

# Effects of dexamethasone on human lens epithelial cells in culture

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**Purpose:** Treatment with glucocorticoids is a well known risk factor for cataract development, although the pathogenic mechanism has not been elucidated. The aim of the study was to investigate the effects of glucocorticoids in cultured human lens epithelial cells.

**Methods:** Human lens epithelial cells (HLECs) were exposed to dexamethasone for 24 h. The number of viable cells was determined using the 3-[4, 5-dimethylthiazolyl-2]-2, 5-diphenyltetrazolium bromide (MTT) assay, and proliferation was quantified using Ki-67. Apoptosis was investigated by measuring caspase-3 activity and by evaluating nuclear morphology of cells stained with Hoechst 33342. Mitochondria depolarization was measured using the potential-sensitive color, JC-1. Cells were assayed for changes in superoxide production using dihydroethidium (HET), for alterations in peroxide production using dichlorofluorescein diacetate (DCFH-DA), and for glutathione (GSH) variations using monochlorobimane (MCB). Caspase-3 activity was also measured in HLECs simultaneously exposed to dexamethasone and the glucocorticoid antagonist, RU486.

**Results:** Low doses of dexamethasone (0.1  $\mu$ M) resulted in increased proliferation of HLECs. Apoptosis was increased in HLECs exposed to 1  $\mu$ M, 10  $\mu$ M, and 100  $\mu$ M of dexamethasone as revealed by nuclear morphology studies. Apoptosis was also confirmed by measuring caspase-3 activation. No effect on superoxide production by dexamethasone was seen. There were no effects on GSH levels or mitochondrial depolarization either. Only the highest concentration of dexamethasone (100  $\mu$ M) caused an increase in peroxide production. In HLECs incubated with the glucocorticoid antagonist, RU486, apoptosis was induced at a lower concentration of dexamethasone (0.1  $\mu$ M) than with dexamethasone alone.

**Conclusions:** Low doses of dexamethasone cause a moderate increase in proliferation of cultured HLECs. Slightly higher but still physiologically relevant concentrations of dexamethasone result in a dose-dependent increase in apoptosis. Dexamethasone-induced apoptosis in HLECs does not seem to involve oxidative mechanisms. The proapoptotic effect of dexamethasone does not appear to act through the glucocorticoid receptor. Effects on proliferation and/or dysregulation of apoptosis in lens epithelial cells may be an important factor in human steroid-induced posterior subcapsular cataract.

It has been well recognized among ophthalmologists for decades that long-term cortisone therapy, commonly used in disorders like asthma, allergy, and rheumatoid arthritis or as an immunosuppressant after transplantation, greatly enhances the risk of cataract. More specifically, it increases the risk of posterior subcapsular cataract (PSC), a type of cataract that often causes substantial impairment of visual acuity being located centrally on the posterior side of the lens.

It was Black et al. [1], in 1960, who first described the association between long-term topical and systemic steroid use and increased occurrence of PSC. Since then, several reports, both clinical and experimental, have implicated cataract formation after prolonged glucocorticoid use [2-4]. Solumedrol (methyl prednisolone sodium succinate), acting on in vitro incubated rat lenses, resulted in PSC, but an addition of vitamin E shifted the site of the damage to the lens

equator [5]. More recently, intravitreal injections with steroids such as triamcinolone have become frequent in the treatment of macular edema. A single dose of intravitreal steroids has been shown to induce PSC [6,7].

Despite the fact that steroid-induced cataract has been and still is a common clinical condition, the mechanism behind this type of iatrogenic lens opacification has not been elucidated. It has long been a matter of debate among lens researchers whether glucocorticoid receptors (GR) exist in the lens or not, a prerequisite for a receptor-mediated mechanism to cataract development [8-10]. Several other receptor-independent mechanisms for steroid-induced cataractogenesis have also been suggested. Studies have demonstrated cataract formation by binding of glucocorticoids to lens proteins, resulting in changes of normal protein structure [11,12], which theoretically may result in aggregate formation and subsequent light scatter. Other reports found that covalent binding of steroids was unlikely to be responsible for steroid-induced cataract [10, 13]. Furthermore, glucocorticoids have been shown to cause

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ion imbalance in the lens [14], but contradictory data exist [13].

The terminal differentiation of lens epithelial cells into lens fibers is essential for development and growth of the lens, a process in which growth factors such as basic fibroblast growth factor (bFGF) and transforming growth factor beta TGF- $\beta$  play an essential role. Disturbances in the differentiation process can lead to loss of lens transparency and thus to cataract development. Studies have suggested that steroids induce changes in growth factor production in the eye, leading to an altered impact of these growth factors on lens epithelial cell differentiation [15].

Another hypothesis states that glucocorticoids may affect protective antioxidative systems in the lens, thereby making the lens more susceptible to oxidative stress [13,15,16]. Dickerson et al. [13] found a decrease in glutathione (GSH) levels after glucocorticoid exposure. Another study demonstrated protection by vitamin E against damage caused by a soluble corticosteroid (methyl prednisolone sodium succinate; solumedrol) in cultured rat lenses [5].

In the present study, we have examined the effect of dexamethasone on cultured human lens epithelial cells (HLECs) with respect to proliferation, apoptosis, oxidative stress, and steroid receptor inhibition.

## METHODS

**Human lens epithelial cell culture:** Human lens epithelium specimens were obtained from lenses during cataract surgery at the Eye Clinic, Sahlgrenska University Hospital (Möln dal, Sweden) after obtaining informed consent. The study was approved by the Gothenburg University Ethics Committee, and the tenets of the Declaration of Helsinki were followed. The human lens epithelium specimens, usually 5 mm in diameter, were placed into Eppendorf tubes, which contained culture medium (RPMI-1640; Sigma Chemical, St Louis, MO) that was supplemented with 10% fetal calf serum, 100 U ml<sup>-1</sup> penicillin, 0.1 mg ml<sup>-1</sup> streptomycin, and 2 mM L-glutamine, immediately after surgery. The lens epithelium specimens were later transferred from the Eppendorf tubes to 24 well culture dishes (TPP, Trasadingen, Switzerland) in a humidified CO<sub>2</sub> incubator at 37 °C to allow the capsule to attach to the bottom of the culture well. After one to two weeks, HLECs on the capsules and cells that had migrated onto the bottom of the culture well were detached by trypsinization and seeded in cell culture flasks (75 cm<sup>2</sup>). At semi-confluency, cells were subcultured by 0.25% trypsin/EDTA treatment.

For experiments, HLECs from one individual, passage IV-VIII, were subcultured in medium RPMI-1640 with 10% fetal calf serum, 100 U ml<sup>-1</sup> penicillin, 0.1 mg ml<sup>-1</sup> streptomycin, and 2 mM L-glutamine in a white 96 well plate with a transparent bottom (Costar Corp., Cambridge, MA) with approximately 10<sup>5</sup> cells per well to yield a confluent

monolayer. For Hoechst 33342 labeling, the cells were seeded in chamber slides with a cover glass bottom (Lab-Tek™ Nalge Nunc International, Rochester, NY). The chamber glasses were coated with collagen (0.3 mg/ml; Sigma Chemical, St Louis, MO) for 15 min after which the slides were allowed to air dry.

**Cell proliferation detection by immunohistochemistry:** HLECs cultured in chamber slides were exposed to dexamethasone for 24 h. After subsequent rinsing in PBS, the cells were fixed in 4% paraformaldehyde (pH 7.4). The cells were rinsed in PBS and permeabilized by digitonin (30  $\mu$ g/ml in PBS for 10 min at room temperature; Sigma Chemical, St Louis, MO). Following standard protocols for immunohistochemistry, the cells were marked with monoclonal mouse anti-proliferating cell protein, Ki-67 (Sigma), and visualized by streptavidin-FITC (Vector Laboratories, Burlingame, CA). Anti-Ki-67 binds to the Ki-67 nuclear antigen, which is strictly associated with cell proliferation. The Ki-67 antigen is expressed by proliferating cells in all phases of the active cell cycle (G<sub>1</sub>, S, G<sub>2</sub>, and M phase) but absent in resting (G<sub>0</sub>) cells.

The cells were viewed using a fluorescence microscope (Nikon Eclipse TE300; Nikon, Tokyo, Japan), and the Ki-67 positive cells were counted. The mean and SEM were calculated from three separate chamber slides and two different cell lines.

**Effect of dexamethasone on cell viability:** MTT (3-[4, 5-dimethylthiazolyl-2]-2, 5-diphenyltetrazolium bromide) is a yellow tetrazolium salt, which is cleaved by mitochondrial dehydrogenases to purple water-insoluble formazan crystals in metabolically active cells. The formazan crystals can be solubilized with acidified isopropanol, and the intensity can be measured colorimetrically at a wavelength of 570 nm where the optical density was proportional to the number of viable cells.

HLECs were cultured in medium RPMI-1640 without phenol red to 80% confluency followed by exposure to dexamethasone for 24 h at 37 °C in a humidified 5% CO<sub>2</sub> incubator. MTT diluted 1:10 from a stock solution of 5 mg/ml in RPMI-1640 without phenol red was added, and the cells were incubated for 4 h.

Absorption was measured at 570 nm in a microplate reader (E-max, Molecular Devices, Sunnyvale, CA) using SOFTmax version 2.01 (Molecular Devices Sunnyvale, CA) as software, and the difference in optical density between the sample wavelength (570 nm) and the reference wavelength (650 nm) was calculated.

**Nuclear morphology with Hoechst 33342:** To detect and to quantify apoptosis, HLECs were seeded in collagen coated chamber slides with a cover glass bottom and incubated with dexamethasone (0.1  $\mu$ M, 1  $\mu$ M, 10  $\mu$ M, and 100  $\mu$ M) in serum-free RPMI-1640 for 24 h at 37 °C in a humidified 5% CO<sub>2</sub> incubator. The cells were then fixed in 4%

paraformaldehyde in PBS (pH 7.4) for 30 min. After fixation, the cells were washed twice in PBS after which cell-permeable Hoechst 33342 (Hoechst, Frankfurt Germany) was added (final concentration  $5 \mu\text{g ml}^{-1}$  in a HEPES buffer) for 15 min. After being washed in PBS, glycerol was added to cover the cells. The cells were immediately viewed using a fluorescence microscope (Nikon Eclipse TE300; Nikon).

At least 300 cells in three different fields were counted, and the percentage of apoptotic nuclei in relation to the total number of cells was determined. The mean and SEM were calculated from three separate coverslips, and the experiments were performed in triplicates.

*Caspase-3 activity in dexamethasone-exposed human lens epithelial cells:* HLECs were incubated with dexamethasone (0.1  $\mu\text{M}$ , 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 100  $\mu\text{M}$ ) in serum-free RPMI-1640 that was supplemented with 100 U  $\text{ml}^{-1}$  penicillin, 0.1  $\text{mg ml}^{-1}$  streptomycin, and 2 mM L-glutamine for 24 h at 37 °C in a humidified 5%  $\text{CO}_2$  incubator. After the incubation period, the cells were centrifuged at 350 g for 5 min. The medium was removed, and the well plates were immediately frozen at -152 °C for at least 30 min. The frozen HLECs were thawed, and 100  $\mu\text{l}$  of 0.2% CHAPS-containing buffer including the protease inhibitors, trypsin inhibitors from chicken egg white (final concentration  $5 \mu\text{g ml}^{-1}$ ), pepstatin (0.5  $\mu\text{g ml}^{-1}$ ), leupeptin (1.25  $\mu\text{g ml}^{-1}$ ), and PMSF (0.5 mM; all from Sigma Chemical) were added. The cells were incubated with the inhibitor-containing CHAPS buffer for 30 min at room temperature after which 20  $\mu\text{l}$  were removed for protein determination.

The synthetic fluorogenic substrate, Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC; from Bachem, Bubendorf, Switzerland), used for caspase-3 determination was diluted from a 10 mM stock solution in water to 50  $\mu\text{M}$  in Tris-HCl (pH 7.3), 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, and 3 mM  $\text{NaN}_3$ , yielding a final concentration of 25  $\mu\text{M}$  Ac-DEVD-AMC in the assay. Dithiothreitol was added to a final concentration of 2 mM.

At the start of the proteolytic assay, 100  $\mu\text{l}$  of substrate was added to the 96 well plate containing 100  $\mu\text{l}$  of cell lysate in CHAPS buffer. The fluorescent cleavage product of the substrate was measured over time at 37 °C, excitation wavelength 380 nm, emission wavelength 460 nm, using a microplate spectrofluorometer (SPECTRAMAX GEMINI, Molecular Devices, Sunnyvale, CA). Protease activity was measured for 2 h, and  $V_{\text{max}}$  was determined in the linear interval using SOFTmax PRO version 2.6 as software (Molecular Devices). Proteolytic activity is expressed as the increase in relative fluorescence units per second and gram of protein ( $\text{RFU s}^{-1}\text{g}^{-1}$ ).

*Protein determination:* Aliquots of 20  $\mu\text{l}$  HLECs lysate with 0.2% CHAPS buffer or hypotonic buffer were taken for protein determination using the BCA protein assay reagent (Pierce Perbio Science UK Limited, Cheshire, UK) with BSA

as the standard. Absorption was measured at 570 nm in a microplate reader (E-max, Molecular Devices) using SOFTmax version 2.01 as software.

*Superoxide production:* HLECs were exposed to dexamethasone (0.1  $\mu\text{M}$ , 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 100  $\mu\text{M}$ ) for 24 h. After subsequent rinsing in PBS, the cells were preloaded with 5  $\mu\text{M}$  dihydroethidium (HET) (Sigma Chemical) for 10 min at 37 °C. HET was removed, the cells rinsed in PBS, and RPMI-1640 was added. In cells, HET is oxidized by superoxides to the fluorescent substance ethidium, and changes in ethidium production can hence be used as a measure of superoxide production. Changes in superoxide production were continuously measured over time at 37 °C for 30 min (excitation wavelength 510 nm, emission wavelength 600 nm).  $V_{\text{max}}$  was determined in the linear interval and is expressed as the increase in relative fluorescence units per second and gram of protein ( $\text{RFU s}^{-1}\text{g}^{-1}$ ).

*Peroxide production:* Changes in peroxide production in dexamethasone exposed (0.1  $\mu\text{M}$ , 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 100  $\mu\text{M}$ ) cells were measured using the nonfluorescent DCFH-DA (20  $\mu\text{M}$ ). In the cell, DCFH-DA is cleaved by esterases yielding polarized nonfluorescent dichlorofluorescein (DCFH). Through peroxides, DCFH is oxidized to fluorescent DCF. Therefore, the amount of formed DCF is a measure of the peroxide production in the cell. The cells were incubated with DCFH-DA for 30 min at 37 °C in a 5%  $\text{CO}_2$  atmosphere. Peroxide production was measured (excitation wavelength 490 nm, emission wavelength 535 nm) using the SPECTRAMAX GEMINI spectrofluorometer. Changes in peroxide production are expressed as the increase of relative fluorescence units per second and gram of protein ( $\text{RFU s}^{-1}\text{g}^{-1}$ ).

*Glutathione levels in dexamethasone-exposed human lens epithelial cells:* Monochlorobimane (MCB; 25  $\mu\text{M}$ ) in serum free RPMI-1640 was added to dexamethasone-exposed (0.1  $\mu\text{M}$ , 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 100  $\mu\text{M}$ ) cells, and changes in the level of glutathione (GSH) were measured immediately after the addition of MCB and after 2 h (excitation wavelength 380 nm, emission wavelength 460 nm). Changes in GSH levels are expressed as  $\text{RFU g}^{-1}$ .

*Mitochondrial membrane potential in dexamethasone-exposed human lens epithelial cells:* After subsequent rinsing in PBS, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; 1  $\mu\text{M}$ ; Molecular Probes, Eugene, OR) was added to cells exposed to dexamethasone (0.1  $\mu\text{M}$ , 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 100  $\mu\text{M}$ ) for 24 h, and the cells were incubated for 15 min at 37 °C. The cationic dye, JC-1, exhibits potential-dependent accumulation in the mitochondria. It indicates mitochondria depolarization by a decrease in the red to green fluorescence intensity ratio. After incubation with JC-1, the dye was removed; the cells were rinsed in PBS after which serum-free RPMI-1640 was

added. The green JC-1 signal was measured at the excitation wavelength of 485 nm and the emission wavelength of 535 nm, the red signal at the excitation wavelength of 540 nm and the emission wavelength of 590 nm, using a SPECTRAMax microplate spectrofluorometer.

Dexamethasone-exposed JC-1 labeled HLECs were also studied by confocal microscopy using an argon laser (excitation wavelength 488 nm; Nikon C1, Nikon).

Fluorescence was plotted against time and the linear interval defined. Proteolytic activity was expressed as relative fluorescence units per second and gram protein ( $\text{RFU s}^{-1}\text{g}^{-1}$ ).

**Apoptotic response after glucocorticoid receptor inhibition:** The synthetic GR antagonist, RU486 (10  $\mu\text{M}$ ), was added to HLECs 2 h before the addition of dexamethasone. The cells were incubated for 24 h at 37 °C in a 5%  $\text{CO}_2$  atmosphere after which the substances were removed. Some cells were exposed to RU-486 alone. After rinsing in PBS, the cells were frozen at -152 °C for caspase-3 determination as previously described.

**Statistics:** Data shown are the mean and SEM from triplicate samples. Each experiment was run at least twice to confirm reproducibility. Statistical analysis was performed using ANOVA with Dunnett's as post-hoc. A p value of less than 0.05 was considered statistically significant. As statistical software, SPSS 11.0.4 (SPSS Inc. Chicago, IL), was used.

## RESULTS

**Immunohistochemical analysis of cell proliferation and cell viability after dexamethasone exposure:** The number of Ki-67 positive cells was increased in HLECs exposed to 0.1  $\mu\text{M}$  dexamethasone for 24 h whereas higher concentrations had no significant effect on cell proliferation. However, a trend toward increased proliferation with increasing dexamethasone concentration was evident (Figure 1). Exposure to dexamethasone significantly decreased the number of viable HLECs as revealed by the MTT assay (Figure 2).

**Dexamethasone exposure leads to apoptosis in human lens epithelial cells:** When studying nuclear morphology using Hoechst 33342, increased apoptosis was seen already at 1  $\mu\text{M}$  dexamethasone and the percentage of apoptotic cells increased in a dose-dependent manner with higher dexamethasone concentrations (Figure 3A,B). Dexamethasone at a concentration of 100  $\mu\text{M}$  significantly increased caspase-3 activity in HLECs (Figure 3C). All incubation periods were for 24 h.

**Superoxide/peroxide production and GSH levels in dexamethasone-treated human lens epithelial cells:** Superoxide production in HLECs was not significantly changed after exposure to dexamethasone (Figure 4A) for 24 h. Cells treated with 100  $\mu\text{M}$  dexamethasone showed increased peroxide production, which was not present at lower concentrations (Figure 4B). No effect of dexamethasone on

GSH levels was evident in HLECs after 24 h exposure (Figure 4C).

**Mitochondrial membrane potential:** No depolarization of the mitochondrial membrane potential occurred after incubation with dexamethasone for 24 h as revealed with the JC-1 assay by quantifying the red to green fluorescence ratio (Figure 5).

**Effect of dexamethasone on caspase-3 activity after glucocorticoid receptor inhibition:** HLECs were incubated with increasing concentrations of dexamethasone for 24 h with and without 10  $\mu\text{M}$  of the glucocorticoid receptor inhibitor RU-486, and apoptosis was determined by

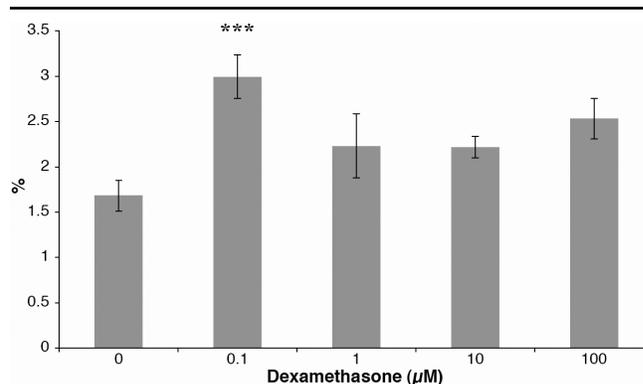


Figure 1. Determination of cell proliferation in dexamethasone-exposed human lens epithelial cells. Quantitative determination of cell proliferation showed an increase in the number of Ki-67 positive cells in human lens epithelial cells exposed to 0.1  $\mu\text{M}$  dexamethasone for 24 h whereas higher concentrations of dexamethasone did not significantly affect cell proliferation. At least 300 cells in three different fields were counted. The mean and SEM were calculated from three separate chamber slides ( $n=3$ ). The experiment was repeated twice with different cell lines, obtaining similar results.

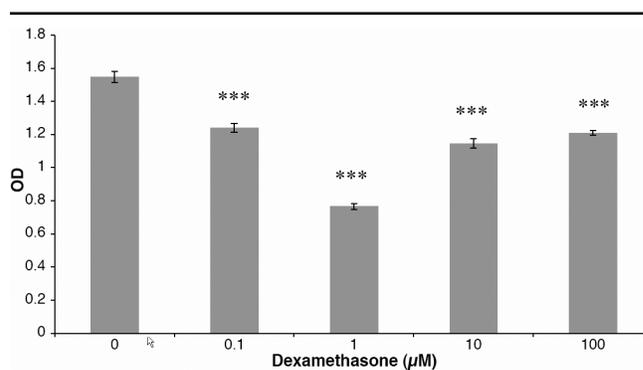


Figure 2. Number of viable cells after dexamethasone exposure. The number of viable human lens epithelial cells as determined by the MTT colorimetric assay was significantly decreased after exposure to dexamethasone for 24 h. The difference in optical density between the wavelength of the sample (570 nm) and the reference wavelength (650 nm) was calculated. Experiments were performed three times in triplicates ( $n=3$ ). One representative experimental run is shown. Mean $\pm$ SEM are given; the triple asterisk indicates a  $p<0.001$ .

measuring caspase-3 activity (Figure 6). This concentration of RU-486 (10  $\mu\text{M}$ ) was chosen since it didn't increase apoptosis when incubated with HLECs alone. At higher

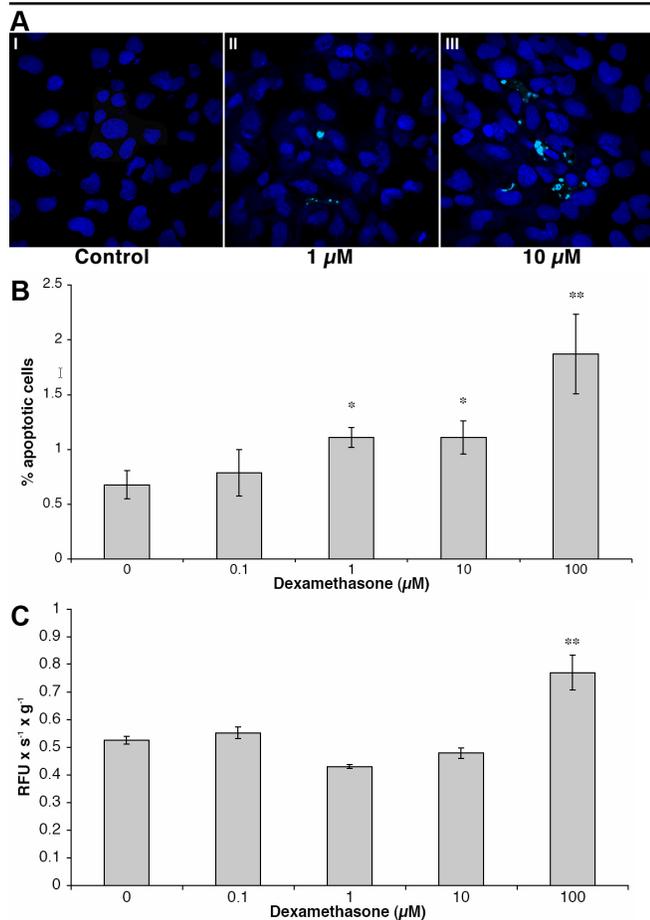


Figure 3. **A:** Apoptotic morphology of human lens epithelial cells after dexamethasone exposure. Cells that were exposed to dexamethasone exhibited morphologic changes typical of apoptosis such as shrinkage, chromatin condensation, and nuclear fragmentation (II and III). Cultured human lens epithelial cells are shown stained with the nuclear dye Hoechst 33342. Control cells (I) and cells exposed to 1  $\mu\text{M}$  (II) and 100  $\mu\text{M}$  (III) dexamethasone are shown. Original magnification 600X. **B:** Showing increased number of apoptotic cells after dexamethasone exposure. Human lens epithelial cells were exposed to dexamethasone at different concentrations during 24 h after which the cell nuclei were stained with Hoechst 33342. The percentage of apoptotic cells increased in a dose-dependent manner with higher dexamethasone concentrations. At least 300 cells from three different chamber slides were counted. Mean  $\pm$ SEM are given; the asterisk indicates a  $p < 0.05$  and the double asterisk indicates a  $p < 0.01$ . **C:** Increased Caspase-3 activity after incubation with dexamethasone. The caspase-3 activity in cultured human lens epithelial cells was significantly increased after the administration of 100  $\mu\text{M}$  dexamethasone for 24 h. A representative experimental run from three experiments with similar results is shown. Caspase-3 activity is expressed as relative fluorescence units per second and gram protein (RFU s<sup>-1</sup>g<sup>-1</sup>). Mean  $\pm$ SEM from 3 separate culture wells are shown; the double asterisk indicates a  $p < 0.01$ .

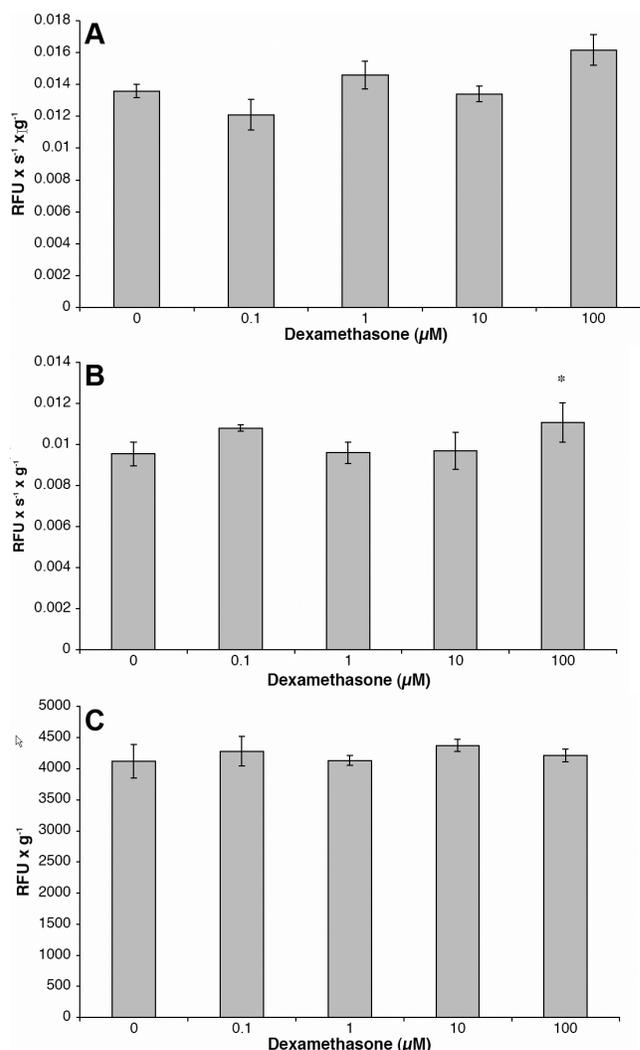


Figure 4. **A:** Superoxide production in human lens epithelial cells exposed to dexamethasone. Dexamethasone exposure to human lens epithelial cells for 24 h had no effect on superoxide production. Superoxide production was measured over time and expressed as relative fluorescence units per second and gram protein (RFU s<sup>-1</sup>g<sup>-1</sup>). The experiment was repeated twice with similar results. **B:** Peroxide production in human lens epithelial cells exposed to dexamethasone is also demonstrated. Exposure of human lens epithelial cells to dexamethasone for 24 h resulted in increased peroxide production only at the highest concentration of 100  $\mu\text{M}$  used. The experiment was repeated twice with similar results. Peroxide production is expressed as relative fluorescence units per second and gram protein (RFU s<sup>-1</sup>g<sup>-1</sup>). Mean  $\pm$ SEM from three separate culture wells are shown; the asterisk indicates a  $p < 0.05$ . **C:** Glutathione in human lens epithelial cells exposed to dexamethasone. Dexamethasone did not affect the GSH level in human lens epithelial cells after incubation with dexamethasone for 24 h. One representative experiment of three is shown. The level of GSH was measured after 2 h and expressed as relative fluorescence units and gram protein (RFU  $\times$  g<sup>-1</sup>). Mean  $\pm$ SEM from 3 separate culture wells is shown.

concentrations, RU-486 caused apoptosis in HLECs by itself, i.e. without the addition of dexamethasone (not shown). No protection of HLECs against dexamethasone-induced apoptosis was seen by simultaneous incubation with RU-486. On the contrary, the GR inhibitor, RU-486, increased the proapoptotic effect of dexamethasone, leading to even more apoptosis.

## DISCUSSION

It is now generally accepted that prolonged therapy with systemic glucocorticoids can cause PSC [1,17]. However, it is still a matter of debate whether these steroid-induced cataracts are dose dependent [18-21]. Both topically applied as well as inhaled steroids have also been shown to induce cataract [22-25].

Dysregulation of proliferation as well as defect differentiation of lens epithelial cells and migration of dysplastic cells with retained nuclei and organelles to the posterior pole have been discussed as pathogenic mechanisms behind PSC formation [26,27]. Studies have shown that lens differentiation as well as proliferation is controlled by growth factors in the vitreous humor [28]. Alterations of these growth factors by corticosteroids have been proposed to disturb lens differentiation, providing a possible mechanism for steroid-induced effects on lens fiber differentiation [15]. In this context, it is interesting to note the study by Creighton et al. [5] where rat lenses incubated with methyl prednisolone developed PSC but the addition of vitamin E changed the location of the opacity to the equator of the lens. This result implies that steroids interfere with the differentiation process.

The expression of the human Ki-67 protein is strictly associated with cell proliferation. It is present during all active phases of the cell cycle ( $G_1$ , S,  $G_2$ ) and during mitosis but is absent from resting cells ( $G_0$ ) [29]. When studying

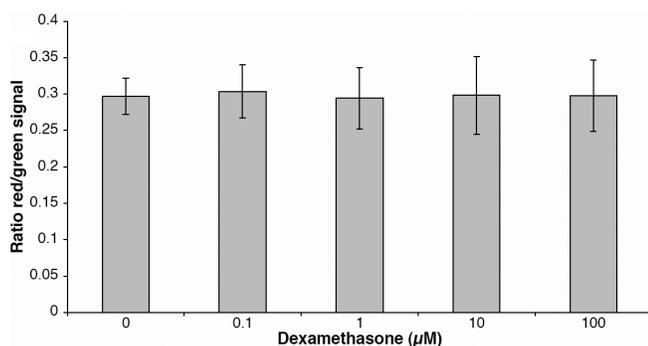


Figure 5. Mitochondrial membrane potential after dexamethasone exposure. Mitochondrial membrane potential in dexamethasone-exposed human lens epithelial cells did not differ significantly from the control group. The experiment was repeated eight times with identical results. Changes in mitochondrial membrane potential are expressed as the ratio of red signal (Ex 540 nm, Em 590 nm) to green signal (Ex 485 nm and Em 535 nm). Mean  $\pm$ SEM are given.

proliferation immunohistochemically with Ki-67, we found increased proliferation in cell cultures exposed to dexamethasone at low concentrations. Pro-proliferative effects of steroids have previously been reported [30,31], although data are contradictory [32,33]. The increase in proliferation as determined by Ki-67 expression in this study is in contrast to the decrease of viable cells seen in dexamethasone-exposed HLECs as examined by the MTT assay. However, the MTT method detects all metabolically active cells including cells in the  $G_0$  phase and is therefore a measure of the total number of viable cells regardless of the proportion of proliferating cells. Hence, increased apoptotic response after dexamethasone exposure may be responsible for the decreased number of viable cells as measured by the MTT assay.

Previous studies in other cell types report conflicting data on steroid-induced apoptosis. Dexamethasone has been shown both to induce apoptosis by activation of caspases (proteases activated during apoptosis) [34] as well as to inhibit apoptosis [35]. This study uses two methods to measure apoptotic changes in HLECs, studies of nuclear morphology by Hoechst-staining and determination of caspase-3 activity, both demonstrating an increase of apoptosis in dexamethasone-exposed cells.

Cultured human lens epithelial cells incubated with dexamethasone did not exhibit significantly increased superoxide production, and peroxide production was only

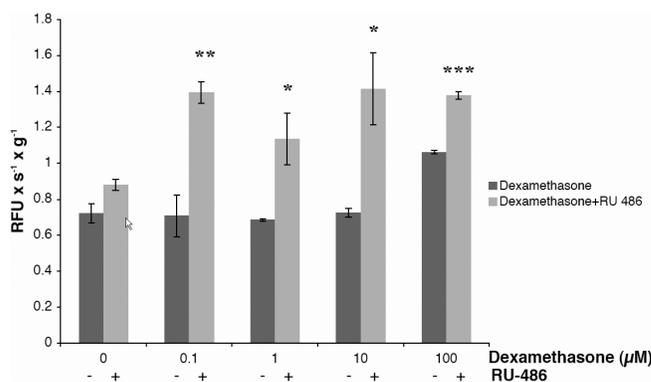


Figure 6. Effect of dexamethasone plus or minus the glucocorticoid receptor antagonist RU486. HLECs were incubated with dexamethasone at concentrations ranging from 0 to 100  $\mu$ M for 24 h with/without 10  $\mu$ M of the glucocorticoid receptor antagonist RU-486, after which apoptosis was determined by the caspase-3 assay. No protection of human lens epithelial cells against dexamethasone-induced apoptosis was seen by 10  $\mu$ M RU-486. Instead, simultaneous incubation with RU-486 significantly increased the proapoptotic effect seen by dexamethasone. Cells exposed to 10  $\mu$ M RU-486 alone showed no increase in caspase-3 activity. Mean  $\pm$ SEM from 3 separate culture wells are shown from one of four experiments with similar results; the asterisk indicates a  $p < 0.05$ , the double asterisk indicates a  $p < 0.01$ , and the triple asterisk indicates a  $p < 0.001$ .

altered when exposed 100  $\mu$ M dexamethasone. Furthermore, GSH levels in dexamethasone-exposed HLECs were not affected. These findings indicate that the increase of apoptosis by dexamethasone was not primarily due to oxidative stress. This is in contrast to a previous study by Dickerson, Dotzel, and Clark [13] who found that dexamethasone significantly decreased GSH levels in cultured rat lenses already at exposure to 1  $\mu$ M dexamethasone. In addition, Creighton et al. [5] have shown that the addition of the antioxidative vitamin E decreases cataract induced by methyl prednisolone in cultured rat lenses, further supporting oxidative mechanisms in the formation of steroid cataract. Different species and different experimental conditions may explain the discrepancies in results as compared with the present study. It is also likely that the use of whole lenses versus cultured lens epithelial cells may explain this difference since the lens epithelium is the most metabolically active part of the lens, containing by far more antioxidative capacity than the rest of the lens. Oxidative insults to an intact lens must hence rely to a high degree on the epithelium for antioxidative defense.

When using lens epithelial cells to study mechanisms for steroid cataract as in this study, one must bear in mind that there are only lens epithelial cells on the anterior side of the lens, and PSC, which is the cataract phenotype associated with glucocorticoid exposure, is located on the posterior side of the lens. However, the present study does not claim that cultured HLECs exposed to dexamethasone should constitute a model for steroid cataract, but they provide a useful way of studying intracellular effects of glucocorticoids on cultured lens epithelial cells. Of course, future studies on cultured whole lenses and whole animals are necessary to determine the pathogenic effects of steroids in a more complex biological system. However, when using intact lenses, it is important to monitor the integrity of the lenses, preferably by checking for protein leakage as described both by Tumminia et al. [36] and in a previous study from our group [37]. Other factors to take into consideration when setting up models for cataract formation is that *in vivo* cataract development is a very slow process and physiologically/clinically relevant models should also develop during an extended period. Nagai et al. [15] used systemic pulse administration to obtain steroid-induced cataract in brown Norway rats, a process that took 10 months before any visible opacification of the lens could be seen. Several investigators have also used cocataractogenic effects by including additional cataractogenic factors in combination with steroids in their cataract models. For instance, Shui et al. [38] developed a steroid cataract model employing a combination of long-term prednisolone exposure and a minimum of X-irradiation. This seems like a relevant approach since cataract in humans is considered a multifactorial disease.

It is well known that oxidative stress, among other effects, causes depolarization of the mitochondria. We have previously shown that the mitochondrial membrane potential

in HLECs is disrupted by hydrogen peroxide treatment [39]. However, exposure to dexamethasone up to 100  $\mu$ M for 24 h did not affect the mitochondrial membrane potential, which further supports that oxidative stress is not an important mechanism for dexamethasone-induced apoptosis in HLECs. It might be of interest to note that another steroid (estradiol at 1  $\mu$ M) may prevent the collapse of the mitochondrial potential during oxidative stress [40,41]. Estradiol has shown antioxidative properties in several studies [42-44] whereas the opposite has been reported for glucocorticoids [13,16].

The presence of a glucocorticoid receptor (GR) in the mammalian lens has long been questioned [15]. However, more recent studies have confirmed the existence of an active GR in the lens [8,9,45-47]. Still, the mechanism behind activation of the GR and its contribution in steroid cataract formation has not been elucidated.

In the present study, we investigated if inhibition of the GR by 10  $\mu$ M of the GR antagonist, RU-486, protected against dexamethasone-induced apoptosis. No such anti-apoptotic effect was seen by simultaneous incubation with dexamethasone and RU-486. Instead, the results indicated that RU-486 enhanced the proapoptotic effect of dexamethasone. The concentration of RU-486 used (10  $\mu$ M) was chosen after performing dose-response experiments in cells subjected to RU-486 alone. These experiments showed no apoptotic effect of RU-486 at 10  $\mu$ M or lower whereas higher concentrations were proapoptotic by itself (data not shown). This finding was also confirmed by microscopic examination of the cells (not shown). However, several studies in cell types other than lens epithelial cells have found a proapoptotic effect of RU-486 as well as an inhibitory effect on cell proliferation [48-50]. Navo et al. [50] proposed that the increase in apoptosis may be due to p53 activation rather than hormone receptor inactivation. It is possible that several signaling pathways are involved in RU-486-mediated apoptosis as well as in glucocorticoid response. In this study, the addition of RU-486 did not rescue from dexamethasone-induced apoptosis, but instead, apoptosis occurred at a lower concentration of dexamethasone when this GR inhibitor was present. This may indicate increased vulnerability of the HLECs to the proapoptotic effects of dexamethasone when simultaneously exposed to RU-486.

Since there is a direct link between prolonged steroid use and PSC, the present findings are of great importance for elucidating the mechanism behind cortisone cataract. Dysregulation of apoptosis may also be a mechanism linking corticosteroids to disturbances in lens fiber differentiation since the differentiation process at the lens equator is considered a limited form of apoptosis where the nuclei and organelles are degraded but the fibers persist [51-54]. Incomplete or defect differentiation of the lens fibers have previously been implied in the formation of PSC [26] so factors that affect the apoptotic process may contribute to or

lead to PSC. As this study demonstrated increased apoptosis in HLECs by dexamethasone, the present data supports the hypothesis that apoptotic dysregulation may be an important pathogenic mechanism in the formation of steroid-induced posterior subcapsular cataract.

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