIMP-2 \(( R = .517, p = .002)\) (Fig. 2h), and tended to be positively correlated with the levels of fibronectin \(( R = .347, p = .065)\) (Fig. 2i). The levels of neuropilin-2 were positively correlated with the levels of MMP-9 \(( R = .563, p < .001)\) (Fig. 2j) and total laminin \(( R = .370, p = .048)\) (Fig. 2k). Additionally, the levels of VEGF-C were positively correlated with the levels of VEGF-A \(( R = .485, p = .005)\) (Fig. 2l) and MMP-9 \(( R = .357, p = .041)\) (Fig. 2m). The levels of CD31 were positively correlated with the levels of MMP-9 \(( R = .372, p = .036)\) (Fig. 2n) and TIMP-2 \(( R = .368, p = .038)\) (Fig. 2o). The levels of COL12A1 were positively correlated with the levels of fibronectin \(( R = .496, p = .006)\) (Fig. 2p). However, no significant correlation was found between the levels of COL8A2, MMP-2, and galectin-8, and the levels of any other neovascularization-related factors analysed.

3.4. Neovascularization-related factors in LPS-treated HCEps, HCSCs, and HCECs detected by immunoblotting

Compared to HCEps/HCSCs/HCECs, HCEps/HCSCs/HCECs treated with LPS had higher expression levels of inflammatory factors, IL-6 and TNF-α, suggesting that inflammation was successfully induced.

Next, the expression levels of neovascularization-related factors in HCEps/HCSCs/HCECs treated with LPS were detected by immunoblotting (Fig. 3). Compared to HCEps, HCEps treated with LPS had higher expression levels of MMP-2, galectin-8, neuropilin-2, Bcl-2, and MMP-9, and lower expression levels of TIMP-2 and TIMP-1. Compared to HCSCs, HCSCs treated with LPS had higher expression levels of MMP-2 and CRYAA, and lower expression levels of neuropilin-2, TIMP-2 and TIMP-1. Compared to HCECs, HCECs treated with LPS had higher expression levels of MMP-2, galectin-8, neuropilin-2, TIMP-2, TIMP-1, and COL8A2.

3.5. Correlation of neovascularization-related factors in HCEps, HCSCs, and HCECs

To identify the regulation correlations of various factors, the cell lysates of HCEps/HCSCs/HCECs either transfected with MMP-2, CRYAA, galectin-8, Bcl-2, neuropilin-2, MMP-9 plasmid, or treated with rhFN were analysed for the expression of neovascularization-related factors by immunoblotting.

In HCEps (Fig. 4a), transfection with the MMP-2 plasmid upregulated the expression level of Bcl-2 but downregulated the expression levels of MMP-9, neuropilin-2, fibronectin, and Bax; transfection with the galectin-8 plasmid upregulated the expression levels of neuropilin-2 and Bax, but downregulated the expression levels of MMP-9, fibronectin, and Bcl-2; transfection with the Bcl-2 plasmid downregulated the expression level of MMP-2 but downregulated the expression level of Bax; transfection with the MMP-9 plasmid upregulated the expression level of TIMP-2, but downregulated the expression levels of MMP-2, galectin-8, and fibronectin; treatment with rhFN upregulated the expression level of MMP-9, but downregulated the expression levels of galectin-8, COL8A2, and TIMP-2.

In HCSCs (Fig. 4b), transfection with the MMP-2 plasmid upregulated the expression levels of fibronectin and COL8A2, but downregulated the expression levels of galectin-8 and Bcl-2; transfection with...
the CRYAA plasmid upregulated the expression levels of galectin-8; transfection with the galectin-8 plasmid upregulated the expression levels of MMP-2 and neuropilin-2, but downregulated the expression level of MMP-9; treatment with rhFN upregulated the expression level of neuropilin-2, but downregulated the expression level of galectin-8.

In HCECs (Fig. 4c), transfection with the MMP-2 plasmid downregulated the expression levels of MMP-9, galectin-8, fibronectin, and TIMP-2; transfection with the galectin-8 plasmid upregulated the expression level of neuropilin-2, but downregulated the expression level of fibronectin, COL8A2, and Bcl-2; transfection with the Bcl-2 plasmid upregulated the expression level of MMP-2; transfection with the neuropilin-2 plasmid downregulated the expression level of MMP-2; treatment with rhFN upregulated the expression levels of galectin-8, neuropilin-2, Bcl-2, and TIMP-2.

Fig. 1. Neovascularization-related factors in corneal tissues identified by immunoblotting and ELISA. (a) Detection of MMP-2, fibronectin, COL8A2, CRYAA, COL12A1, laminin α5, galectin-8, neuropilin-2, Bcl-2, MMP-9, Bax, TIMP-2, TIMP-1, VEGF-A, CD31, VEGF-C, and beta-actin in corneal extracts by immunoblotting. C1–C5, vascularized corneal tissues of patients; N1–N3, normal corneal tissues. (b) Detection of MMP-2, TIMP-2, galectin-8, VEGF-C, VEGF-A, COL8A2, Bcl-2, MMP-9, total laminin, COL12A1, fibronectin, CD31, Bax, and neuropilin-2 in corneal extracts by ELISA. Values represent the mean ± SD. Vessel (+), vascularized corneal tissues of patients; Vessel (−), non-vascularized corneal tissues of patients; Normal, normal control corneal tissues. N = 33 for vessel (+) group; n = 21 for vessel (−) group; n = 9 for normal group.

Fig. 2. Correlation of neovascularization-related factors in vascularized corneal tissues. Pearson’s analysis was used to determine the correlation of various factors in vascularized corneal tissues (n = 33). Data are presented as dot plots with fitted regression lines. The R and p represent the Pearson correlation coefficient and the two-tailed p-value, respectively.
3.6. Neovascularization influenced by HCEps, HCSCs, and HCECs treated with LPS

Endothelial tube formation and sprouting of HUVECs and/or HLECs were analysed to evaluate the influence of the LPS-treated HCEps, HCSCs, and HCECs supernatants on neovascularization. Using tube formation assays with HUVECs, the tube length of LPS-treated HCEps, HCSCs, and HCECs were found to be significantly higher compared to that of the non-treated controls, and the change of tube length stimulated by LPS was most significant in HCSCs (Fig. 5a). Similar results were obtained in the tube formation assay with HLECs, however, the change of tube length stimulated by LPS was most significant in HCEps (Fig. 5b). Moreover, using the spheroid sprouting assay, the levels of HUVECs sprouting in presence of LPS-treated HCEps, HCSCs, and HCECs were also found to be significantly higher compared to that in the non-treated controls (Fig. 5c).

3.7. Neovascularization-related factors in HUVECs and HLECs treated with HCEps/LPS supernatants

The key proteins and signalling pathways of HCEps-LPS, HCSCs-LPS, and HCECs-LPS supernatants in neovascularization were evaluated. The cell lysates of HUVECs and HLECs treated with HCEps-LPS, HCSCs-LPS, and HCECs-LPS supernatants were analysed for the detection of neovascularization-related factors by immunoblotting. The results are summarized in Fig. 6.

Compared to HUVECs treated with HCEps supernatant, HUVECs treated with HCEps-LPS supernatant had higher expression levels of fibronectin, Bcl-2, and p-NF-κB, but a lower expression level of TIMP-1.
Compared to HUVECs treated with HCSCs supernatant, HUVECs treated with HCSCs-LPS supernatant had higher expression levels of MMP-2, COL8A2, CRYAA, galectin-8, MMP-9, and p21, but a lower expression level of TIMP-1 (Fig. 6a). Compared to HUVECs treated with HCECs supernatant, HUVECs treated with HCECs-LPS supernatant had higher expression levels of fibronectin, Bcl-2, p-NF-κB, MMP-2, and p21, but a lower expression level of TIMP-1 (Fig. 6a).

Compared to HLECs treated with HCEps supernatant, HLECs treated with HCEps-LPS supernatant had a higher expression level of p21, but a lower expression level of TIMP-1 (Fig. 6a). Compared to HLECs treated with HCSCs supernatant, HLECs treated with HCSCs-LPS supernatant had higher expression levels of MMP-2, neuropilin-2, and p-p38, but lower expression levels of p21, TIMP-1, COL8A2, MMP-9, TIMP-2, and p38 (Fig. 6b). Compared to HLECs treated with HCECs supernatant,
HLECs treated with HCECs-LPS supernatant had higher expression levels of p21, COL8A2, TIMP-2, fibronectin, and galectin-8, but a lower expression level of TIMP-1 (Fig. 6b).

3.8. Neovascularization influenced by MMP-2, CRYAA, galectin-8, neuropilin-2, MMP-9, and fibronectin

To further confirm the influence of the factors secreted by HCEps, HCSCs, and HCECs on neovascularization, we performed spheroid sprouting assays using HUVECs stimulated by the supernatant of HCEps, HCSCs, and HCECs either transfected with MMP-2, CRYAA, galectin-8, neuropilin-2, MMP-9 plasmid, or treated with rhFN. The HUVECs sprouting in presence of MMP-9 plasmid transfected HCEps was significantly higher compared to that in the controls (Fig. 7a). Likewise, the HUVECs sprouting in presence of MMP-2, CRYAA, galectin-8, and MMP-9 plasmid transfected HCSCs were significantly higher compared to that in the controls (Fig. 7b). The HUVECs sprouting in presence of MMP-2, galectin-8, and MMP-9 plasmid transfected HCECs, and rhFN-treated HCECs were significantly higher compared to that in the controls (Fig. 7c).

3.9. CRYAA overexpression in rat vascularized corneal stromal layer detected by immunoblotting

To further confirm the involvement in neovascularization of CRYAA in corneal stromal cells, we established a fungus-induced corneal neovascularization model in vivo. In the corneas of rats infected with fungus, fungus infection was observed at day 2 and robust neovascularization was observed at day 7 (Fig. 6a). The immunoblotting results showed that the CRYAA expression level in rat vascularized corneal stromal layer lysate was significantly higher than that in the normal control (p = .002) (Fig. 8b, c).

3.10. Potential mechanism of corneal host cells involved in neovascularization under inflammatory stimulation

The in vivo and in vitro data suggest a possible mechanism by which corneal epithelial, stromal, and endothelial cells participate in the development of neovascularization under inflammatory stimulation. As shown in Fig. 9, in corneal epithelial cells, MMP-9 is the key protein promoting neovascularization. Overexpressed MMP-9 was found to downregulate MMP-2 and galectin-8, while overexpressed MMP-2 or galectin-8 also downregulated MMP-9. In corneal stromal cells, MMP-2 and CRYAA were found to be the key proteins promoting neovascularization. Overexpressed CRYAA upregulated galectin-8, which in turn upregulated MMP-2, while overexpressed MMP-2 downregulated galectin-8. In corneal endothelial cells, MMP-2 and galectin-8 were identified as the key proteins promoting neovascularization. Overexpressed MMP-2 downregulated galectin-8, while overexpressed galectin-8 upregulated neuropilin-2, which in turn downregulated MMP-2.

4. Discussion

In this study, MMP-2, CRYAA, and galectin-8 were found to be upregulated and MMP-9 was downregulated in full-thickness vascularized corneal tissues compared to that in the normal controls. The expression of MMP-9 in corneal epithelial cells, CRYAA in corneal stromal cells, galectin-8 in corneal epithelial and endothelial cells, and MMP-2 in all three layers of corneal cells was upregulated under infectious stimulus. The correlations of these factors were subject to the different types of corneal cell. All three kinds of corneal host cells were found to experience enhanced neovascularization under infectious stimulus, although to varying degrees. Finally, MMP-9 in corneal epithelial cells, MMP-2 and CRYAA in stromal cells, and MMP-2 and galectin-8 in endothelial cells were found to be the key proteins in promoting corneal inflammatory neovascularization. As such, these could be used as potential therapeutic targets for the treatment of corneal inflammatory neovascularization.

Although various corneal proteomes have been previously identified [40–43], to the best of our knowledge, this study is the first to report on the proteome of vascularized corneae.

MMP-9 is located in the basement membrane of the corneal epithelium and in the superficial stroma. MMP-9 has been previously reported to be upregulated in corneas with herpes simplex virus [44] fungal [45] and bacterial [46] infection, while the inhibition of MMP-9 with TIMP-1 has been found to result in reduced angiogenesis in herpes simplex keratitis [44]. However, in the present study, although MMP-9 was found to be downregulated in human full-thickness vascularized corneal tissues, MMP-9 expression in vascularized corneal tissues was positively correlated with VEGF-A expression, consistent with the previously reported literature [47]. The overexpression of MMP-9 in LPS-treated HCEps, as well as the enhanced neovascularization in the LPS-treated HCEps supernatant and MMP-9 plasmid transfected HCEps supernatant suggest...
that inflammation may enhance corneal neovascularization by inducing MMP-9 overexpression in corneal epithelial cells.

MMP-2 is one of the main mediators responsible for corneal neovascularization [48]. MMP-2 was previously found to immunolocalize to the epithelium and stroma of normal corneas [49], and MMP-2 was mainly produced by the corneal stromal cells and neutrophils in the rabbit fungal keratitis corneas [50]. Our own previous report showed that a selective MMP-2 and -9 inhibitor (SB-3CT) could inhibit lymphangiogenesis in a suture-induced corneal neovascularization model [51], which indicates that MMP-2 and MMP-9 play positive roles in corneal lymphangiogenesis. In the present study, the overexpression of MMP-2 in full-thickness vascularized corneal tissues and LPS-treated HCSCs, as well as the enhanced neovascularization in the LPS-treated HCSCs supernatant and MMP-2 plasmid transfected HCSCs supernatant suggest that inflammation may enhance corneal neovascularization by inducing MMP-2 overexpression in corneal stromal cells. Similar results also indicated that inflammation may enhance corneal neovascularization by inducing MMP-2 overexpression in corneal endothelial cells.

Fig. 7. Neovascularization influenced by MMP-2, CRYAA, galectin-8, neuropilin-2, MMP-9, and fibronectin. HUVEC spheroids formed from 4000 cells were embedded in collagen and overlaid with the supernatants of HCEps (a), HCSCs (b), and HCECs (c) treated with condition medium, blank vector, MMP-2 plasmid, CRYAA plasmid, galectin-8 plasmid, neuropilin-2 plasmid, MMP-9 plasmid, or rhFN. The sprouting degree was measured after 30 h. The representative spheroids are shown. Scale bar = 200 μm.

Fig. 8. CRYAA overexpression in rat vascularized corneal stromal layer detected by immunoblotting. (a) Slit-lamp photographs of Sprague-Dawley rat corneas for normal control group (left) and fungus infection group at day 2 (middle, arrow indicates fungus infection) and day 7 (right, arrows indicate the new-born blood vessels) after fungus injection. (b) Detection of CRYAA and beta-actin in vascularized stromal layer extracts and normal control by immunoblotting. Vessel (+), fungus-induced vascularized corneal stromal layer lysate; normal, normal control stromal layer lysate. The representative images from three independent assays are shown. (c) Data represent the mean ± SD. *p < .05, **p < .01, ***p < .001.
CRYAA is a major structural protein in the lens [52]. An increasing number of studies have demonstrated that CRYAA expression is upregulated in diabetic retinopathy [53], inflammatory eye diseases [54,55], and tumour angiogenesis [56]. However, research concerning the role of CRYAA in corneal neovascularization is rare and contradictory. The CRYAA gene has been previously found to be significantly upregulated in suture- or chemical burn-induced corneal neovascularization models by microarray analysis [57]. In another study, CRYAA was found to promote angiogenesis using CRYAA siRNA and CRYAA-knockout mice [58]. However, another research has found no difference in the development of corneal neovascularization between CRYAA-deficient mice and wild-type mice, but has demonstrated that a subconjunctival injection of CRYAA significantly attenuates suture-induced corneal neovascularization [59]. In the present study, CRYAA was found to be highly expressed in human full-thickness vascularized corneal tissues for the first time, located mostly in corneal stromal cells after LPS stimulation and in vascularized corneal stromal layer after fungus infection. These results suggest that inflammation may enhance corneal neovascularization by inducing CRYAA expression in corneal stromal cells.

Galectin-8 was expressed in both corneal epithelial and stromal cells under normal condition [60], and robust galectin-8 immunoreactivity was mainly detected in the stromal matrix of inflamed human and thermal cautery or AgNO3 cautery-treated mouse corneas, while immunolocalization of galectin-8 was mostly found in lymphatic vessels but not in blood vessels [10]. In the present study, the overexpression of galectin-8 in full-thickness vascularized corneal tissues and LPS-treated HCECs, as well as the enhanced neovascularization in the LPS-treated HCECs supernatant and galectin-8 plasmid transfected HCECs supernatant, suggest that inflammation may enhance corneal neovascularization by inducing galectin-8 overexpression in corneal endothelial cells.

The overexpression of MMP-9 in LPS-treated HCEPs, the subsequent promotion of neovascularization effects, and the unchanged expression levels of MMP-9 in LPS-treated HSCCs and HCECs suggest that treatment of corneal inflammatory neovascularization with MMP-9 inhibitors may only be effective in HCEPs. Similarly, the treatment of corneal inflammatory neovascularization with CRYAA and galectin-8 inhibitors may only be effective in HSCCs and HCECs, respectively. Although the treatment of corneal inflammatory neovascularization with MMP-2 inhibitors may be effective in both HSCCs and HCECs, it may cause side effects in HCEPs, considering the inhibition effects of MMP-9 expression by MMP-2. In this study, a total of 16 neovascularization-related factors were investigated. After screening, four factors were found to play the most significant roles: MMP-2, CRYAA, galectin-8, and MMP-9. However, the remaining 12 factors were found to also contribute to neovascularization, either directly or indirectly, by regulating MMP-2, CRYAA, galectin-8, and MMP-9 expression in corneal host cells.

In clinical terms, corneal neovascularization could invade from just below the corneal epithelium to the deep layer of stroma, which coincides with our finding that corneal stromal and epithelial cells showed stronger abilities to promote neovascularization than corneal endothelial cells. In addition, in the HUVECs and HLECs stimulated by the supernatants of LPS-treated HCEPs/HSCCs/HCECs, the expression of MMP-2, CRYAA, galectin-8, and MMP-9, as well as some signalling proteins, were influenced, however, the exact mechanism and signalling pathways will need to be studied further.

Inflammatory neovascularization is an unregulated phenomenon associated with the influx of activated leukocytes that extravasate/transport from within the blood vessels to the tissue parenchyma. The infiltrating leukocytes along with corneal host cells, including corneal epithelial and stromal cells, can all play intricate roles in angiogenesis via releasing a series of factors, such as IL-1β, TNF-α, TGFβ, VEGF, MMP-2, MMP-9, platelet derived growth factor, basic fibroblast growth factor [361–63], which shift the balance toward corneal neovascularization [64]. However, the leukocytes did not appear to play a significant role in inflammatory neovascularization. Specifically, depletion studies revealed the progressive development of neovascularization despite a significant loss of infiltrating leukocytes [19–21]. In contrast, the neovascularization-related factors likely produced by corneal host cells under inflammatory conditions were found to be significantly elevated following virus clearance [65]. These findings indicate that infiltrating leukocytes and corneal host cells might occur in parallel promoting inflammatory neovascularization. As such, corneal host cell-based anti-neovascularization therapies could be used as complementary treatments for leukocyte-based anti-neovascularization therapies.

In conclusion, corneal epithelial, stromal, and endothelial cells participated to varying degrees in corneal neovascularization. In particular, MMP-2, CRYAA, galectin-8, and MMP-9 were found to be key proteins for the regulation of corneal inflammatory neovascularization, although their involvement varied according to the different corneal cell types. These results provide compelling evidence for the host cell-based therapy of corneal inflammatory neovascularization. Since corneal neovascularization is often restricted to some certain corneal layer, the current study will benefit the targeted treatment and prevention of corneal neovascularization based on the affected layer, which will be helpful to reduce side effects. Our study highlights the importance of the role of corneal host cells in corneal inflammatory neovascularization and...
provides a theoretical basis for the targeted drug development for the treatment of corneal vascularization.

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Declaration of interests

The authors have no conflicts of interest to declare.

Author contributions

P.L., X.L.T., X.G.L., and H.Q. designed this study. H.Y.Y., L.Y.S., J.C., Y.L., Y.Y., K.W., F.Q.S., W.T.J., Y.F.L., and H.Y.G. performed the experiments. X.G.L. and H.Q. guided and supervised this study. H.Y.Y., X.G.L., and H.Q. wrote the manuscript. P.L. and X.L.T. revised the manuscript.

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