The Role of LOX and LOXL2 in Scar Formation After Glaucoma Surgery

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PURPOSE. The aim of this study was to elucidate the role of lysyl oxidase (LOX) and lysyl oxidase like (LOXL) 2 in pathologic wound healing after glaucoma surgery. We therefore investigated the expression of LOX and LOXL2 and evaluated the therapeutic potential of anti-LOX (GS-639556, formerly M64) and anti-LOXL2 (GS-607601, formerly AB0023) antibodies in a rabbit model of glaucoma trabeculectomy.

METHODS. Ocular expression of LOX and LOXL2 was investigated by immunohistologic staining at different time points after trabeculectomy. Treatment with GS-639556 or GS-607601 was initiated in rabbits immediately after trabeculectomy by giving both intracameral and subconjunctival injections. Thereafter, the antibodies were given twice a week subconjunctivally until day 30 after surgery (day of euthanization). Treatment outcome was studied by clinical investigation of the bleb and by immunohistochemical analysis of angiogenesis, inflammation, and collagen deposition.

RESULTS. LOX and LOXL2 were both upregulated in Tenon’s capsule and the conjunctiva after glaucoma surgery. Repeated administration of LOX- or LOXL2-targeting monoclonal antibodies increased bleb area and bleb survival. Analyses of immunohistochemical stainings showed that both antibodies significantly decreased fibrosis, whereas the anti-LOXL2 antibody also significantly reduced blood vessel density and inflammation.

CONCLUSIONS. Targeting LOXL2 with an inhibitory monoclonal antibody (GS-607601) reduced pathologic angiogenesis, inflammation, and fibrosis. These results suggest that LOXL2 could be an appealing target for treatment of scar formation after glaucoma surgery, and point to the potential therapeutic benefits of simtuzumab, a humanized monoclonal antibody derived from GS-607601.

Keywords: lysyl oxidase, fibrosis, inflammation, angiogenesis

The lysyl oxidase family is an important class of extracellular matrix (ECM) crosslinking enzymes. Lysyl oxidase (LOX) is the best characterized member of this family and is known as an amine oxidase that catalyzes crosslinking of collagen and elastin in the ECM via generation of aldehydes on lysine residues.¹,² Several additional lysyl oxidase family members have been identified (LOXL1, -2, -3 and -4) and are fully functional.³ LOX and LOXL proteins are expressed during development and maintenance in the skin, aorta, heart, lung, liver, cartilage, kidney, stomach, small intestine, colon, ovaries, testis, and brain of the mouse.⁴ Given their roles in disease-associated modification of the ECM,¹,² LOX family enzymes have also been described as critical contributors to the development of a variety of fibrotic-related diseases, including liver and lung fibrosis,⁵ tumor progression,⁵,⁶ and neurodegenerative and cardiovascular diseases.⁷-⁹ Targeting LOXL2 with an inhibitory monoclonal antibody⁹ has been shown to be efficacious in rodent models of cancer, as well as in liver and lung fibrosis models.⁵ Inhibition of LOXL2 resulted in a marked reduction in activated fibroblasts and endothelial cells, decreased production of growth factors and cytokines, and decreased TGF-β pathway signaling.⁵ In healthy ocular tissues, lysyl oxidase activity is present in the vitreous, iris, ciliary body, lens, choroid, retinal pigment epithelium, and retina.² Although a majority of blinding ocular diseases are associated with a disruption of the tissue architecture in the eye caused by vascular leakage and fibrosis,¹⁰ little information is available about LOXL involvement in ocular diseases. The vitreous levels of lysyl oxidase activity were significantly decreased in proliferative diabetic retinopathy and rhegmatogenous retinal detachment. These reduced lysyl oxidase levels might be associated with an inadequate cross-linking that causes ECM changes, known to be present during these diseases.² An indirect effect of LOX in corneal wound healing is suggested by Schultz et al., who showed that administration of TGF-β on human ocular fibroblasts could increase LOX expression as well as synthesis of ECM components, resulting in ECM remodeling.¹¹ Recent studies showed that polymorphisms of LOXL1 are associated with a significantly increased risk of exfoliation glaucoma,¹² suggesting a potential role for the LOX family in the pathogenesis of this disease. Indeed, a very recent study showed that LOX expression was
upregulated in trabecular meshwork (TM) cells derived from glaucoma patients. This suggests that increased LOX activity might be responsible for an increased aqueous humor outflow resistance by inducing deposition of ECM material within the TM. Progressive fibrosis is not only associated with the pathogenesis of glaucoma, but can also be a consequence of surgical treatment to lower the IOP. Surgical failure is indeed characterized by an excessive postoperative wound healing response with subsequent scarring. The role of LOXL in the process of wound healing after glaucoma surgery, however, is still unknown.

The first aim of this study was to characterize the expression of LOX and LOXL2 in pathologic wound healing after glaucoma filtration surgery. Next, we investigated the therapeutic anti-fibrotic potential of anti-LOX (GS-639556) and anti-LOXL2 (GS-607601) antibodies in a rabbit model of glaucoma filtration surgery. In addition, the possible anti-angiogenic and anti-inflammatory effects of both antibodies were analyzed.

**Materials and Methods**

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Institutional Animal Care and Research Advisory Committee of the Katholieke Universiteit (KU) Leuven approved all experimental animal procedures.

**Rabbit Model of Filtration Surgery**

Female New Zealand rabbits (animal facility of KU Leuven), 12 to 14 weeks of age and weighing 2 to 3 kg, were used. Before surgery, the IOP was measured in both eyes with a tonometer (Tono-Pen; Medtronic Solan, Jacksonville, FL) under 4 mg/mL oxybuprocaine hydrochloride topical anesthesia (Unicaine; Thea Pharma, Schaffhausen, Switzerland). Three recordings per eye were averaged. General anesthesia was induced by intramuscular injection of 1.2 mL ketamine (Ketalar, 50 mg/mL; Pfizer, Ann Arbor, MI) and 0.8 mL xylazine (Rompun, 2%; Bayer Health Care, Pittsburgh, PA). Surgery was performed on both eyes, using a technique previously described. In a first experiment, four rabbits (8 eyes) were used to determine LOX and LOXL2 localization on days 3, 8, 14, and 30 after surgery by immunohistochemistry. In a second experiment of 12 rabbits, six rabbits were treated with the anti-LOX antibody (GS-639556, formerly M64; Gilead Sciences, Foster City, CA), whereas the other six rabbits were treated with the anti-LOXL2 antibody (GS-607601, formerly AB0023; Gilead Sciences), immediately after surgery. In all rabbits, one eye was injected with the antibody and the contralateral eye was used as a control and received an injection of PBS. For each eye, 200 μL (0.6 mg) were injected in the anterior chamber (AC) and 100 μL (0.3 mg) were injected subconjunctivally (SC) into the filtration bleb immediately after surgery. Thereafter, repeated injections (0.3 mg/injection) were given twice a week subconjunctivally until day 30 after trabeculectomy (day of euthanization). Rabbits were clinically examined on day 1 after surgery and then every 2 days until they were killed. IOP bleb area (width × length) and height were measured under topical anesthesia using calipers. Bleb survival was taken as the endpoint of the study; bleb failure was defined as the appearance of a scarred and flat bleb. The examiner of the rabbits was masked to the treatment allocation.

**Immunohistochemistry**

On the day of euthanization, rabbits were killed using a lethal intravenous injection of sedative. Both eyes were enucleated: a superior piece of the bleb (fibrotic conjunctiva) and an inferior part of the conjunctiva and Tenon’s capsule (non-fibrotic) were collected. The tissues were fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Serial sections (7 μm) were cut and subjected to different (immuno) histochemical stainings. Hematoxylin and Eosin (H&E; Merck, Darmstadt, Germany) staining was performed to identify the first serial section to locate the bleb. To investigate LOX and LOXL2 expression at the protein level, immunohistologic stainings were performed. Rabbit sections were incubated overnight at room temperature with a monoclonal mouse anti-human LOX (1:1000) or LOXL2 antibody (1:200). Inflammation was analyzed by anti-CD45 staining and anti-CD31 staining was performed to identify the blood vessels. The samples were incubated overnight with mouse anti-rabbit CD45 antibody (1:100, MCA808; AbDserotec, Oxford, UK) or mouse anti-human CD31 antibody (1:200, M0825; Dakocyto-mation, Copenhagen, Denmark), respectively. The following day, all sections were incubated with peroxidase-labeled rabbit anti-mouse secondary antibodies and tyramide signal amplification (Renaissance TSA Indirect, NEL700; PerkinElmer, Waltham, MA) with diaminobenzidine (DAB, 32750; Sigma-Aldrich, St. Louis, MO) as a chromogen. Deposition of collagen was analyzed in both groups by Sirius Red staining.

**Imaging and Analysis**

Images were obtained using a microscope (Leica Microsystems, Wetzlar, Germany) with a digital camera (Axioimac Mr5; Carl Zeiss, Oberkochen, Germany) at a magnification of 20× and a resolution of 2584 × 1956 pixels. Morphometric analyses were performed using commercial software (Axiovision; Carl Zeiss). The density of blood vessels was determined by calculating the CD31-positive area (area of neovascularization) as a proportion of the total wound area of the bleb. The density of leukocytes was quantified by counting the CD45-positive cells, expressed in number per millimeter squared of the total area. LOX and LOXL2 expression was quantified by calculating the LOXL2 positive area as a percentage of the total area. Fibrosis was evaluated using Sirius Red staining and was determined by measuring the percentage of the area of mature collagen fibers to the total wound area. Polarized light was used to distinguish mature from immature collagen fibers. Mature type I collagen fibers appear bright yellow or orange, whereas immature collagen fibers appear green.

**Expression of Different Wound Healing–Related Factors**

Since it is known that different factors in the aqueous humor can influence the process of ocular wound healing, the expression of a variety of such molecules was measured by multi-analyte bead-based immunoassay. Rabbit aqueous humor samples (200 μL) were obtained from rabbits treated with either antibody, at 30 days after surgery. All samples were immediately transferred to and stored at −80°C until analysis. Samples were analyzed by Myriad RBM (Austin, TX) with their Human Inflammation MAP 1.0 panel, whereby levels of various markers including IL-1α/β, IL-1 receptor antagonist (IL-1ra), matrix metalloproteinase (MMP)-3, and Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) were determined.

**Statistical Analysis**

All histologic data were analyzed using the Student’s t-test for independent samples. Data at individual time points were analyzed using mixed model analysis for repeated measures and overall P values were calculated (GraphPad
Prism 5; GraphPad Software, San Diego, CA). Kaplan-Meier survival analysis was performed for bleb failure using the logrank test (GraphPad Prism 5). Spearman correlation coefficient ($r$) was calculated to evaluate the strength of association between expression of wound healing-related factors and the bleb area on day 30 (GraphPad Prism 5). $P$ values less than or equal to 0.05 were considered to be statistically significant. Data are represented as mean ± SEM.

**RESULTS**

**Ocular Expression of LOX and LOXL2**

To investigate the localization and the expression of LOX and LOXL2 proteins in the rabbit eye, immunohistochemical staining was performed at different time points after glaucoma filtration surgery in rabbits (*$P < 0.05$).

**Figure 1.** Quantification of the level of LOX and LOXL2 in Tenon’s capsule and conjunctiva. Images show representative pictures of the immunostainings for LOX (A) and LOXL2 (B) on day 30 after surgery in the superior conjunctiva (conj) and Tenon. Both LOX (C) and LOXL2 (D) proteins were upregulated in the fibrotic samples as compared with the nonfibrotic tissues (control) on different time points after glaucoma filtration surgery in rabbits (*$P < 0.05$).

**Figure 2.** Macroscopic postoperative photographs of rabbit eyes after surgery. Macroscopic photographs of rabbit eyes at day 30, after repeated anti-LOX (B) or anti-LOXL2 (D) antibody injections show surviving blebs that remain diffusely elevated compared with their respective controls (A, C).
LOX and LOX2 were both upregulated in Tenon’s capsule and conjunctiva after trabeculectomy. Importantly, since LOX and LOXL2 levels were upregulated as early as day 3 after glaucoma surgery, both proteins could play an important role in the early stages of postoperative wound healing.

**Effect of GS-639556 and GS-607601 in a Rabbit Trabeculectomy Model**

To determine whether inhibition of LOX or LOXL2 impacted glaucoma surgery outcome in vivo, antibodies targeting each protein were assessed for efficacy in preservation of bleb area and height in a surgical rabbit trabeculectomy model. One group of rabbits received repeated injections of the anti-LOX antibody (GS-639556; 0.6 mg AC and 0.3 mg SC immediately after surgery, 0.3 mg twice a week thereafter). Another group of rabbits was treated with repeated injections of the anti-LOXL2 antibody (GS-607601; 0.6 mg AC and 0.3 mg SC immediately after surgery, 0.3 mg SC twice a week thereafter).

Bleb area, height, and survival were analyzed as a measure of filtration surgery outcome. The animals were killed at day 30 after trabeculectomy.

Figure 2 shows the typical appearance of the bleb after treatment on postoperative day 30. GS-639556 and GS-607601 administration were associated with a large bleb (Figs. 2B, 2D, respectively) compared with a flat and scarred bleb in their respective control groups (Figs. 2A, 2C). Treatment with GS-
639556 resulted in a postsurgical increase in bleb area \( (N = 6; P < 0.0001; \text{Fig. 3A}) \) and bleb height \( (N = 6; P < 0.0001; \text{Fig. 3B}) \) from day 17 onwards. Repeated injections of GS-639556 significantly prolonged bleb survival after filtration surgery, compared with control, as shown in the Kaplan-Meier survival curve \( (N = 6; P = 0.0007; \text{Fig. 3C}) \). All blebs had failed in the control group by day 23, whereas all blebs survived until day 30 \( (\text{day of euthanization}) \) in the GS-639556 group. There was no evidence that inhibition of LOXL2 affected the IOP, as the pressure in both groups remained similar over a period of 30 days \( (P = \text{NS}; \text{data not shown}) \). The immunohistochemical stainings on day 30 showed a significant reduction of 21\% in collagen deposition in the bleb of the treated eyes compared with control \( (N = 6; P = 0.0009; \text{Figs. 4C, 4G}) \). There were no significant differences in blood vessel density and inflammation within the treated and control eyes \( (N = 6; \text{Figs. 4A, 4B, 4G}) \). In the group treated with GS-607601, antibody administration significantly increased bleb area \( (N = 6; P < 0.0001; \text{Fig. 3D}) \) and bleb height \( (N = 6; P < 0.0001; \text{Fig. 3E}) \) from day 19 onwards. Anti-LOXL2 antibody prolonged bleb survival in treated eyes compared with control eyes, as shown in the Kaplan-Meier survival curve \( (N = 6; P = 0.0005; \text{Fig. 3F}) \). All blebs had failed in the control group by day 23, whereas 83\% of the blebs survived until day 30 in the GS-607601 treated group \( (\text{one of the 6 blebs failed at day 27}) \). Thirty days after surgery, immunohistochemical stainings showed a significant reduction of 44\% in blood vessel density \( (N = 6; P = 0.01; \text{Figs. 4D, 4G}) \), 32\% decrease in inflammation \( (N = 6; P = 0.01; \text{Figs. 4E, 4G}) \), and 16\% reduction of collagen deposition \( (N = 6; P = 0.01; \text{Figs. 4F, 4G}) \) in the blebs of the treated eyes compared with controls. Of note, in both groups the inferior conjunctiva showed no difference in blood vessel density, inflammation, or collagen deposition in the treated and control eyes \( (P < 0.05) \). (G) The images show representative pictures of immunostainings of eyes treated with the anti-LOX or anti-LOXL2 antibody or control eyes, at 30 days after surgery (magnification of ×20).

**Figure 4.** Neovascularization, inflammation, and collagen deposition in rabbit eyes. The density of blood vessels and leukocytes was determined by calculating the CD31-positive area and by counting the CD45-positive cells, respectively, as a proportion of the total wound area of the bleb. Fibrosis was stained by Sirius Red and was determined by measuring the percentage of the area of mature collagen fibers in the total wound area of the bleb using polarized light. (A–C) Treatment with anti-LOX antibody (GS-639556) showed no significant differences in neovascularization and inflammation compared with PBS-treated eyes. The process of collagen deposition was significantly decreased (by 21\%) after inhibition of LOX at the filtration site on day 30 as compared with the control eyes. (D–F) Treatment of anti-LOXL2 (GS-607601) showed significant reductions of 44\% in neovascularization, 32\% in inflammation, and 16\% in collagen deposition in the bleb of the treated eyes compared with controls. In both groups the inferior conjunctiva showed no difference in blood vessel density, inflammation, or collagen deposition in the treated and control eyes \( (P < 0.05) \).
whereas anti-LOXL2 antibody significantly reduced blood vessel density, inflammation, and collagen deposition in the blebs at postoperative day 30.

**Upregulation of Different Wound Healing–Related Factors in Aqueous Humor**

On day 30, aqueous humor was collected from both rabbit eyes after treatment. The expression level of different factors related to wound healing in aqueous humor was analyzed by multi-analyte bead-based immunoassay. Anti-LOX treatment increased the expression of β-2 microglobulin (1.26-fold; \(P = 0.002\)), ICAM-1 (2.18-fold; \(P = 0.04\)), and RANTES (2.10-fold; \(P = 0.04\)) in the rabbit aqueous humor compared with control. Beta-2 microglobulin (1.18-fold; \(P = 0.04\)), ICAM-1 (3.60-fold; \(P = 0.02\)), α1-antitrypsin (5.17-fold; \(P = 0.02\)), IL-1β (7.15-fold; \(P = 0.02\)), IL-1ra (3.49-fold; \(P = 0.04\)), and MMP-3 (4.62-fold; \(P = 0.03\)) were upregulated after anti-LOXL2 treatment (GS-607601) compared with control eyes.

Although an upregulation of different factors was seen in the antibody-treated groups, several treatment groups had a large distribution of the results, whereas the PBS-treated eyes had more tightly outcomes. Moreover, this variation was also seen in the values of the bleb area, especially in the anti-LOXL2 treated eyes (Fig. 3D). Therefore, Spearman correlation coefficient (\(r\)) was calculated to evaluate the strength of association between expression of wound healing related factors and the bleb area on day 30 after anti-LOXL2 treatment. Significant correlations between different factors and bleb features were found. Wound healing promoting factors were negatively correlated with the bleb area. Thus, eyes with a high/low expression of wound healing promoting factors were correlated to small/large blebs, respectively. The wound healing inhibitory factors were positively correlated with the bleb area after anti-LOXL2 treatment. Thus, eyes with a high/low expression of wound healing inhibitory factors were correlated to large/small blebs, respectively. The significant correlations between bleb area on day 30 and expression of different wound healing factors (β-2 microglobulin, ICAM-1, α1-antitrypsin, IL-1β, IL-1ra, and MMP3) are illustrated in Figure 6.

Thus, treatment with LOX- or LOXL2-targeting antibodies led to an aqueous upregulation of different factors after glaucoma surgery, which can influence the process of wound healing. Moreover, significant correlations between different factors and bleb features in the anti-LOXL2–treated eyes were found.

**DISCUSSION**

Filtering surgery (trabeculectomy) is the most effective treatment for glaucoma, and is therefore a crucial procedure...
inhibitory factors were positively correlated with the bleb area after anti-LOXL2 treatment: $r = -0.92$, $P = 0.02$. ICAM-1 was negatively correlated with the bleb area: $r = -0.87$, $P = 0.03$. (C-F) The wound healing inhibitory factors were positively correlated with the bleb area after anti-LOXL2 treatment: $\alpha_1$-antitrypsin ($r = 0.94$, $P = 0.02$), IL-1$\beta$ ($r = 0.92$, $P = 0.02$), IL-1ra ($r = 0.94$, $P = 0.02$), and MMP-3 ($r = 0.94$, $P = 0.02$).

Karalekas et al. demonstrated that the presence of several molecules in the aqueous humor can influence the process of wound healing, which may increase the risk of scarring after filtration surgery. Therefore, aqueous humor was collected at day 30 and the levels of these proteins after treatment were analyzed by multi-analyte bead-based immunoassay. From literature, we know that aqueous levels of $\beta$-2 microglobulin, $\alpha_1$-antitrypsin, IL-1, and MMP are upregulated after glaucoma surgery (Rodriguez-Agirretxe I, et al. IOVS 2012;53:ARVO E-Abstract 2514). Therefore, we believe that those levels were likely induced in the PBS group over naive rabbits due to surgery. However, anti-LOX antibody treatment further increased the expression of $\beta$-2 microglobulin, ICAM-1, and RANTES, factors that are known to stimulate the process of wound healing by increasing inflammation and/or blood vessel formation. $\beta$-2 microglobulin and ICAM-1 were also upregulated after anti-LOXL2 treatment. However, other inhibitory factors, such as $\alpha_1$-antitrypsin, IL-1$\beta$, IL-1ra, and MMP-5 also increased, and could have had opposing effects on the wound healing process by inhibiting blood vessel, elastin, and collagen formation. Moreover, a significant correlation was observed between the expression of these factors and bleb features after anti-LOXL2 treatment. Eyes with high/low expression of wound healing promoting factors were correlated to small/large blebs, respectively, whereas eyes with a high/low expression of wound healing inhibitory factors were correlated to large/small blebs, respectively. Therefore, these data provide one possible explanation for the difference in efficacy between the two monoclonal antibodies. Targeting LOX did not affect blood vessel density and inflammation, since treatment with GS-639556 primarily increased the expression of wound healing inhibitory factors. On the other hand, GS-607601 (anti-LOXL2 antibody) primarily induced inhibitory factors that inhibit wound healing and therefore could be improving surgical outcome by inhibiting the angiogenic, inflammatory, and profibrotic component of the wound healing process.
CONCLUSIONS

We showed that LOX and LOXL2 play an important role in wound healing after glaucoma filtration surgery. Targeting LOXL2 with an inhibitory monoclonal antibody (GS-607601) had a broader efficacy than targeting LOX, reducing angiogenesis and inflammation, as well as fibrosis. These results render LOXL2 an appealing target for scar formation after glaucoma surgery, and point to the potential therapeutic benefits of the clinical candidate monoclonal antibody simtuzumab.

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