

The study aims to identify other collagen genes that may significantly define subconjunctival fibrosis following experimental glaucoma filtration surgery.

Methods: Subconjunctival scarring was induced using a mouse model of glaucoma filtration surgery (GFS). The early and late phases of wound healing were analyzed by RNA sequencing (RNA-seq). The top ten highest expressed collagen genes in the late phase were validated by quantitative polymerase chain reaction (qPCR). Immunoblotting and immunolocalization were performed to verify and determine the expression profiles of the top three highest expressed collagen genes. Mouse and human conjunctival fibroblasts were treated with TGF- β 2 to determine the inducibility of the collagen transcripts. Conjunctival tissues, collected from 20 and 15 patients requiring initial and repeat GFS respectively were also analyzed by qPCR.

Results: RNA-seq identified *Col8a1* (70-fold), *Col11a1* (40-fold) and *Col8a2* (20-fold) as the three most highly expressed collagen genes in the late phase conjunctival transcriptome. These collagens were also induced at the protein level in late phase tissues. Type VIII collagen co-localized with type I collagen in fibrous structures and in ACTA-2-positive pericytes, appearing to fill gaps where type I collagen was low. Type XI collagen showed little co-localization with both collagens but was associated with the presence of macrophages. TGF- β 2 induced the top ten collagen genes in both mouse and human conjunctival fibroblasts. Conjunctival tissues from eyes undergoing repeat trabeculectomy surgery expressed 3.60-fold and 2.78-fold increase in type VIII and I collagen transcripts respectively compared to conjunctival tissues from primary trabeculectomy.

Conclusions: The high induction and unique expression profiles of types VIII and XI collagen suggest that together with collagen I, form a group of collagen biomarkers for the evaluation of fibrosis in the mouse model of GFS and post trabeculectomies.

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Presentation Time: 8:30 AM–10:15 AM

Utility of purified collagenase (Xiaflex®) as a possible aid in glaucoma surgery: A pilot study

Robert A. Honkanen¹, Kevin Kaplowitz¹, Edward Yung¹, Alan G. Fong¹, Jonathan P. Wright². ¹Ophthalmology, SUNY Stony Brook, Stony Brook, NY; ²School of Medicine, Stony Brook University, Stony Brook, NY.

Purpose: Trabeculectomy ab externo (Trab) remains one of the most commonly performed surgeries targeted at lowering intraocular pressure (IOP). Post-operative episcleral fibrosis over the scleral flap is a common cause of failure. Modulation of wound healing with mitomycin C remains the current gold standard for inhibiting post-operative scarring, and has been shown to extend bleb survival to 30 days post-operatively in rabbits. We hypothesize that the use of a purified collagenase Xiaflex® (Endo Pharmaceutical, {Dublin, Ireland}) given perioperatively may extend the life of glaucoma filtering surgery and re-establish filtration post-operatively in a rabbit model of glaucoma filtering surgery.

Methods: 13 eyes of New Zealand White rabbits underwent limbal-based Trab without the use of antifibrotic agents by the same surgeon (RH). Purified collagenase was delivered peri-operatively and post-operatively by subconjunctival injections of 12.5 μ l volumes at varying concentrations. Post-operative filtration was assessed using Moorfield grading of the bleb on examination and photographs, IOP measurements via TonoPen®, fluorescein transmission through the

bleb, and ultrasound biomicroscopy (UBM). Clinical bleb failure was defined as increase in IOP to baseline or failure of fluorescein transmission. Eyes undergoing surgery without the administration of collagenase were used as controls.

Results: Four eyes underwent both peri-operative and post-operative injections of Xiaflex, with improved survival time by IOP and fluorescein transmission compared to controls. Three of the four eyes demonstrated clinical bleb survival beyond 30 days post-operatively. Two of five eyes that underwent only post-operative injections demonstrated an improved survival time compared to controls. Four eyes in the control group demonstrated clinical bleb failure between 11 and 14 days. Adverse events included subconjunctival hemorrhage, eyelid ecchymosis, corneal ectasia and pannus, and conjunctival breakdown. Moorfield grading and UBM was found to be of limited value.

Conclusions: This pilot study preliminarily demonstrates that Xiaflex can extend the life of glaucoma filtering surgeries. Best results were obtained with peri-operative and post-operative injections of the medication. Further work must be done to optimize dosage and develop a delivery protocol prior to a formal efficacy study.

Commercial Relationships: Robert A. Honkanen; Kevin Kaplowitz, None; Edward Yung, None; Alan G. Fong, None; Jonathan P. Wright, None

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Novel MRTF/SRF inhibitors prevent conjunctival scarring after glaucoma filtration surgery: An ex vivo and in vivo study

Cynthia Yu-Wai-Man³, Richard M. Lee³, Scott Larsen¹, Richard Neubig², Peng T. Khaw³. ¹Vahlteich Medicinal Chemistry Core, College of Pharmacy, University of Michigan, MI; ²Department of Pharmacology and Toxicology, Michigan State University, MI; ³National Institute for Health Research (NIHR) Biomedical Research Centre at Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology, London, United Kingdom.

Purpose: Post-surgical scarring remains the main cause of failure of glaucoma filtration surgery and current antimetabolites carry the risk of potentially blinding complications. There is increasing evidence that the Myocardin-related transcription factor/Serum response factor (MRTF/SRF) pathway plays a pivotal role in myofibroblast activation. We thus hypothesised that inhibiting the MRTF/SRF pathway would reduce scarring in an aggressive rabbit model of conjunctival fibrosis.

Methods: Ex vivo segments of rabbit conjunctiva were cultured in 100 μ M MRTF inhibitor 1 or PBS control and imaged for tissue area changes over 30 days. We validated our results using a randomised, prospective, masked-observer study of 24 New Zealand White female rabbits undergoing glaucoma filtration surgery. The animals received either intraoperative 0.2mg/ml mitomycin-C (MMC) [N=6] or postoperative subconjunctival injections of 100 μ M MRTF inhibitor 1 [N=6] or 100 μ M MRTF inhibitor 2 [N=6] or PBS [N=6]. Bleb morphology and intraocular pressure were recorded over 30 days. Tissue sections were immunohistochemically graded on day 30. We analysed our results using Kaplan-Meier curve Log-rank test and Student's t-test.

Results: Ex vivo conjunctival tissue contraction was significantly reduced by 35%(day 6), 39%(day 15), 48%(day 21) and 68%(day 30) with inhibitor 1 compared to PBS (Fig 1). In vivo, bleb survival was significantly improved with inhibitor 1 ($p=0.01$) and inhibitor 2 ($p=0.0005$) compared to PBS (Fig 2). The mean day of bleb failure was 28.8 (range=24-30) for MMC, 28.5 (range=24-30) for

inhibitor 2, 24.5 (range=15-30) for inhibitor 1, and 14 (range=12-18) for PBS. IOP also remained significantly lower with inhibitor 1 ($p=0.027$) and inhibitor 2 ($p=0.0005$) compared to PBS. MMC treatment led to thin avascular blebs with destruction of the epithelial layer. MRTF inhibitors however were not toxic and significantly reduced conjunctival scarring using H&E, picrosirius red, Gomori's trichrome, and alpha-smooth muscle actin staining ($p<0.05$).

Conclusions: Novel MRTF inhibitors significantly improved bleb survival and prevented conjunctival scarring. MRTF inhibitor 2 had more potent anti-scarring effects than inhibitor 1. They were safe for subconjunctival delivery and less destructive to local tissue than MMC. MRTF inhibitors show potential as a novel class of anti-fibrotic agents in glaucoma filtration surgery.

Fig 1. Ex vivo model of conjunctival tissue contraction: (A) Representative conjunctival tissue segments cultured in 100 μ M MRTF inhibitor 1 or PBS control over 30 days; (B) Conjunctival tissue contraction was significantly reduced by 35%(day 6), 39%(day 15), 48%(day 21) and 68%(day 30) with inhibitor 1 compared to PBS control, each condition was performed in triplicates and results shown are mean \pm SEM.

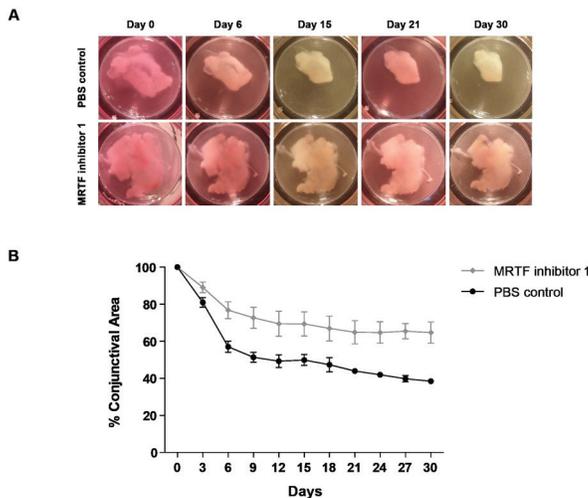
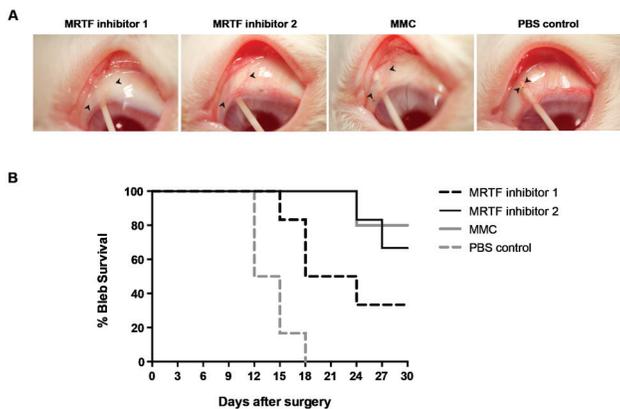


Fig 2. In vivo model of experimental Glaucoma filtration surgery: (A) At day 30, the blebs that survived remained diffusely elevated with the MRTF inhibitors while all the PBS-treated blebs were flat, scarred and vascularised, Arrows: edges of the blebs; (B) Kaplan-Meier curve showing that bleb survival rates were significantly improved after treatment with MRTF inhibitor 1 ($p=0.01$, log rank) and MRTF inhibitor 2 ($p=0.0005$, log rank) compared to PBS.



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Effects of rho-associated protein kinase inhibitor Y-27632 on scarring formation after glaucoma filtration surgery

Hideaki Okumichi, Wakana Iwata, Satoshi Okimoto, Ji-Ae Ko, Yoshiaki Kiuchi. Department of Ophthalmology and Visual Science, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan.

Purpose: Glaucoma filtration surgery usually fails because of post surgical scarring, a process in which fibroblasts play a prominent role. To elucidate the effects of rho-associated protein kinase (ROCK) inhibitor Y-27632 in post surgical scarring (fibrosis), we have now investigated the molecular mechanism with human tenon fibroblasts.

Methods: Human tenon fibroblasts were cultured with Y-27632 or various antiglaucoma drugs for indicated periods. After cultivation, we have prepared total RNA and protein samples from tenon fibroblasts. Using multiple RT-PCR array, we examined the factors respond to Y-27632. And, we have studied the expression of factor(s) of relating scarring formation using RT-PCR, immunoblot and immunofluorescence analysis. Also, we have examined the three-dimensional collagen gels cultivation for gel contraction by various antiglaucoma drugs.

Results: Collagen gel contraction by tenon fibroblasts was blocked in the presence of Y-27632. In multiple RT-PCR array using fibrosis-related genes, the expression of MMP-3 was down-regulated in tenon fibroblasts by additional Y-27632. Furthermore, immunoblot and immunofluorescence analysis revealed that the expression of fibrosis markers was down-regulated in the presence of Y-27632.

Conclusions: These results suggest that the ROCK inhibitor Y-27632 may block scarring formation with interaction MMP-3 after glaucoma surgery. And, it will be possible that ROCK inhibitors and MMP-3 may have potential to be developed for treatment of glaucoma and other ocular diseases.

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Effects of ripasudil (K-115), a Rho kinase inhibitor, on the activation of human conjunctival fibroblasts

Akiko Futakuchi, Toshihiro Inoue, Tomokazu Fujimoto, Miyuki M. Inoue, Hidenobu Tanihara. Ophthalmology, Kumamoto University Hospital, Chuo-ku, Kumamoto city, Japan.

Purpose: Ripasudil, a selective Rho kinase inhibitor, is an ophthalmic solution which was approved in Japan for the twice-daily treatment of glaucoma and ocular hypertension in 2014. The purpose of this study is to assess the effects of ripasudil on the activation of human conjunctival fibroblasts.

Methods: Human conjunctival fibroblasts were pretreated with or without different concentrations of ripasudil (25 and 50 μ M) for 1 hour and subsequently stimulated with 5 ng/ml TGF- β 2 for 48 hours. The effects of ripasudil on α -smooth muscle actin (α -SMA) expression and extracellular matrix (ECM) expression were analyzed by Western blot analysis. Contractile activity was evaluated by collagen gel contraction assay. Cell viability and cytotoxicity were assessed using WST-8 assay and Hoechst 33342/propidium iodide (PI) dual staining, respectively. The human monocytic cell line THP-1 were differentiated into M1- and M2-like macrophages, and fibroblasts were treated with conditioned medium derived from these macrophages in the presence or in the absence of 50 μ M ripasudil to quantify the α -SMA expression level.