The Effect of Dexamethasone on Synthesis of Collagen, Fibronectin and \( \alpha \)-smooth Muscle Actin in Cultured Human Trabecular Meshwork Cells

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Abstract

**Purpose:** Steroids cause increased extracellular matrix (ECM) deposition in trabecular meshwork (TM) leading to increased aqueous outflow resistance. Since, the same is also observed in primary open angle glaucoma, steroid-treated TM cells are commonly used as *in vitro* model of glaucoma. However, the time-dependent effects of steroids on ECM production by TM cells have not been described. Hence, we investigated the time-dependent effects of dexamethasone on collagen type I (COLI), collagen type III (COLIII), collagen type IV (COLIV), fibronectin and \( \alpha \)-smooth muscle actin (SMA) production by cultured human TM cells.

**Methods:** TM cells were cultured in DMEM with or without dexamethasone (100 nM) for 7, 14 and 21 days. At the end of each time point, cells morphology was observed and cell viability was assessed by MTS assay. Cells and media were collected to measure the protein expression of COLI, COLIII, COLIV, fibronectin and SMA using Elisa and immunocytochemistry.

**Results:** Secretion of all 3 collagen subtypes and fibronectin in the dexamethasone-treated cells were significantly greater (p<0.001) than untreated cells. Similarly, SMA protein expression was increased about 2-fold compared to untreated cells. Additionally, COLI, COLIII and COLIV secretion was significantly greater on days 14 and 21 compared to day 7 (p<0.05).

**Conclusion:** Dexamethasone significantly increased synthesis of COLI, COLIII, COLIV, fibronectin and SMA by TM cells. The effect of dexamethasone on the synthesis of COL I, III and IV, in particular, was found to be time-dependent.

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(Received on April 12, 2015)
Introduction

Glaucoma, the most common cause of irreversible visual loss, is characterized by loss of retinal ganglion cells (RGCs) and optic nerve damage. Elevated intraocular pressure (IOP), the most important risk factor for progression of glaucoma, depends on the balance between the rate of aqueous humor inflow into the anterior chamber of eye and rate of its outflow from the anterior chamber. Trabecular meshwork (TM) is the major dynamic regulator of aqueous humor outflow and accounts for the most aqueous outflow resistance (Grant 1963). It consists of a complex of endothelium-like cells, which are surrounded by extracellular matrix (ECM). Structural and functional changes in TM tissue that lead to increase in outflow resistance are known to be associated with primary open angle glaucoma (POAG) (Rohen et al. 1963; Tektas and Lutjen-Drecoll 2009). Although, the cause and nature of changes in TM tissue in POAG are not fully understood, increased deposition of ECM in TM as one of the primary structural change is widely accepted (Yue 1996; Alexander et al. 1998; Pang et al. 2003; Agarwal et al. 2009). Moreover, the extent of optic nerve damage in glaucoma is correlated with the extent of ECM deposition (Gottanka et al. 1997).

Studies have shown that in TM, among the several ECM proteins, collagen types I (COLI), III (COLIII) and IV (COLIV) are extensively expressed (Tengroth et al. 1985; Hann et al. 2001). COLI and COLIII form the standard collagen fibrils, which are dispersed throughout the center of TM beams and juxtacanalicular region (Acottand Kelley 2008). COLIV appears to be the connecting fibrils between juxtacanalicular and the cribiform elastic-like plexus that permeate the juxtacanalicular region (Ueda et al. 2002). Extensive distribution of fibronectin has also been described in TM (Acottand Kelley 2008). TM is also rich is elastic tissue. The contractile properties of TM are attributed to the presence of microfilaments, which are made up of α-smooth muscle actin. (Gipson and Anderson 1979; Grierson and Rahi 1979).

Glucocorticoid-induced glaucoma is similar to POAG with regards to its clinical presentation as well as pathological changes in TM including the ECM changes (Wilson et al. 1993; Wordinger et al. 1999; Tawara et al. 2008; Razali et al. 2015). Several studies have shown that treatment with glucocorticoids such as dexamethasone results in excessive ECM deposition in TM (Lo et al. 2003; Rozsa et al. 2006; Razali et al. 2016). Hence, glucocorticoid-treated TM cells are considered a good representative in vitro model for glaucoma related research. Although, glucocorticoid-induced changes in ECM deposition have widely been investigated, most studies describe the effects of only a short-term treatment with glucocorticoids and the time-dependent effects of glucocorticoids on human TM cells have not been studied. Since, glucocorticoid treated TM cells are commonly used as in vitro model of glaucoma, it is extremely important to elucidate how secretion of ECM is affected by glucocorticoids in a time-dependent manner. Time-dependent pathological effects are of great significance particularly when evaluating extent of change in the pathology in response to change in experimental condition, experimental substances or other stimuli over a period of time. Hence, we carried out this study to demonstrate the time-dependent effects of glucocorticoids on the production of COLI, COLIII, COLIV, fibronectin and α-smooth muscle actin by TM cells over a prolonged period of 21 days.

Methods

Cell culture

Primary human TM cells (Scien Cell Research Laboratories) were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal bovine serum, L-glutamine, penicillin and streptomycin (Grand Island, NY, USA). Cells were incubated at 37°C in humidified atmosphere with 5% CO₂ and 95% air. The cells were passaged at 3 to 4 day interval and were allowed to grow to approximately 90% confluence. Cells from passages 3rd up to 5th were used in this study. Once the TM cells reached 90% confluence, they were divided into 3 groups. Group 1 consisted of normal TM cells cultured in DMEM. In group 2, TM cell were cultured in DMEM containing 100 nM DEX dissolved in 0.1% dimethyl sulfoxide (DMSO) (Raghunathan et al. 2015).
TM cells in group 3 were cultured in DMEM containing 0.1% DMSO. The cells were then incubated for 7, 14 and 21 days. At the end of each time point, cells were observed for morphological changes under inverted microscope and cell viability assay was done. Cells and media were collected for estimation of the expression of COLI, COLIII, COLIV, fibronectin and α-smooth muscle actin using ELISA and immunocytochemistry. All experiments were repeated 3 times with 3 technical replicates.

Cell viability assay (MTS assay)

Cell viability assay was carried out to determine time-dependent effects of dexamethasone on cell viability. In this study, MTS assay was used to determine the viability of cells. This assay determines the number of viable cells based on quantitation of the ATP, which indicates the metabolic activity in viable cells. The MTS assay was done using the Cell Titer 96 AQueous MTS Assay System (Promega, USA) as described before (Birke et al. 2010). Twenty μL of MTS solution was added to each well of the 96 well plate and cells were incubated for 4 hour at 37°C. Subsequently, the absorbance was recorded at 490 nm.

Expression of collagens, fibronectin and α-smooth muscle actin: Elisa

The concentration of COLI, COLIII, COLIV, fibronectin and α-smooth muscle actin were measured using commercially available ELISA kits (Elabscience Biotechnology Co.). For the estimation of fibronectin, COLI, COLIII and COLIV, media from the HTM cell cultures was collected at the end of each time point and stored till further use. For estimation of the α-smooth muscle actin, cell lysate was used. To prepare cell lysate, cells were scrapped from the surface of flask in PBS and then were transferred to a 15 mL centrifuge tube. The cells were centrifuged for 5 minutes at 1000 rpm at 4°C and washed with ice-cold phosphate buffer saline (PBS) for 3 times. 500 μL of RIPA buffer was then added to each of the tubes containing cells pellet and mixed thoroughly. The samples were then sonicated for 1 minute on ice. This was followed by centrifugation at 10000 rpm for 20 minutes at 4°C to pellet cell debris. The supernatant was then collected for further processing. The protein concentration of the lysate was estimated using A280 or BCA assay. All estimations were done in duplicates.

For assay, 100 μL of samples, blank and standard were added to the designated wells. Plates were then incubated for 90 minutes at 37°C. The solution was then removed from each well and 100 μL of Detection Ab working solution was immediately added and incubated for 1 hour at 37°C. Solution was then aspirated out of each well followed by washing for 3 times. After the last wash, remaining wash buffer was removed. 100 μL HRP conjugate working solution was then added to each well and incubated for 30 minutes at 37°C. After the incubation time, the wash process was repeated for 5 times. Then, 90 μL of substrate solution was added to each well and incubation was done for 15 minutes at 37°C. To stop the reaction, 50 μL of stop solution was added to each well. The optical density of each well was then determined using micro-plate reader set to 450 nm.

Expression of collagens, fibronectin and α-smooth muscle actin: immunofluorescence

Cells were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature and then were washed three times with ice cold PBS (pH7.5). The cells were then incubated in PBS containing 0.1% Triton-X for 10 minutes and this was followed by three times washing with PBS. Subsequently, cells were incubated with 1% bovine serum albumin (BSA) in PBST (PBS + 0.1% Tween 20) for 30 minutes to block unspecific binding of the antibodies and then incubation was done with primary mouse monoclonal antibodies against fibronectin, α-smooth muscle actin, COLI, COLIII and COLIV (Abcam, USA) in 1% BSA in PBST overnight at 4°C. After the incubation period, the solution was decanted and cells were washed three times in PBS. This was followed by incubation with the goat anti-mouse immunoglobulin Alexa 488, 555 and 594- conjugated secondary antibodies in 1% BSA for 2 hours at room temperature in dark. Alexa Flour 488 was used to tag fibronectin and α-SMA, Alexa Flour 555 to tag COLI and COLIII and Alexa Fluor 594 for COLIV (Abcam, USA). The secondary antibody solution was
then aspirated and cells were again washed three times with PBS in dark. Cells were now counterstained with DAPI for 15 minutes followed by rinsing with PBS and mounting. Observations were made using inverted fluorescence microscope Olympus IX81. The fluorescence signal was quantified using Image J software (Image J 1.31, National Institutes of Health, Bethesda, MD, USA). For quantification, the background was removed and fluorescent areas were highlighted. The intensity of fluorescence was then read after setting the threshold. All measurements were done in 3 biological and 3 technical replicates by two masked investigators independently. For each replicate, 3 randomly selected fields of view were visualized.

**Statistical analysis**

The data is presented as mean±SD. Statistical comparisons among groups were done based on absolute values using one-way ANOVA by SPSS software version 22. *P*≤0.05 was considered significant. Values are presented as percent of corresponding control, which was set to 100%.

**Results**

**Effect of dexamethasone on HTM cells morphology and viability**

TM cells cultured in vehicle (DMSO) were similar in morphology and size as the cells in DMEM. However, dexamethasone-treated cells showed different morphology and more random orientation. These cells had increased size and intercellular space. Additionally, cells were elongated and showed bulging at the edges (Fig. 1).

![Image](image_url)  
**Fig. 1**: Effect of treatment with dexamethasone on the morphology of TM cells. After treatment for 7, 14 and 21 days, cells appeared larger, elongated and with thickened bulging edges.
MTS assay was done to assess the effects of dexamethasone on cell viability. The assay showed that the TM cells survival was close to 100% in both the DMSO or DEXA groups. Culture upto 21 days had no significant effect on the cell viability in any of the groups (Fig. 2).

**Expression of collagens, fibronectin and α-smooth muscle actin**

Treatment with dexamethasone for 21 days resulted in significantly higher levels of COLI, COLIII and COLIV by TM cells. COLI expression in the culture media was 4.72, 6.26 and 5.21 folds higher compared to corresponding mean values for cells cultured in DMEM at the end of days 7, 14 and 21, respectively. The extent of COLI secretion on days 14 and 21 was significantly higher than that on day 7 (p<0.05). No significant difference was observed in the expression of COLI on days 14 and 21 by dexamethasone-treated cells (Fig. 3A). Concentration of COLIII on days 7, 14 and 21 of dexamethasone treatment increased by 1.63, 1.67 and 2.74 folds, respectively, compared to DMEM group and the COLIII level on days 14 and 21 was significantly higher than that on day 7 (p<0.05 and p<0.01, respectively). However, no difference was observed between days 21 and 14 (Fig. 3B). COLIV levels in culture media were 1.07, 1.65 and 1.66 folds higher in group treated with dexamethasone compared to that cultured in DMEM alone. No significant differences were observed at three time points (Fig. 3C).

Secreted fibronectin in dexamethasone treated group was 1.33, 1.35 and 1.38 fold higher compared to corresponding DMEM groups and no significant difference were observed at 3 time points (Fig. 4A). α-smooth muscle actin was measured in the cell lysate and its concentration was found to be 1.64, 1.60 and 1.94 fold higher in cells treated with dexamethasone compared to cells that were cultured in DMEM alone at days 7, 14 and 21, respectively. Although, on the day 21, there was a trend toward higher mean value, the difference did not reach the significant level (p=0.08) (Fig. 4B).

**Expression of collagens, fibronectin and α-smooth muscle actin using immunofluorescence**

Immunofluorescent staining was done to visualize the extent of deposition of COLI, COLIII, COLIV, fibronectin and α-smooth muscle actin. Since no
differences were observed between DMEM and DMSO groups. DMEM group was taken as control for representing immunofluorescence expression of COLI, COLIII, COLIV, fibronectin and α-smooth muscle both qualitatively and quantitatively. Production of all tested ECM components at all time points was higher in dexamethasone treated group compared to group cultured in media without dexamethasone. According to observations made in Elisa, immunofluorescent staining also showed time-dependent effects on the deposition of COLI and COLIII with greater staining for these 2 proteins on days 14 and 21 compared to day 7 of dexamethasone exposure. Additionally a similar effect was observed
for COLIV, whereas no differences were observed for fibronectin and alpha smooth muscle actin over the same period of dexamethasone exposure (Figs. 5–9). The quantitative representation of the expression of COLI, COLIII, COLIV, fibronectin and alpha smooth muscle actin on days 7, 14 and 21 of dexamethasone exposure is shown in figure 10 as mean percentage of fluorescence intensity compared to corresponding controls. In line with the observations made using Elisa, COL I expression was 5.88 and 5.62 folds higher on days 14 and 21 compared to control (DMEM) while the same was 3.44 folds higher on day 7 of dexamethasone exposure. COLIII expression was 2.88, 3.77 and 3.79 folds higher compared to control on days 7, 14 and 21 of dexamethasone exposure, respectively. Dexamethasone also increased the expression of COLIV by 1.36, 2.89 and 2.86 folds compared to control on days 7, 14 and 21 respectively. Comparison of mean fluorescence among groups showed that the expression of all the tested types of COL was higher on days 14 and 21 compared to day 7 of dexamethasone treatment. The fibronectin expression was increased by 1.10, 1.27 and 1.35 folds whereas that of \( \alpha \)-smooth muscle actin increased by 1.31, 1.50 and 1.67 folds on days 7, 14 and 21 respectively.

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Fig. 5: Effect of treatment with dexamethasone on the expression of COLI over 21 days. Relatively greater COLI deposition was observed at all time points compared to corresponding controls as indicated by immunofluorescent staining.

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Fig. 6: Effect of treatment with dexamethasone on the expression of COLIII over 21 days. Immunofluorescent staining showed greater COLIII deposition at all time points compared to corresponding controls.
Fig. 7: Effect of treatment with dexamethasone on the expression of COLIV over 21 days. Greater COLIV deposition was observed in dexamethasone treated group at all time points compared to corresponding controls.

Fig. 8: Effect of treatment with dexamethasone on the expression of fibronectin over 21 days. Immunofluorescent staining showed greater fibronectin deposition in response to treatment with dexamethasone at all time points compared to corresponding controls.

Fig. 9: Effect of treatment with dexamethasone on the expression of α-smooth muscle actin over 21 days. Immunofluorescent staining showed greater α-smooth muscle actin expression in dexamethasone treated group at all time points compared to corresponding controls.
Discussion

Disturbances of ECM homeostasis are known to occur in glaucomatous eyes. Clinical studies have shown increased amounts of collagens in the aqueous humor of glaucoma patients compared to control (González-Avila et al. 1995; Tuulonen et al. 1996). Kim et al. (1992) showed significantly high level of fibronectin in the aqueous humor of glaucoma patients. Vesaluoma et al. (1998), however, showed that aqueous humor fibronectin levels are elevated in patients with exfoliative glaucoma and not those with POAG. In these clinical studies the source of ECM proteins, however, remains unclear. Since, the steroid-induced glaucoma resembles POAG with regards to increased aqueous outflow resistance; steroid-treated TM cells are commonly used as in vitro model for glaucoma.

Dexamethasone exerts a variety of effects on cultured TM cells, such as changes in gene expression (Polansky et al., 1991), TM cell and nuclear size (Clark et al., 1994; Steely et al., 1992), ECM deposition (Synder et al., 1993), protease activity (Samples et al., 1993), phagocytic function (Shirato et al., 1988) and cytoskeletal organization (Wilson et al., 1993). These effects of dexamethasone on TM cells contribute to changes in cell morphology and functions. Accordingly, in our study morphological changes were observed in TM cells over 21 days of treatment with dexamethasone despite no changes in cell viability. Thickening at the edges of the cells could be attributed to dexamethasone-induced reorganization of actin filaments at the cellular periphery (Liu et al. 2003). Similar changes in TM cell morphology have earlier been described by Wang et al. (2011) after 1 week of treatment with dexamethasone. No change in cell viability observed in our study, however, is contrary to the observation by Yu et al. (2010) who showed reduced TM cell proliferation after treatment with dexamethasone $10^{-5}$–$10^{-7}$ M for 3 days. This difference may be attributed to the difference in the cell line and the concentration of dexamethasone used.

Among the ECM proteins, COLI, COLIII and COLIV are distributed throughout the trabecular beams and juxtacanalicular region (Murphy et al. 1987; Zhou et al. 1998). These collagens provide tensile strength to TM. The amount of COLI, COLIII and IV has been shown to be significantly increased in glaucomatous eyes (Lütjen-Drecoll et al. 1989; Zhou et al. 1998). Another ECM protein, fibronectin is also abundantly...
expressed in TM and is particularly associated with basement membranes. It produces fibrous bands with considerable elasticity (Acott and Kelley 2008). TM cells are known to possess smooth muscle like properties, which are attributed to the abundant $\alpha$-smooth muscle actin contents of these cells (Grierson and Rahi 1979; Gipson and Anderson 1979).

In the current study, increased secretion of all 3 types of collagens and fibronectin by TM cells and increased $\alpha$-smooth muscle actin within TM cells was observed in response to treatment with dexamethasone. Additionally, we observed a progressive increase in the secretion of COLI, COLIII and COLIV over a period of 21 days by dexamethasone treated cells compared to cells that were not exposed to dexamethasone. Measurements done by Elisa as well as immunofluorescence showed a similar trend of higher expression of COLI and COLIII on days 14 and 21 compared to day 7 of dexamethasone treatment. For COLIV, immunofluorescence showed higher expression on days 14 and 21 compared to day 7 of treatment; however, Elisa did not show the same difference despite a similar trend in mean values. Hence, despite slight differences observed among the mean values of collagen expression by Elisa and immunofluorescence, collectively the results indicate that the treatment of HTMC with dexamethasone increases the secretion of 3 subtypes of collagen over a period of 21 days in a time-dependent manner and the extent of change in the expression of all three types of collagen varies. Since, different subtypes of collagen are differently distributed in the ECM of TM and have distinct functions, it could be extrapolated from the results that dexamethasone causes extensive changes in the structure and function of ECM in TM.

Steely et al. (1992) have shown that treatment of TM cells with dexamethasone for 17 days results in doubling of cell associated and secreted fibronectin. Wang et al. (2011) have shown that treatment of TM cells with dexamethasone for just 1 week causes modest increase in extracellular fibronectin. In line with these observations, Elisa as well as immunofluorescence used in the current study showed that dexamethasone causes increased secretion of fibronectin as early as one week and the same continues up to 21 days with no significant difference during this treatment period. The $\alpha$-smooth muscle actin level in TM cells after treatment with dexamethasone was significantly greater at all 3 time points compared to cells that were cultured only in DMEM. Elisa and immunofluorescence both techniques showed higher expression of smooth muscle actin at day 7, 14 and 21 compared to control group. Raghunathan et al. (2015) have shown that treatment of TM cells with dexamethasone results in over expression of $\alpha$-smooth muscle actin as early as 3 days. Using immunofluorescence, Clark et al. (1994) have demonstrated that when TM cells are treated with dexamethasone over a period of 21 days, the actin stress fibers are reorganized as cross-linked actin networks that resembled polygonal lattices and these effects were reversible upon withdrawal of dexamethasone. However, this study did not show the effects of dexamethasone at shorter treatment durations. Raghunathan et al. (2015), however, have shown that the treatment of TM cells with dexamethasone caused their stiffness to increase by 2 folds as early as 3 days post-treatment indicating cytoskeletal changes early during the course of treatment. It is likely that $\alpha$-smooth muscle actin expression increases early in the course of dexamethasone treatment but the reorganization of fibers takes longer time. None of the studies, however, have described how the effect of dexamethasone changes with continued treatment.

In conclusion, the current study has demonstrated that exposure of TM cells to dexamethasone for 21 days results in morphological changes, although the cell viability remains unchanged. Dexamethasone also causes significant increase in the secretion of COLI, COLIII, COLIV, fibronectin and $\alpha$-smooth muscle actin. Secretion of all three types of collagen increased progressively up to day 14 of dexamethasone treatment and was maintained at the same level thereafter up to day 21. The effect of dexamethasone on fibronectin and $\alpha$-smooth muscle actin remained comparable at 7, 14 and 21 days of treatment. These findings suggest that exposure to dexamethasone does not affect the production of
fibronectin and α-smooth muscle actin beyond the early effects during first 7 days whereas effects on collagens continue up to 2-3 weeks. The results of this study are particularly important in view of widely used dexamethasone treated TM cells as in vitro model for glaucoma related mechanistic, biochemical and pathophysiological studies.

Acknowledgments

We acknowledge the financial support from the Ministry of Education, Malaysia, under grant numbers 600-RMI/FRGS TD 5/3 (2/2015), 600-RMI/FRGS 5/3 (110/2014) and 600-RMI/FRGS 5/3 (24/2014).

References


