Angiopoietin-Like Protein 2 Is a Potent Hemangiogenic and Lymphangiogenic Factor in Corneal Inflammation

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Corneal avascularity is essential for optimal optical clarity. However, a wide variety of corneal inflammatory insults, such as infections and chemical injuries, can cause capillary invasion and, thus, result in corneal neovascularization (NV). Vascular ingrowth leads to further scarring, and subsequently results in the loss of corneal transparency. Although corneal NV is a severe debilitating condition that results in visual impairment, to our knowledge therapy for corneal NV has not been fully established yet and keratoplasty (corneal transplantation) remains the mainstay of surgical treatment of vascularized corneas. However, as the immune privilege of the cornea is compromised, the severely vascularized corneas are at a high risk for graft rejection.1–4 Furthermore, in the vascularized cornea, there is a parallel outgrowth of blood and lymphatic vessels.5–9 As afferent lymphatic vessels are the route through which antigen-presenting cells (APCs) migrate to the regional lymph nodes and lymphoid organs, the inhibition of corneal lymphangiogenesis, in addition to hemangiogenesis, may be the ideal therapeutic strategy for avoiding rejection.8–10 However, the mechanisms that induce hemangiogenesis and lymphangiogenesis in the cornea after inflammation have not been fully clarified yet, to our knowledge.

Recently, members of the angiopoietin-like proteins family (Angptls) have been implicated in diverse physiologic activities, including lipid metabolism, angiogenesis, and inflammation.6–9,11–14 Angptls are members of a family of secreted proteins possessing an amino-terminal coiled-coil domain and a carboxy-terminal fibrinogen-like domain, which also represents the common primary structure of angiopoietins (Anglts).11,12,15,16 In contrast to Anglts, Angptls do not bind to either of the receptor tyrosine kinases of the Tunica internal endothelial cell kinase family (Tie2 or Tie1).8,11–13 Among the seven members of the Angptls family, the expression of Angptl2 was reported to be elevated particularly in inflammatory diseases.5,17–24 Moreover, Angptl2 signaling via integrins was shown to activate inflammatory cascade in vascular endothelial cells and monocytes/macrophages via integrin signaling.15,18,20 Constitutive activation of Angptl2 in skin epithelial cells of transgenic mice induced inflammation, including inflammation of the vasculature characterized by abundant attachment of leukocytes to the vessel walls and increased vascular permeability.5,8,13,18,20,23,25–26

In view of the above, the objective of our study was to investigate the plausible functional role of Angptl2 in inflam-
Angptl2 in Corneal Inflammation

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They investigated the expression of Angptl2 and interleukin 1β in normal and vascularized corneas at mRNA and protein levels by real-time RTPCR and immunohistochemistry. Further, they assessed corneal hemangiogenic and lymphangiogenic responses, and macrophage infiltration by immunofluorescent microscopic studies using specific antibodies against CD51 (a panendothelial marker), LYVE-1 (a lymphatic marker), and F4/80 (a macrophage marker), and compared to their corresponding background strains.

**Materials and Methods**

**Animals and Reagents**

All animal experiments were approved by the University of Tokyo Hospital Animal Care Committee, and conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. All surgical procedures were performed under general anesthesia. Animals (male 20–24-week-old mice) were injected with xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (35 mg/kg). Angptl2 knockout mice on C57BL/6 background (Angptl2+/−) and K14-Angptl2 transgenic mice on Balb/c background were gifted by one of the coauthors of this study (YO). The corresponding wild-type littermates were used as control mice. The transgenic mice overexpressed Angptl2 under the control of the keratinocyte-specific promoter (K14 promoter; K14-Angptl2).18,27,28 The offspring from the F10 or F11 generations were used in this study. For gene therapy, 10/0 nylon suture was placed intrastromally 1 mm away from the limbal vessel. Ofloxacin ophthalmic ointment was instilled immediately after suture. Balb/c background (Angptl2+/−/C0) and K14-Angptl2 transgenic mice on Balb/c background were gifted by one of the coauthors of this study (YO). The corresponding wild-type littermates were used as control mice. The transgenic mice overexpressed Angptl2 under the control of the keratinocyte-specific promoter (K14 promoter; K14-Angptl2).18,27,28 The offspring from the F10 or F11 generations were used in this study. For gene therapy, 10/0 nylon suture was placed intrastromally 1 mm away from the limbal vessel. Ofloxacin ophthalmic ointment was instilled immediately after suture. Balb/c background (Angptl2+/−/C0) and K14-Angptl2 transgenic mice on Balb/c background were gifted by one of the coauthors of this study (YO). The corresponding wild-type littermates were used as control mice. The transgenic mice overexpressed Angptl2 under the control of the keratinocyte-specific promoter (K14 promoter; K14-Angptl2).18,27,28 The offspring from the F10 or F11 generations were used in this study. For gene therapy, 10/0 nylon suture was placed intrastromally 1 mm away from the limbal vessel. Ofloxacin ophthalmic ointment was instilled immediately after suture.

**Induction of Corneal Inflammatory Hemangiogenesis and Lymphangiogenesis**

We induced corneal neovascularization in 20- to 24-week-old male mice by nylon suturing under microscopy according to a previous study.1,18 Under general anesthesia, 10-0 nylon suture was placed intrastromally 1 mm away from the limbal vessel. Ofloxacin ophthalmic ointment was instilled immediately after suturing.

**Real-Time RT-PCR**

Under a microscope (SMZ745T; Nikon, Tokyo, Japan), normal and vascularized corneal buttons in mice were obtained carefully. Total RNA was isolated from these corneas (Isogen; Nippon Gene, Tokyo, Japan), and cDNA was synthesized using reverse transcriptase (RT; SuperScript II; Invitrogen, San Diego, CA). RT-PCR was performed on the LightCycler (LC; Roche Diagnostics, Basel, Switzerland) instrument. Aliquots of each RT reaction in each LC glass capillary were amplified by PCR in a 20 μL total volume containing 10 μL PCR master mix (0.8 μL SYBR Premix Ex TaqII, final concentration, 0.4 μL Takara Bio, Inc., Shiga, Japan): 1.4 μL each of forward and reverse primers, and 6.4 μL PCR-grade sterile water, and 2 μL templates. LC capillaries were sealed, inserted into the specially designed LC Carousel (Roche Diagnostics), and centrifuged at 740g for 15 seconds. Finally, the LC Carousel was placed into the LC rotor. The following LC experimental run protocol was used: initial denaturation cycle (95°C for 30 seconds); followed by 45 cycles consisting of denaturation at 95°C for 5 seconds, annealing at 60°C for 20 seconds (temperature transition rate 20°C/s), and extension at 72°C for 5 seconds, with a single fluorescence measurement: melting curve cycle (95°C–99°C with a heating rate of 0.1°C/s and continuous fluorescence measurement), and finally a cooling step to 40°C for 20 seconds. LC software (version 3.5; Roche Diagnostics) was used to calculate crossing points (C0) for each transcript, by the automated second derivative maximum method. Relative transcript abundance was normalized to the expression of the housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The sequences of the oligonucleotide primers used for RT-PCR were as follows: mice Angptl2: forward, 5′-GAGGTTGACTGTCATCCAGAG-3′, reverse, 5′-GCGTTGTTGCTGTACGGTA-3′; mice F4/80: forward, 5′-ATGACTGGCCACAGTACGATGTGGG-3′, reverse, 5′-TGCTGGACAGGAAAGCTCGTT-3′; mice Interleukin 1β: forward, 5′-CAGGATAGGACGACATGAGCAC-3′, reverse, 5′-GAAAGCTCACACACGACAGGTTA-3′; mice GAPDH: forward, 5′-CACATTGGGGGTAGGAAAC-3′, reverse, 5′-AAGTTGCGATTGTGGAAG-3′.

**Immunohistochemistry**

Immunohistochemical staining was performed to investigate the protein expression of Angptl2 in the cornea. Mouse eyes were enucleated, embedded in optimal cutting temperature (OCT) compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), snap frozen in liquid nitrogen, and cut into 8 μm thick sections. Subsequently, the sections were blocked with 3% nonfat dried milk bovine working solution (M7409; Sigma-Aldrich) and stained with rabbit antiserum Angptl2 antibody as described previously.5,18 After an overnight incubation, sections were washed and stained for 60 minutes with Alexa Fluor 488 goat antirabbit IgG (H+L, 1:500; Invitrogen).

To detect monocyte-macrophage infiltration, the sections were stained with rat antiserum F4/80 antibody (Abcam, Cambridge, UK). After an overnight incubation, sections were washed and stained for 60 minutes with Alexa Fluor 594 donkey antirabbit IgG (H+L, 1:1000; Invitrogen). The samples were observed under a fluorescence microscope and counted in a masked fashion. Eight serial sections extending through the suture and limbus were studied per cornea. The number of F4/80-positive cells between the corneal peripheral edge (right above the angle) and suture area (1 mm away from the limbus) was determined at a magnification of ×50.

**Quantification of Hemangiogenesis and Lymphangiogenesis**

Hemangiogenesis and lymphangiogenesis were quantified as described previously using double staining with CD31/LYVE-1.1,10 Briefly, the excised corneas following neovascularization were rinsed in PBS, and fixed in acetone for 10 minutes at −20°C. Subsequently, the corneas were washed thrice in PBS, blocked with 3% BSA in PBS for 48 hours at room temperature, stained with rabbit antimouse LYVE-1 antibody (lymphatic vessel marker; Abcam) and rat antiserum CD31 antibody (blood vessel marker; BD Biosciences, Franklin Lakes, NJ), and incubated at 4°C overnight. The sections were washed again with PBS and stained with secondary antibodies (Alexa Fluor 488 goat antirabbit IgG and Alexa Fluor 594 donkey antirat IgG; Invitrogen) for 5 hours at room temperature. Double stained whole mounts were analyzed using a fluorescence microscope (BX-9000; Keyence, Osaka, Japan). The area covered with blood or lymphatic vessels positive for CD31 or LYVE-1 was quantified using National Institutes of Health (NIH) Image software (ImageJ; available in the public domain at http://rsb. NIH.gov).
results are expressed as vessel area/total field.

**Statistical Analysis**

All data were analyzed with the statistical software (ImageJ; available in the public domain at http://rsb.info.nih.gov/ij/). The Mann-Whitney U test was used to compare the differences in the mRNA expression levels of Angptl2 and IL-1β, the extent of corneal hemangiogenic and lymphangiogenic area, and F4/80-positive cell numbers between Angptl2 knockout and K14-Angptl2 transgenic mice with respect to their corresponding background strains (control). Steel Dwass test was used to compare the hemangiogenic and lymphangiogenic responses among PBS, control siRNA, and Angptl2 siRNA, treatments. Values of $P < 0.05$ were considered statistically significant.

**RESULTS**

**Expression of Angptl2 in Suture-Induced Inflammatory Corneal NV Model**

We analyzed Angptl2 expression level in normal and vascularized corneas at the mRNA and protein levels by real-time RT-PCR and immunohistochemistry. In C57BL/6 mice, a significant increase in the Angptl2 mRNA level was observed on days 1, 4, 7, and 10 following surgery, compared to that observed in normal cornea before treatment (Fig. 1A). The Angptl2 mRNA expression was peaked on day 4 following surgery, and was decreased gradually on day 7 following surgery (Fig. 1A). In Balb/c mice, the expression of Angptl2 mRNA was peaked on days 1 following surgery and was decreased gradually on days 4, 7, and 10 following surgery (Fig. 1B).

Immunohistochemical analysis revealed that Angptl2 was expressed strongly in the epithelial and stromal cells of the vascularized corneas on day 7 after surgery, compared to the normal cornea (Fig. 1C). The partial Angptl2-positive cells were stained with anti-CD31 or anti-F4/80, but not with anti-LYVE-1. These results revealed that these positive cells would be mainly keratocytes.

**Activation of Angptl2 Induces Corneal Hemangiogenesis and Lymphangiogenesis and Macrophage Infiltration in K14-Angptl2 Transgenic Mice**

To quantify the early hemangiogenic and lymphangiogenic responses in the inflammatory corneal NV model, corneal whole mounts were double stained using CD31 as a panendothelial marker and LYVE-1 as specific lymphatic vessel marker. To investigate the functional role of Angptl2 in inflammatory corneal NV, the hemangiogenic and lymphangiogenic responses of K14-Angptl2 mice were compared to those of their background strain (Fig. 2A). On day 5 after suturing, the areas of hemangiogenesis and lymphangiogenesis in the corneas of K14-Angptl2 mice were increased significantly compared to those in background strain (Figs. 2B, 2C). As infiltrating macrophages and inflammatory...
cytokines have a crucial role in the development of inflammatory corneal NV, we confirmed the macrophage infiltration by F4/80 immunohistochemical staining, and analyzed for gene expressions of F4/80 and IL-1β at the corresponding time point after suturing in the corneas of K14-Angptl2 mice (Figs. 2D–G). In contrast to the corneas of control mice, the expressions of F4/80 and IL-1β were higher in those of the K14-Angptl2 mice (Figs. 2D, 2G). Notably, there were no statistically significant differences in the number of F4/80-positive cells between wild-type and K14-
Angptl2 mice (Figs. 2E, 2F). However, the number of F4/80-positive cells in K14-Angptl2 mice on day 5 postsurgery was significantly higher than that in the wild-type corneas (Fig. 2E). Therefore, these results suggested that the corneal hemangiogenesis and lymphangiogenesis are driven by overexpression of Angptl2 via macrophage infiltration and IL-1β expression.

**Reduction of Inflammatory Corneal Hemangiogenesis and Lymphangiogenesis in Angptl2−/− Knockout Mice**

The hemangiogenic and lymphangiogenic responses induced by corneal suture in Angptl2−/− knockout mice were compared to those in their background strain (Fig. 3A). On day 7 following suturing, the hemangiogenic and lymphangiogenic
regions in the corneas of Angptl2−/− mice were significantly decreased compared to those in their background strain (Figs. 3B, 3C). Interestingly, the gene expressions of F4/80 and IL-1β in the corneas of Angptl2−/− mice were significantly lower compared to those in the control mice (Figs. 3D, 3G). As expected, the number of F4/80-positive cells in the corneas of Angptl2−/− mice on day 7 postsurgery was found to be comparatively lower than that in the wild-type (Figs. 3E, 3F). Our findings, thus, highlight that Angptl2 may be a potent inducer of inflammatory corneal hemangiogenesis and lymphangiogenesis.

Angptl2 siRNA Inhibited Corneal Hemangiogenesis and Lymphangiogenesis

The hemangiogenic and lymphangiogenic responses induced by corneal suture in mice treated with Angptl2 siRNA were investigated (Fig. 4). The mRNA expressions of Angptl2 were totally inhibited by subconjunctival administration of Angptl2 siRNA, but not by that of control siRNA (Fig. 4A). Corneal hemangiogenesis and lymphangiogenesis were markedly reduced in eyes receiving subconjunctival injections of Angptl2 siRNA compared to eyes treated with the vehicle-siRNA or saline on day 5 after the intrastromal suturing with 10-0 nylon (n = 4 per group). Error bars denote the mean ± SD (*P < 0.05).

DISCUSSION

To our knowledge, our study is the first of its kind to demonstrate the role of Angptl2 in corneal inflammation in vivo using an established suture-induced corneal NV model. While a normal cornea represents an avascular as well as an alymphatic tissue, the vascularized cornea displayed a parallel outgrowth of blood and lymphatic vessels from the limbal vascular arcade.6–9,13,14 Although the function of newly formed lymphatic vessels in cornea has not been resolved fully, studies using mouse models of keratoplasty have shed light on the underlying mechanisms of immune privilege of corneal allografts. It has been shown that APCs migrate through the corneal lymphatic vessels to reach the regional lymph node in the corneal limbus. On the basis of this observation, earlier studies have demonstrated that the inhibition of new lymphatic vessels drastically limited the allograft rejection in the mouse keratoplasty model.15,16 Furthermore, as inflammation-induced lymphangiogenesis in the cornea arises from macrophages,8,13 the regulation of macrophages in corneal inflammation may inhibit hemangiogenesis as well as lymphangiogenesis.

In our study, the gene as well as the protein expression of Angptl2 was upregulated in the vascularized cornea compared to the normal cornea (Fig. 1). Strikingly, the areas of corneal hemangiogenesis and lymphangiogenesis were markedly reduced in eyes receiving subconjunctival injections of Angptl2 siRNA compared to eyes treated with the vehicle-siRNA or saline on day 5 after the intrastromal suturing with 10-0 nylon (Figs. 4B, 4C).

Figure 4. Corneal hemangiogenesis and lymphangiogenesis are inhibited by Angptl2 siRNA. (A) Angptl2 mRNA expression was reduced completely by subconjunctival injection of Angptl2-specific siRNA (n = 4 per group, *P < 0.01). (B, C) Surface areas of corneal hemangiogenesis (B) and lymphangiogenesis (C) were quantified (vascularized area/total corneal area). Corneal hemangiogenesis and lymphangiogenesis were markedly reduced in eyes receiving subconjunctival injections of Angptl2-specific siRNA compared to eyes treated with the control-siRNA or PBS on day 5 after the intrastromal suturing with 10-0 nylon (n = 4 per group). Error bars denote the mean ± SD (*P < 0.05).
activities in the inflamed cornea. This is strengthened further by the observation that the deletion of macrophages following the subconjunctival injection of clodronate liposomes nearly completely inhibited corneal hemangiogenic and lymphangiogenic responses in experimental models. In our study, the expressions of the macrophage marker F4/80 and the typical pro-angiogenic factor II-1β were increased significantly in the corneas of K14-Angptl2 mice compared to the expressions in their background strain (Fig. 2). It has been reported that the extravasation of monocyte-macrophage lineage cells from limbal vessels to corneal stroma is regulated through integrin α5β1, which is a functional receptor of Angptl2. Further, Angptl2 was shown to activate an inflammatory cascade in endothelial cells and promote their migration through integrin α5β1 receptor, thereby inducing the chemotaxis of monocytes and macrophages. Furthermore, integrin α5β1 signaling activated NF-κB cascade and accelerated various inflammatory cytokines, including IL-1α. Taken together, our results highlighted the functional role of Angptl2-integrin α5β1 interaction in corneal inflammation. Corroborating the results of a previous study by Muehler et al., who reported that integrin α5β1 is upregulated during corneal angiogenesis, and that its inhibition by antagonist caused prevention and regression of corneal NV, our study demonstrated a significant reduction in the expression of the F4/80 and II-1β in the corneas of Angptl2−/− mice compared to their background strains (Fig. 3). Interestingly, Dietrich et al. reported that corneal lymphatic vessels expressed α5 integrin and corneal lymphangiogenesis also was mediated through α5 integrin. Because monocyte-macrophage lineage cells participate in the neovascularization process, fewer F4/80-positive cells in the corneas of Angptl2−/− mice may, at least in part, explain the promotion mechanisms of inflammatory corneal hemangiogenesis and lymphangiogenesis by Angptl2.

Although the trigger of Angptl2 expression is hitherto unknown, earlier studies have shown that the expression of Angptl2 was stimulated by several pathologic conditions involving hypoxia and sunlight exposure. It has been reported that most contact lens-induced changes to the cornea are caused by hypoxia, which further leads to corneal NV in long-term contact lens users. Further, chronic UV irradiation was shown to be the causal factor of pterygium, which is characterized by generative ocular surface NV disorders. The animal model also showed that UV irradiation displayed corneal inflammatory neovascularization. Future studies focusing on the plausible role of Angptl2 in human ocular surface pathology are warranted.

In summary, our findings provided evidence that Angptl2 is upregulated in corneal inflammation, and highlight that Angptl2 is crucial to inflammatory hemangiogenesis and lymphangiogenesis in the corneas. Further, Angptl2 may represent a novel therapeutic target for preventing blindness.

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