Corneal Collagen Cross-Linking With Riboflavin and UVA Regulates Hemangiogenesis and Lymphangiogenesis in Rats

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PURPOSE. The purpose of this study was to determine whether corneal collagen crosslinking (CXL) inhibits hemangiogenesis and lymphangiogenesis during acute corneal inflammation in an in vivo rat model.

METHODS. Inflammatory corneal neovascularization was induced by suture placement into a rat cornea. At day 3 after suture, a CXL protocol using riboflavin and UVA was administered after mechanical epithelial debridement. Hemangiogenesis and lymphangiogenesis were analyzed morphometrically. CD45 and CD68 immunostaining evaluated corneal leucocyte and macrophage immune cell infiltration, respectively. A TUNEL assay detected stromal cell apoptosis. Quantitative RT-PCR analysis identified angiogenic and lymphangiogenic genes as well as proinflammatory cytokine expression. Western blot analysis characterized vascular endothelial cell CD31 and lymphatic vessel endothelial hyaluronan receptor (LYVE-1) protein expression.

RESULTS. CXL treatment significantly reduced corneal pathologic suture-induced hemangiogenesis and lymphangiogenesis 7 days after suture emplacement, but this procedure failed to affect hemangiogenesis and lymphangiogenesis 14 days after suture. Increased cell apoptosis and reduced CD45+ and CD68+ cell infiltration were evident in CXL-treated rats on days 7 and 14 after suture emplacement. CXL treatment significantly decreased angiogenic and lymphangiogenic mRNA expression levels and both CD31 and LYVE-1 protein expression levels, whereas it increased proinflammatory cytokine levels on day 7 after suture emplacement. However, on day 14 after corneal neovascularization, angiogenic and lymphangiogenic mRNA gene expression levels were upregulated along with hematic CD31 and lymphatic LYVE-1 protein expression.

CONCLUSIONS. CXL treatment only temporarily inhibits corneal inflammatory-associated hemangiogenesis and lymphangiogenesis in vivo. Such insight suggests that future studies are warranted to develop novel CXL strategies with longer-lasting effectiveness in attenuating hemmetic- and lymphatic-related corneal diseases.

Keywords: CXL, cornea, hemangiogenesis, lymphangiogenesis

Corneal avascularity and immune privilege are both necessary for preserving tissue transparency and normal vision. However, this “angiogenic and lymphangiogenic privilege” can be compromised by various inflammatory pathologic conditions induced by infection, inflammation, trauma, and hypoxia, leading to corneal opacification and reduced visual acuity. Another change associated with these different stresses can include matrix metalloproteinase upregulation, which results in enzymatic disruption of tissue integrity and stromal extracellular matrix remodeling, as well as capillary endothelial cell migration and proliferation. Corneal neovascularization is a more pervasive response to immune cell activation and infiltration than it is in the retina or choroid. Lymphatic vessels provide a conduit for the afferent limb of the immune response. Thus, corneal graft rejection after keratoplasty is a very likely outcome if hemangiogenesis, in particular lymphangiogenesis, is induced by any of the aforementioned stresses.

UVA exposure modulates systemic immunity by reducing the number of epidermal Langerhans cells, as well as upregulating mucosal immunoglobulin A levels in the intestine. Furthermore, short-term UVA exposure has been shown to downregulate proinflammatory and macrophage-releasing cytokine release by limbal epithelial cells and fibroblasts. UVA alleviates the pro(lymph) angiogenic milieu via downregulating macrophage-releasing cytokine levels.

Corneal collagen crosslinking (CXL) with riboflavin and UVA has become the preferred treatment modality to strengthen biomechanical stromal properties and halt keratoconus progression. There is considerable experimental evidence showing that this procedure has this outcome because its structural reorganizational effects increase resistance to proteolytic degradation by reducing corneal penetrance. Such changes occur due to increases in collagen fibrillar tortuosity. Other tissue compromising challenges for which performing the CXL procedure can provide a therapeutic benefit include (1) directly inactivating bacterial and fungi by damaging their DNA with UVA and riboflavin; (2) reducing inflammation by suppressing inflammatory and immune cell infiltration with...
riboflavin\textsuperscript{15}; and (3) reducing the likelihood of neovascularization.\textsuperscript{14} Nevertheless, there remains uncertainty as to how CXL provides a therapeutic effect in lessening the impact of corneal-related diseases on tissue function.

A recent study applied CXL in a murine model in which there were corneal, blood, and lymphatic vessels. This procedure caused blood vessels to regress 4 days after being irradiated with 5.4 J/cm\textsuperscript{2}.\textsuperscript{15} Such an effect was attributed to increased stromal stiffness impeding blood vessel invasiveness. However, the therapeutic effect of this procedure is questionable because the energy delivered is too high for being selective in the mouse. The safe upper limit in the mouse is much lower than that in the human because the mouse cornea is much thinner than its human counterpart.\textsuperscript{10} To clarify whether the CXL procedure can be used to selectively suppress neovascularization, the UVA dosage must not be higher than that used to selectively increase stromal biomechanical strength. Similarly, another study found that CXL regressed both preexisting blood and lymphatic vessels significantly via inducing apoptosis in vascular endothelial cells.\textsuperscript{17} Both studies determined the combined effect of CXL with UVA and riboflavin on mature corneal blood and lymphatic vessel infiltration. We evaluated the effects of this procedure on corneal lymphangiogenesis and hemangiogenesis with UVA doses that we previously identified as being safe and effective for use in the rat.

We describe here a safe and effective UVA dose in the CXL procedure\textsuperscript{18} that selectively inhibits rat corneal lymphangiogenesis and hemangiogenesis in vivo. Our results suggest that CXL therapy may be an early preclinical experimental approach to inhibit hemangiogenesis and lymphangiogenesis.

\section*{Materials and Methods}

\section*{Rat Model of Suture-Induced of Corneal Neovascularization}

All procedures were approved by the Animal Care and Ethics Committee of Wenzhou Medical University, Zhejiang, China, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eight- to 10-week-old male Sprague-Dawley rats (weight, 200 to 300 g, \(n = 205\)) were used. Suture-induced corneal neovascularization (SNV) was achieved by using a previously described method.\textsuperscript{19} In brief, animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (50 to 75 mg/kg) and xylazine hydrochloride (5 mg/kg), supplemented with topical anesthesia (0.5\% proparacaine hydrochloride, Alcaine eye drops; Alcon Laboratories, Inc., Fort Worth, TX, USA). Under an operating microscope, three single 10-0 nylon sutures were placed through the corneal stroma approximately 1.5 to 2 mm from the temporal limbus at 4, 8, and 12 o’clock positions in the right eye. The vessel response was examined every day using slit-lamp microscopy characterized by rapid new vessel corneal ingrowth between days 3 and 7 after suture. Day 3 was used to evaluate the effects of suture placement in subsequent experiments. Untreated eyes serve as a normal control (NC). Blood and lymphatic vessels were normally absent and ignored if present in areas undergoing suture-induced neovascularization and sampled for quantitative RT-PCR (qRT-PCR) analysis.

\section*{Animal Treatment}

On day 3 after suture emplacement, the right eyes underwent epithelial debridement in all rats. Sutures were left in place for 14 days and were then removed before qRT-PCR and Western blot assays. They were randomized to receive either CXL with UVA and riboflavin (SNV+UVA) group) or irradiation with UVA alone (SNV+UVA group) or no treatment for control (SNV group) (Fig. 1). The UVA irradiation alone corneas received 9 mW/cm\textsuperscript{2} UVA for 4 minutes (total energy dose, 2.16 J/cm\textsuperscript{2}) after epithelial removal. The CXL procedure delivered 9 mW/cm\textsuperscript{2} UVA for 4 minutes (total energy dose, 2.16 J/cm\textsuperscript{2}) after being instilled with isotonic 0.22\% riboflavin eye drops (10 mg riboflavin in 4.5 ml 20\% dextran T-500 solution; Peschke GmbH, Nürnberg, Germany) every 3 minutes for 30 minutes. All of the irradiation protocols were performed according to our previously determined safe and effective CXL protocols established for rat use.\textsuperscript{15} Following treatment, Tobramycin eye drops (Tobradex; Alcon Laboratories, Inc.) were administered to both groups.

\section*{Evaluation of Corneal Inflammation}

Following treatments, corneas were examined every day with a slit lamp. Inflammatory index values were assigned based on summing the individual scores of the different described parameters to evaluate inflammation on days 1, 3, 7, and 14.\textsuperscript{20} Inflammatory index values were assigned based on the following evaluation: ciliary hyperemia (absent, 0; present but extending less than 1 mm, 1; hyperemia extending between 1 and 2 mm, 2; present and extending more than 2 mm, 3); central corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present without visible pupil, 3); peripheral corneal edema (absent, 0; present with visible iris details 1; present without visible iris details, 2; present without visible iris, 3).

\section*{Corneal Neovascularization Assay}

Corneal neovascularization was observed on day 3, 7, 10, and 14 after suture emplacement, and three photographs of each cornea were taken using a digital camera (EOS 5200D; Canon, Tokyo, Japan) dissecting microscope at 16× magnification (10 corneas per group); 360° area of corneal new vessels was analyzed at the same magnification using Image J software (National Institutes of Health, Bethesda, MD, USA). The area between the innermost new vessel and the limbus was defined as the vascularized area. The degree of neovascularization was determined by dividing the vascularized area by the total corneal area (measured in pixels). This image analysis system was modified based on previous studies.\textsuperscript{21–23} Photographs were analyzed in a random order by two double-blinded investigators to minimize observer bias.

\section*{Morphologic Analysis of Lymphangiogenesis in Flat Mounts}

One and 2 weeks after the suturing, rats were euthanized, and their eyes were enucleated and fixed in 4\% paraformaldehyde. Five corneas in each group were used for flat mounts. Fixed tissues were stained overnight at 4°C with rabbit anti-mouse LYVE-1 antibody (1:100; sc-28190; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Tissues were washed three times...
and incubated with secondary goat anti-rabbit antibody (1:100; Abcam, Cambridge, MA, USA). All corneas were mounted with Vector Shield mounting medium (Vector Laboratories, Burlingame, CA, USA). LYVE-1 expression was photographed with a laser scanning confocal microscope (LSM710; Zeiss with krypton-argon and He-Ne laser; Carl Zeiss Meditec, Sartrouville, Germany). The area covered by lymphatic vessels was determined using ImageJ software (National Institutes of Health).

**Immunofluorescent Staining**

Five eyes from each group were excised andembedded in optimal cutting temperature compound and frozen in liquid nitrogen and then cut into 8-μm-thick sections. To observe inflammatory cells in detail, sections were stained using leukocyte marker anti-CD45 antibody (dilution 1:100; ab33984; Abcam) and CD68 (dilution 1:200, ab125212; Abcam) overnight. Alexa Fluor 488–conjugated secondary antibody, goat anti-mouse IgG (ab96879; Abcam; 1:300) was applied at room temperature for 1 hour. 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen; 1:1000). DAPI was added for 5 minutes. Expression of CD45 and CD68 was photographed with a laser scanning confocal microscope (LSM710; Zeiss with krypton-argon and He-Ne laser; Carl Zeiss Meditec) at 200× magnification.

**TUNEL Assay**

To detect keratocyte apoptosis, DNA fragmentation was measured with the fluorescence-based TUNEL assay (In Situ Cell Death Detection Kit; Roche Applied Science, Indianapolis, IN, USA) and was measured in frozen sections according to the manufacturer’s recommendations. Sections were counterstained with DAPI (Invitrogen; 1:1000).

**Cell Counting**

As previously described, for the TUNEL assay and immunofluorescent staining for CD45 and CD68 cells, all stained cells were counted in five nonoverlapping areas (0.1 × 0.1 mm²) that extended from the anterior stromal surface to the posterior stroma.24,25 The columns used to count positive cells were randomly selected from the central cornea or peripheral cornea at the edge of each specimen. In each cornea, the mean numbers of positive cells were calculated by counting the number of cells in five nonoverlapping areas of three individual corneal sections, separated from each other by at least 0.2 mm to avoid overlap.

**Table.** Rat Primer Sequences Used for qRT-PCR

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<th>Gene</th>
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**RNA Isolation and qRT-PCR**

Total RNA from two corneas in each group was extracted (RNasey mini kit [50×]; Qiagen, Crawley, UK) according to the manufacturer’s instructions. Each experiment was repeated three times with a total of six corneal samples in each group. The RNA concentration and integrity were measured based on its optical density at the 260/280-nm ratio and stored at −80°C before use. qRT-PCR was performed on Biosystems 7500 Real-Time PCR System (Applied Biosystems, Paisley, UK) using the Power SYBR Green PCR Master Mix (Applied Biosystems). The specific gene products were amplified using designated primer pairs listed in the Table. Results were analyzed by the comparative threshold cycle (Ct) method, normalized with GAPDH as an endogenous reference and calibrated against the SNV group.

**Western Blot Assay**

Western blot analysis evaluated CD31 (endothelial cell marker) and LYVE-1 protein expression levels on days 7 and 14 after the treatment. Three groups were included to compare across groups and experimental conditions: NC cornea, SNV cornea, SNV cornea with CXL treatment (i.e., SNV+CXL), or SNV cornea with UVA irradiation (i.e., SNV+UVA). Nine corneas were pooled from each group. Western blot assays were performed as previously described.26 Total proteins were extracted from rat corneas with cold RIPA buffer containing a proteinase inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Equal amounts of rat corneal protein were subjected to electrophoresis on 10% SDS-PAGE. The standard Western blot assay protocol was applied. Specific primary antibodies (CD31, LYVE-1, and GAPDH) and secondary antibodies (horseradish peroxidase–conjugated goat anti-rabbit IgG) were used. Finally, the specific bands were detected by an enhanced chemiluminescence reagent. The images were recorded and assessed by a gel imaging system (Molecular Imager ChemiDoc XRS; Bio-Rad, Hercules, CA, USA). Each quantified band was normalized to the corresponding GAPDH level.

**Statistical Analysis**

Data are expressed as mean ± SEM where appropriate. The Mann-Whitney U test, Kruskal-Wallis test, and 1-way ANOVA were performed for multiple comparisons among groups. SPSS 19.0 software (IBM SPSS Statistics; IBM Corporation, Chicago, IL, USA) was used for analyses. P values were determined. P < 0.05 was considered statistically significant.
RESULTS

CXL Suppresses Suture-Induced Corneal Hemangiogenesis

Suturing induced very apparent inflammation, new vessel, and edema, as well as opacification, which persisted up to day 14 (Figs. 2A–2I). On day 7 after suture, SNV+UVA-treated corneas had significantly more neovascularization, whereas SNV+CXL-treated corneas had significantly less neovascularization than that in the SNV untreated corneas ($P < 0.01$). The vascularized area of the SNV corneas was $45.3 \pm 5.6\%$ on day 7 and $65.2 \pm 4.93\%$ on day 14, respectively. The vascularized area of the SNV+UVA corneas was $61.3 \pm 5.3\%$ on day 7 and $82.7 \pm 5.9\%$ on day 14, respectively. The vascularized area of the SNV+CXL corneas was $33.6 \pm 3.1\%$ on day 7 and $64.2 \pm 5.32\%$ on day 14, respectively (Fig. 2J). Inflammatory index was significantly increased in the SNV+UVA group from day 10 to 14 ($P < 0.01$). However, the mean changes in inflammatory index on days 7 and 14 did not differ significantly between the SNV+CXL and SNV groups (Fig. 2K).

CXL Suppresses Suture-Induced Corneal Lymphangiogenesis

Lymphatic vessel endothelial hyaluronan receptor-1–stained corneal flat mounts had less lymphangiogenesis in the SNV+CXL-treated corneas on day 7 compared with those in the SNV untreated corneas (i.e., lymphatic coverage area expressed as a percentage of whole-mounted cornea; Figs. 3A–3H). The lymphatic coverage area of the SNV corneas was $21.1 \pm 2.8\%$ on day 7 and $27.1 \pm 1.9\%$ on day 14, respectively. In the SNV+UVA corneas, it was $32.5 \pm 3.5\%$ on day 7 and $51.6 \pm 3.6\%$ on day 14, respectively. In the SNV+CXL corneas, it was $11.4 \pm 1.8\%$ on day 7 and $26.6 \pm 3.8\%$ on day 14, respectively. However, lymphangiogenesis in SNV+CXL-treated and SNV corneas on day 14 no longer significantly differed between the two groups ($P < 0.01$; Fig. 3I).

Inflammatory Cell Infiltration

To evaluate whether the CXL procedure reduces lymphangiogenesis, immune cell activation and infiltration were evaluated in...
the SNV untreated and SNV+UVA- and SNV+CXL-induced groups. Accordingly, we probed for CD45$^+$ and CD68 immunostaining to evaluate leukocyte and macrophage infiltration, respectively (Figs. 4A–4P, 5A–5P). Both patterns were almost exclusively limited to the peripheral cornea and the limbus in the NC group. On day 7 after suture emplacement, CD45 and CD68 expression was significantly more in the SNV+UVA-treated group, whereas it was less in SNV+CXL-treated group. This decline was indicative of fewer leukocytes and macrophages in the SNV+CXL-treated corneas than in the SNV untreated cornea ($P < 0.05$). However, their relative expression levels were not significantly different between these two groups on day 14 (Figs. 4Q, 5Q).

**Apoptosis**

TUNEL-positive cells were localized in the surface cells of the NC group. In the SNV+CXL group, TUNEL-positive cells dramatically increased in the stroma relative to those in the SNV and SNV+UVA groups on days 7 and 14 ($P < 0.05$; Figs. 6A–6D).

**CXL Treatment Decreases VCAM-1, CD31, VEGFR2, VEGFR3, VEGFC, and LYVE-1 Gene Expression Levels in the SNV-Induced Cornea**

To determine whether CXL inhibits corneal hem- and lymphangiogenesis, we used real-time PCR analysis to assess the changes in expression of representative angiogenic and lymphangiogenic genes. This assessment included evaluating VCAM-1, CD31, VEGFR2, VEGFR3, VEGFC, and LYVE-1 levels on days 7 and 14 in the corneas following suture emplacement. As shown in Figures 7A–7F, VCAM-1, CD31, VEGFR2, and VEGFR3 expression levels in the SNV+CXL corneas were significantly downregulated compared with the SNV untreated group on day 7 after suture emplacement ($P < 0.05$ and $P < 0.01$, respectively). VEGFC and LYVE-1 expression levels in the SNV+CXL corneas were lower than the SNV corneas. However, the expression levels of VCAM-1, CD31, VEGFR2, VEGFR3, and LYVE-1 in the SNV+CXL corneas increased significantly compared with the SNV-untreated corneas 14 days after suture emplacement ($P < 0.05$ and $P < 0.01$, respectively). At both time points, those gene expression levels in SNV+UVA-treated corneas were significantly upregulated compared with the SNV group ($P < 0.05$). This decline indicates that CXL may temporarily inhibit corneal hem- and lymphangiogenesis. Low CD31 and LYVE-1 protein expression levels were found in the unsutured rat cornea, whereas their levels increased considerably after suture emplacement. Similarly, in SNV+UVA group, CD31 and LYVE-1 protein levels increased compared with the SNV group on days 7 and 14 after suture treatment (Figs. 8A–8C). The CD31 and LYVE-1 levels were reduced in the SNV+CXL group on day 7 compared with those in the SNV group ($P < 0.01$), whereas on day 14 after suture, these expression levels were significantly higher compared with those in the SNV+CXL group and those in the SNV group ($P < 0.05$ and $P < 0.01$, respectively; Figs. 8D–8F).

**Proinflammatory Cytokine Gene Expression Levels in Cornea**

qRT-PCR analyzed changes in the proinflammatory factors TNF-$
\alpha$, IFN-$\gamma$, monocyte chemotactic protein 1 (MCP-1), and matrix metalloproteinase-9 (MMP-9). The TNF-$\alpha$ mRNA expression levels in the SNV+CXL corneas were significantly lower compared with the SNV corneas on days 7, whereas the levels were significantly higher in the SNV+UVA corneas ($P < 0.05$ and $P < 0.01$, respectively). IFN-$\gamma$, MCP-1, and MMP-9 mRNA expression levels increased significantly in the SNV+UVA and SNV+CXL groups on days 7 and 14 after suture ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively; Figs. 7G–7J).

**DISCUSSION**

We show here for the first time that CXL temporarily suppresses suture-induced corneal hemangiogenesis and lymphangiogenesis in an in vivo rat model. With the rat-specific CXL protocol, suture-induced increases in corneal inflammation were only transiently suppressed during transient rises in blood and lymphatic vessels up to 7 days. However, after 14 days, this decline underwent a complete reversal. The correspondence between the time-dependent immunostaining pattern changes and the variations in gene expression levels of the markers of hemangiogenesis and lymphangiogenesis confirm that CXL treatment only temporarily suppressed these two suture-induced responses in rat corneas.

UVA exposure can induce mutagenesis arising from DNA and protein damage. Such alterations can induce cancer, and even dysregulated immunity. Previous work has shown that the prolymphangiogenic effect on human limbal epithelial cells and proangiogenic effect on human limbal fibroblast cells were eliminated after short-term UVA irradiation. Moreover, it was suggested that its antilymphangiogenic effect at the cell level is in part induced by short-term UVA irradiation. The impact has thus far been poorly studied of exposing corneas in vivo to UVA. Our results clearly show that UVA irradiation alone damages the epithelium and stroma, which is followed...
by inflammatory and (lymph)angiogenic effects in these tissue layers.

Riboflavin is a naturally occurring compound and an essential human nutrient. It reduces the lipopolysaccharide-induced synthesis of the inflammatory cytokines TNF-α, IL-1, and IL-6. Riboflavin acts as a useful photosensitizer to inactivate pathogens in plasma, platelet, and red cell products because of its nucleic acid specificity and its limited tendency...
FIGURE 5. (A–P) CXL inhibits CD68 marked macrophage infiltration (red) after corneal suture at days 7 and 14. Immunostaining analysis for CD68 of the corneal stroma in normal control (A, B, I, M), SNV corneas (C, D, J, N), SNV+UVA corneas (E, F, K, O), and SNV+CXL-treated corneas (G, H, L, P). (Q) Quantification of infiltrating CD68 macrophages per field. Significant difference was evident between CD68-positive macrophage infiltration in SNV, SNV+UVA corneas, and SNV+CXL-treated corneas at day 7 (*P < 0.05, **P < 0.01 versus SNV group; data are shown as mean ± SEM).
toward indiscriminate oxidation. UV light activated riboflavin oxidizes guanine in nucleic acids, preventing replication of the pathogen’s genome. Riboflavin/UVA combination treatment may damage nucleic acids by direct electron transfer, production of singlet oxygen, and generation of hydrogen peroxide leading to formation of hydroxyl radicals.

Blood and lymphatic vessels start growing outwards from the limbal vascular arcade as early as day 2 after an inflammatory insult. Hem- and lymphvascularization peaked approximately day 14 after corneal suture emplacement. In another rat alkali injury model, it was shown that the predominantly lymphangiogenic factor, VEGFC, was dramatically upregulated at day 3 after a corneal alkali injury and that corneal lymphangiogenesis began to develop at this point. For this reason, we chose to perform the CXL procedure on day 3 and evaluate its effects on hemangiogenesis and lymphangiogenesis on days 7 and 14.

LYVE-1, a hyaluronan receptor related to CD44, is a definitive marker of lymphatic structure and function. The leukocyte adhesion molecule VCAM-1 facilitates leukocyte adhesion to activated vascular endothelial cells. The VEGF family members are generally considered as the main regulators of lymphangiogenesis through binding to their cognate receptors, the VEGFRs. Both blood endothelial cells and lymphatic endothelial cells express VEGFR2. VEGFC is the main prolymphangiogenic factor that acts through activating its cognate receptor, VEGFR3, expressed on lymphatic endothelial cells.

qRT-PCR analysis shows that all of the detected representative proangiogenic and prolymphangiogenic cytokines were temporarily downregulated following performing the CXL protocol. The downregulated proangiogenic and prolymphangiogenic cytokines include VCAM-1, CD31, VEGFR2, VEGFR3, VEGFC, and LYVE-1. Western blot analysis of CD31 and LYVE-1 expression showed that their levels underwent a small but significant decrease in the CXL-treated corneas on day 7 after suture emplacement. It is noteworthy that the CD31 and LYVE-1 gene expression levels declined in parallel with one another in the SNV+CXL group relative to those in the SNV group. It is likely such declines in these master regulators of angiogenesis and lymphangiogenesis mirrored reductions in their protein expression levels.

Consistent with the increases in the corneal vascularized area after day 7, the proangiogenic and prolymphangiogenic cytokine expression levels also rose after a temporary decline in neovascularization. A previous study reported that significant changes occurred in the levels of cytokines, chemokines, enzymes, and growth factors in tear samples after CXL in keratoconus corneas. At day 4 after performing CXL, IL-6, and IL-8 expression levels increased compared with their levels prior to this treatment. Another finding showed that CXL triggers transient increases for 5 hours in basic fibroblast growth factor (bFGF) release by isolated keratocous kerocytes.

Blood and lymphatic vessel formation are regulated by local growth factors and cytokines. The standard CXL procedure used in the clinic involves removing the central epithelium. Stromal edema typically develops during the first few weeks or months after surgery combined with stromal keratocyte apoptosis. It is estimated that corneal re-epithelialization requires at least 4 days for completion and up to 3e months for qualitative recovery of the normal epithelial cell mosaic in humans. As it was shown before in the cornea, persistent inflammatory response, epithelial damage, or traumatic injury can disrupt the balance between angiogenic and antiangiogenic factors and tilt the balance toward promoting angiogenesis. These findings may explain, at least in part, why declines in hemangiogenesis and lymphangiogenesis were transient rather than sustained following CXL treatment. However, the mechanisms underlying corneal lymphangiogenesis are still not clear. Further study is needed to clarify how CXL alters regulation of hemangiogenesis and lymphangiogenesis.

We measured the expression of CD45 (a receptor expressed broadly by bone marrow–derived leukocytes) and found it significantly decreased following CXL treatment 7 days after suture emplacement. This decline is in agreement with a corresponding decline in CD68-labeled cell infiltration following performing the CXL procedure. Previous study demonstrated that CXL induced anterior stromal collagen fiber compaction. It is possible that this tightening of collagenous architecture provides a defense mechanism suppressing inflammatory cell infiltration. Even though CXL may be used to prevent or reduce inflammatory cell infiltration and immunologic reactions, all of its effects on these responses to suture emplacement underwent a full reversal after 14 days. This temporary therapeutic effect warrants further study to hopefully identify a more long-lasting reversal in experimental animal models before it can used in a clinical setting.

Severe injury activates inflammatory cells, which in turn elicit chemotaxtrant release amplifying immune cell infiltration into a wound site. During this process, keratocytes and other stromal resident cell types upregulate VEGF expression levels, which are key stimulators of neovascularization. To investigate the impact of UVA and CXL treatment on its angiogenic effect in corneas, inflammatory and macrophage-recruiting cytokines were evaluated. Analysis by PCR showed that both UVA irradiation alone and CXL upregulated proinflammatory IFN-γ, MCP-1, and MMP-9 cytokine expression levels. Conversely, TNF-α increased in SNV/UVA corneas only, whereas it was downregulated in SNV+CXL corneas.
Figure 7. Quantitative real-time PCR results show significant declines in CD31 (A), VCAM-1 (B), VEGFR2 (C), and VEGFR3 (D) in the SNV+CXL-treated corneas on day 7 after suture, whereas all of these angiogenic and lymphangiogenic cytokine gene expression levels significantly increased by day 14. The expression levels of VEGFC (E) and LYVE-1 (F) in the SNV+CXL corneas were lower than the SNV corneas on day 7, whereas both gene expression levels increased by day 14. All of gene expression levels in SNV+UVA-treated corneas were significantly upregulated compared to the SNV group on days 7 and 14 after suture. Significant increases in IFN-γ (G), MCP-1 (I), and MMP-9 (J) in the SNV+UVA-treated and SNV+CXL-treated corneas on days 7 and 14 after suture, whereas TNF-α (H) expression levels decreased in the SNV+CXL-treated corneas.
Moreover, CXL treatment of inflamed tissue caused in the exposed area massive keratocyte apoptosis and decellularization. Accordingly, the transient losses we observed in resident stromal cell populations may account for why the declines in inflammation were only temporary. Taken together, these observations may explain the recurrence of hem- and lymphangiogenesis 14 days after suture emplacement.

It has been confirmed that no obvious endothelial cell damage was noted after CXL using 9 mW/cm² for 4 minutes in the in vivo rat model. We observed that the corneas in the SNV+CXL group appeared transparent at 14 days after suture emplacement. In a histopathologic evaluation of neovascularization in human corneas, new vessels were usually associated with edema and inflammatory cell infiltration. These vessels were commonly located in the upper and middle third of the stroma. Using the standard CXL method, the cytotoxic effect was observable down to a depth of 300 μm. This suggests that CXL therapy would be an early preclinical experimental approach to inhibit hemangiogenesis and lymphangiogenesis.

To ensure that CXL therapy is effective in selectively suppressing neovascularization in thin corneas, the classical CXL operation sequence requires further modification.

In conclusion, we demonstrate that the rat specific CXL procedure only temporarily inhibits pathologic corneal hemangiogenesis and lymphangiogenesis. Additional studies are required to determine whether this procedure can be used to decrease ocular inflammation prior to performing keratoplasty by suppressing the extension and infiltration of both endothelial cell tubes and lymphatic vessels into an inflamed cornea.

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