ASSESSING THE EFFECTS OF SIMVASTATIN ON REDUCING ACTION OF TGF-β IN CORNEAL CELLS FOR THE PREVENTION OF POST-OPERATIVE HAZE

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BACKGROUND AND SIGNIFICANCE

Excessive scarring in tissues following injury can lead to various pathological conditions. For example, excessive scarring in the cornea after surgery, trauma or infection leads to corneal haze, which is a major cause of impaired vision. Substantial laboratory and clinical data indicate that transforming growth factor beta (TGF-β) plays a major role in stimulating excessive scarring in corneal tissue and in other tissues throughout the body. (1) Furthermore, recent experiments have demonstrated that TGF-β up-regulates synthesis of connective tissue growth factor (CTGF), and CTGF mediates most of the effects of TGF-β on collagen synthesis and formation of myofibroblasts. (2) Thus, therapies that reduce the intracellular signaling pathway of TGF-β, and thereby reduce synthesis of CTGF, might be clinically effective in reducing corneal scarring.

Results of experiments with cultures of lung fibroblasts indicate that a major intracellular signaling pathway that is activated by TGF-β and leads to synthesis of CTGF involves the activation of the RhoA cascade. (3) RhoA is a member of the GTPase family of cytosolic signaling proteins. RhoA is activated by isoprenylation which anchors the RhoA protein to the intracellular side of the plasma membrane. An anchored RhoA is then phosphorylated and mediates the intracellular signaling cascade for cellular activation by CTGF (Figure 1). Furthermore, it was shown that direct inactivation of Rho using an exotoxin C3 resulted in nearly complete loss of CTGF expression induced by TGF-β. (4) Therefore, RhoA is a key target protein for the modulation of TGF-β up-regulation of CTGF expression.

Simvastatin is a member of the statin family of HMG CoA reductase inhibitors, and is a widely used drug for the control of cholesterol. However, Simvastatin is now recognized to indirectly affect multiple cellular actions via its inhibition of the synthesis of farnesylpyrophosphate (FPP) and geranylgeranyl-pyrophosphate (GGPP). Both FPP and GGPP are used in the post-translation modification and activation of the Rho family of intracellular signaling proteins. Furthermore, recent research has shown that members of the statin family can inhibit fibrosis in human Tenon fibroblasts presumably due to the ability to decrease expression of TGF-β and its down-stream mediator, CTGF (Figure 2). (5)

Figure 1

![Figure 1](image1.png)

Figure 2

![Figure 2](image2.png)
HYPOTHESIS

We hypothesize that Simvastatin will inhibit the TGF-β1-induction of CTGF through the decreased prenylation of Rho proteins, which will decrease synthesis of CTGF mRNA and protein in human corneal keratocytes.

SPECIFIC AIDS

1. Measure the effects of simvastatin exposure on expression of CTGF in cultures of human and rabbit corneal fibroblasts exposed to TGF-β using ELISA and Western blots.

PROGRESS MADE TOWARDS EACH SPECIFIC AIM

CTGF protein expression in rabbit and human corneal fibroblasts was measured using both ELISA and Western Blot analysis. ELISA analysis included conditioned media samples to measure the concentration of secreted CTGF and cell lysate samples to quantify the concentration of CTGF protein within the cell. The samples were pipetted into two plates and detected using a different primary antibody for each plate: US Biological polyclonal IgG antibody, and the specially made monoclonal IgG antibody to the CTGF hinge region. Results from the HCF polyclonal IgG samples (Figure 3a) seems to suggest that simvastatin + TGF-β induced cells have a lower mean CTGF concentration than TGF-β induced cells in extract samples. This trend is not observed in media samples, as mean CTGF concentrations are highest with simvastatin + TGF-β induced cells. However, the small sample size and wide range of recorded CTGF concentrations for each condition makes accurate interpretation of the data impossible. The data was analyzed using ANVOA and Tukey’s HSD post-hoc test and report that all of the current data is not statistically significant. Results from the RCF polyclonal IgG samples (Figure 3b) show that mean CTGF concentrations are not decreased with the addition of simvastatin in either the media or extract samples. Also, it is interesting to note that TGF-β induced cells have the lowest recorded mean CTGF concentration in all of the media sample conditions. This likely indicates that some error occurred in experimental procedure, sample collection, or sample analysis.
Analysis of the data collected from the HCF monoclonal IgG extract samples (Figure 4a) seems to correlate with the results of the HCF polyclonal IgG extract samples. The mean CTGF concentration of samples from simvastatin + TGF-β conditioned cells is lower than all of the other conditions including the control. Also analysis of HCF media samples using the monoclonal antibody detection illustrates a similar trend in CTGF concentration as that found with the polyclonal antibody ELISA. Results of the RCF monoclonal IgG extract and media samples (Figure 4b) seem to conflict with data reported from the RCF polyclonal ELISA. Although it can be noted that the media samples may seem to show a decrease in CTGF concentration in the simvastatin + TGF-β conditioned cells against the TGF-β conditioned cells, this data must be considered with skepticism as some of the data points had to be removed from the data tables used to compose this and the previous graphs as some values were negative and indicated a CTGF concentration lower than the control of 0 ng CTGF. Thus, the graphs and data included are shown as an indication of current work, but with the understanding that our data is incomplete and should not be considered significant for conclusion at this time.

Western Blot analysis was also performed on both media and extract samples of the HCF and RCF cells. RCF media samples (Image 1a) should show an invisible or much lighter band for the Control 3 well than the Simvastatin 1 or 2 wells according to the results of the ELISA; however, the Western Blot shows a very bright band in the Control 3 well and invisible or very light bands in the Simvastatin 1 and 2 wells. The Western Blot performed for the RCF extract samples (Image 1b) seems to better correlate with the data obtained from ELISA. Although, the CTGF standards do not appear to have been diluted correctly, as the 32.5 ng CTGF well does not have a light band and according to the data from the ELISA other wells with ~30 ng CTGF do show a band.
SUMMARY AND CONCLUSIONS

Since analysis of the current data has shown that the results are not statistically significant, it is not possible to comment accurately on the effectiveness of simvastatin in reducing the concentration of CTGF either intracellularly or extracellularly post induction with TGF-β. Thus further pilot studies will be conducted to determine if simvastatin does impact the synthesis and/or excretion of CTGF after cellular insult. However, a few comments should be made on the areas of potential error which may enlighten future experiments. It is believed that the albumin used to serve as a carrier for TGF-β may have interfered with cellular induction to scarring by TGF-β. Previous studies have shown successful results with scar induction by diluting a stock solution of 1µg/ml TGF-β to 5ng/ml directly into cell wells without the use of a carrier protein.

LIST ANY ABSTRACTS OR PUBLICATIONS THAT MAY ARISE FROM THIS WORK:
1. Abstract presented at the 2010 annual meeting of the Association for Research in Vision and Ophthalmology

REFERENCES