An in vitro study of scarring formation mediated by human Tenon fibroblasts:
Effect of Y-27632, a Rho kinase inhibitor.

(Rho-Kinase 阻害剤、Y-27632 の術後瘢痕形成に及ぼす作用と治療への応用)

Glucoma is the second leading cause of blindness in the worldwide. As a leading cause of blindness, glaucoma may cause irreversible blindness. Any treatment, including medication and surgery, can only prevent the progression of vision loss and once the vision lost to glaucoma, it cannot be restored, which becomes the reason why glaucoma is essential to study. Reduction of intraocular pressure (IOP) is the only proven method to prevent glaucoma progression. Drug therapy to reduce IOP is the primary treatment modalities for glaucoma, and among those agents, topical prostaglandin F2α and β-blocker are commonly prescribed as an initial treatment. When medication insufficiently reduces the IOP, surgery is indicated. Glaucoma filtration surgery is regularly performed by creating a channel between anterior chamber to the subconjunctival space for lowering IOP. However, scarring formation due to increase fibroblast activation and proliferation is the primary cause of failure after filtering surgery. Thus, continuing research to find a safer yet effective treatment to suppress fibrosis is necessary to prevent obstruction to the created channel.

Rho-kinase (ROCK) is a major downstream effector of Rho GTPases that have involved in many cellular functions including the regulation of actomyosin cytoskeletal organization, cell adhesion, cell morphology, cell motility, and smooth muscle contraction. ROCK elicits its effect through phosphorylation/activation of their specific substrate, including myosin light chain kinase, LIM kinase 1 (LIMK1), LIMK2, and myosin phosphatase target subunit 1.

This study investigated the effect of ROCK inhibitor, Y-27632, on scarring formation mediated by Human Tenon Fibroblasts (HTFs) with a direct comparison with other anti-glaucoma drugs. HTFs were used and obtained from patients who underwent strabismus surgery. Cells from passages 5 to 6 were used for this study to avoid either morphological changes and increase gap variant among the cells treated. Collagen gel contraction was used for evaluating contraction activity of HTFs induces by several drugs including Y-27632, latanoprost, timolol, and TGF-β (as a positive control, pro-
fibrotic agent) with concentrations 10 μM. The gel that had been used was then observed under phase contrast microscope to evaluate the morphological and changes of fibroblasts induces by certain drugs compare to control. The dose-dependent response of drugs on collagen gel contraction was then tested to determine the optimal concentration of drugs that we used for entire experiment. Given that TGF-β expression has been shown to increase after surgery, this study mimicked the process by inducing the collagen gel with TGF-β for 24 hours before treatment with Y-27632, latanoprost or timolol to examine whether Y-27632 could block contractility effect of TGF-β that secreted after surgery. The effect of Y-27632 for blocking contraction induced by latanoprost and timolol was also tested in this study. Immunofluorescence assay was used to detect and compare the distribution of fibrosis-related marker, α-SMA and vimentin, in HTFs after induction with various drugs that might be mediated the mechanism of the drugs to either inhibit or exhibit the fibrosis. Similar to immunofluorescence, western blot was used to confirm and compare expression of α-SMA and vimentin. The gel that had been induced in several treatments, as described in the gel contraction assay, was then used as a sample for western blot. Western blot was also used for detecting MAPK activation (phosphorylation of ERK 1/2, p38, and JNK) induced by TGF-β and for detecting the ability of Y-27632 for blocking this activation.

This study found that Y-27632 significantly inhibited contraction in collagen gel assay, which was inversely promoted by latanoprost, timolol or TGF-β. Immunofluorescence analysis showed that latanoprost and timolol significantly enhanced the upregulation of α-SMA and vimentin while Y-27632 significantly suppressed those expressions. After observation of the gel under phase-contrast microscope, HTFs treated with Y-27632 induced morphological changes into round shape and suppressed fibroblast proliferation, as indicated by reduction in the number of elongated fibroblasts, while latanoprost, timolol, or TGF-β maintained their native morphology and increased the fibroblast proliferation. Apoptotic detection assay confirmed that collagen gel contraction inhibition by Y-27632 was not correlated with apoptotic/necrotic cells as showed by no increase in the number of apoptotic/necrotic cells compared with control. This data revealed that inhibition of collagen gel contraction by Y-27632 was indeed due to inhibition of fibroblast proliferation, inhibition of transdifferentiation of fibroblast to myofibroblast rather than increase of apoptotic/necrotic cells. Y-27632 significantly blocked the contraction started at concentration 5μM and increased its effect in a dose-dependent manner. Moreover, Y-27632 significantly inhibited the collagen gel contraction induced by TGF-β. Immunofluorescence and western blot analysis confirmed the results. TGF-β induced collagen contraction by the upregulated expression of α-SMA and vimentin in HTFs, while treatment with Y-27632 downregulated these expressions. A similar pattern of results was also shown in cells treated with latanoprost and timolol. Latanoprost and timolol induced collagen gel contraction and upregulated the expression of α-SMA and vimentin, while Y-27632 could significantly block these effects. Y-27632 also blocked TGF-β-induced MAPK activation. These results suggest that ROCK inhibitor may inhibit excessive fibrosis after glaucoma filtering surgery by reducing fibroblast proliferation, suppressing the transdifferentiation of fibroblast into myofibroblast, and inhibiting of TGF-β effects through suppression of MAPK.
pathway.

The effect of Y-27632 in this study on MAPK kinase is the only initial study to determine whether there is substantial evidence that downstream effector of TGF-β may cause a significant effect on fibrosis. Indeed, other signaling pathways including Smad-dependent pathway are planning to be studied in our following research in vivo. In conclusion, these results showed that ROCK inhibitors may have a potential effect as anti-scarring for glaucoma filtration surgery while latanoprost and timolol may induce fibrosis. Based on these results, this dissertation greatly contributes to elucidating the inhibitory mechanism of Rock inhibitor on tenon fibroblast proliferation. Therefore, all the committee members admitted that this dissertation is of sufficient value to confer the Doctor of Philosophy in Medical Science to Diah Gemala Ibrahim.