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Dexamethasone influences FGF-induced responses in lens epithelial explants and promotes the posterior capsule coverage that is a feature of glucocorticoid-induced cataract

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Abstract

Aberrant spreading of lens epithelial cells along the posterior capsule is the basis for development of glucocorticoid (GC)-induced cataract; the resulting foci of nucleated cells at the posterior pole causing disruptions to normal lens cellular architecture. In this study, rat lens epithelial explants were used to assess the effects of dexamethasone (DEX), a widely used synthetic GC, on FGF2-induced lens cell proliferation and elongation as well as the ability of lens cells to spread and cover the posterior capsule. In the presence of FGF2, DEX significantly promoted lens cell proliferation after 48 hours. Cell coverage of the posterior capsule was also enhanced during 5 days culture. In contrast, cell elongation was retarded by the inclusion of DEX. In the absence of FGF2, DEX had no marked effects on any of these cellular processes. Thus, in the presence of FGF2, DEX promoted cell proliferation and posterior capsule coverage but inhibited cell elongation. These results provide insights into the molecular mechanism underlying GC-induced cataract in humans.

Keywords

Dexamethasone; fibroblast growth factor; lens epithelial cells; cell proliferation; cell coverage; cell differentiation; posterior subcapsular cataract

1. Introduction

Long-term treatment with glucocorticoids (GCs) leads to a high risk of cataract, specifically posterior subcapsular cataract (PSC) (Black et al., 1960; Urban and Cotlier, 1986). Generally, a defining characteristic of PSC is the presence of swollen fiber-like cells with retained nuclei that are located in the posterior cortex and towards lens posterior pole.

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Globular and amorphous material and smaller necrotic migrating cells have also been described (Eshaghian and Streeten, 1980; Greiner and Chylack, 1979). This causes disruption of the normally highly ordered lens cellular architecture and leads to light scattering in the affected region. Although GCs have been clearly implicated as causative agents for PSC, the cellular mechanisms behind their effects remain unclear.

The lens consists of two types of cells; a monolayer of epithelial cells overlies a mass of fiber cells, and they are both contained within a thickened basement membrane, the lens capsule (Lovicu and McAvoy, 2005). Lens epithelial cells line the anterior capsule and are normally absent from the posterior subcapsular region. Lens grows by continuous proliferation and differentiation of epithelial cells. Most proliferation takes place in a region above the equator known as the germinative zone. Progeny of cell divisions exit from the cell cycle and migrate below the equator where they differentiate into secondary fibers that are progressively added to the fiber mass throughout life (McAvoy, 1978a, 1978b). Fiber differentiation involves extensive cellular elongation and development of a precise hexagonal cross-sectional shape that facilitates their regular packing. Later in the differentiation process fiber cells lose their nuclei and most intracellular organelles. Precisely coordinated regulation of this sequence of cellular events is important for maintaining highly ordered lens cell architecture and lens transparency (Sugiyama et al, 2011).

Analysis of human GC-induced PSC specimens has shown the presence of aberrant cells associated with the posterior capsule; some with fiber cell characteristics but otherwise swollen and nucleated, and others with morphologies typical of migratory cells (Eshaghian and Streeten, 1980; Greiner and Chylack, 1979). The presence of these cells suggests a disturbance in the regulation of the highly ordered patterns of lens cell proliferation, migration and differentiation in the equatorial region. In an ex vivo cataract model using the synthetic steroid, Dexamethasone (DEX), rat lens epithelial cells spread along the posterior capsule towards the posterior pole. In addition, lesions that formed between fibers and posterior capsule resembled those in patients with GC-induced PSC (Lyu et al., 2003). This is consistent with observations from earlier studies on human steroid cataract (Eshaghian and Streeten, 1980), where the aberrant posteriorly-directed migration of lens epithelial cells away from the lens equator is reported to be an early event in PSC (James, 2007). However, GC-induced disturbance in regulation of cell proliferation and differentiation are also likely to be involved, since the regulation of these sequential cellular events is interrelated (Lovicu et al., 2011).

There is now compelling evidence that members of the FGF growth factor family are key regulators of the processes of proliferation, migration and differentiation in the lens (Chamberlain and McAvoy, 1997; Lovicu et al., 2011; McAvoy and Chamberlain, 1989); therefore GCs may have their effects by disturbing these FGF-induced processes. To assess the effects of GC on FGF-induced lens cell proliferation, migration and differentiation, we applied the lens epithelial explant model (Lovicu and McAvoy, 2008). Previous studies have shown that explanted lens epithelial cells can be induced to proliferate, migrate or differentiate depending on the concentration of FGF in the culture medium (McAvoy and Chamberlain, 1989). Here we show that the presence of the GC, Dexamethasone (DEX), enhances FGF-induced cell proliferation and cell coverage of the capsule. In contrast, the observation that DEX inhibits FGF-induced cell elongation indicates that it disturbs the process of fibre differentiation. This provides new insights into the cellular mechanisms of GCs-induced PSC.

2. Materials and methods

2.1 Preparation and treatment of rat lens epithelial explants

All animal procedures conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement on the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Ethics Committee, University of Sydney. Methodology has been described previously (Chen et al., 2006; Lovicu and McAvoy, 2008). In brief, Wistar rats of postnatal 6–7 days (P6–7) and 19–21 days (P19–21) were sacrificed and their lenses were isolated and collected in serum-free Medium 199, supplemented with 0.5mM L-glutamine, 100 IU/ml penicillin, 100µg/ml streptomycin and 2.5µg/ml Amphostat. For cell coverage assessment, reverse explants were employed. This type of explant was prepared by tearing the anterior capsule, removing the fiber mass, and pinning the posterior capsule onto the base of the culture dish; thereby providing the cell-free posterior capsule for cell coverage. Standard explants were also used in this study for assessment of cell proliferation and elongation. This type of explant was prepared by tearing the posterior capsule, removing the fiber mass, and pinning the anterior capsule onto the base of the culture dish (see Lovicu and McAvoy, 2008 for detailed preparation methods). Fibroblast growth factor-2 (FGF2, R&D Systems, Minneapolis, USA) and Dexamethasone (DEX, cell culture grade, Sigma, St Louis, USA) were immediately added to freshly set up explants. Explants were cultured as follows: 1) no treatment-Control, 2) DEX (100nM), 3) FGF2 (20ng/ml) and 4) DEX (100nM) and FGF2 (20ng/ml). The concentration of 20ng/ml FGF2 was used because it is within the range known to promote rat lens epithelial cell proliferation, migration and elements of differentiation (McAvoy and Chamberlain, 1989). DEX was used at 100nM because, as reviewed by Symonds et al (2006), this concentration was previously shown to be found in aqueous after application of dexamethasone eye drops during cataract surgery and had also been shown to influence the behaviour of cultured lens cells. Explants were cultured at 37 °C in a humidified 5% CO₂ incubator and observed daily by inverted phase contrast microscopy (IX71, Olympus, Tokyo, Japan). Culture medium was changed every 4 to 5 days.

2.2 Assessment of lens cell coverage of posterior capsule

The effect of DEX on lens cell coverage on reverse explants was assessed using Image J 1.62 software (developed by Wayne Rasband, NIH, Bethesda, MD). Measurements of cell-free posterior capsule were generated from 4X digital images using phase contrast microscopy with DPC controller imaging software (IX71, Olympus, Tokyo, Japan). A complete profile of each explant was put together by combining several images using Office PowerPoint 2010 (Microsoft, USA) (insert, Figure 1A). Since most of the explants exhibited a much slower rate of cell coverage after 5 days culture, assessments of cell coverage were performed from day 0 (immediately after explants preparation) to day 5. Preliminary studies also showed that reverse explants prepared from younger pups (P6–7) had higher viability than those prepared from older animals (P19–21). For this reason detailed assessments of cell coverage and phenotypic changes during this process were performed on explants from the P6–7 age group. However, additional experiments were carried out with explants from P19–21 rats to confirm that results from the experiments with the peri-natal rats were relevant to other ages. For the P19–21 age group, cell coverage was graded semi-quantitatively at day 5 of culture by assessing the proportion of the posterior capsule with cell coverage.

2.3 Morphological assessment of posteriorly spreading cells

To assess phenotypic characteristics of posteriorly spreading cells; E-cadherin, β -crystallin and acetylated-tubulin were immunolocalized in reverse explants. E-cadherin is a reliable marker for the lens epithelial phenotype as it is present at the margins of all lens epithelial

cells and is lost at the onset of fiber differentiation at the lens equator (Xu et al., 2002). β -crystallin is expressed early in the fiber elongation process and is a commonly used marker for fiber differentiation (McAvoy, 1978a). Acetylated-tubulin localizes to stabilized microtubules and is a useful way of delineating elongated cells. For immunofluorescent labelling, explants were fixed in 100% methanol for 45 seconds at room temperature followed by 4 successive washes with phosphate buffered saline (PBS). Non-specific cellular sites were blocked with the addition of normal donkey serum (1:10) in 0.1% BSA in PBS with incubation for 1 hour at room temperature. Primary antibodies (1:350–1:1000 dilution) were diluted in 0.1% BSA in PBS with normal donkey serum (1.5:100) and applied overnight at 4°C. On the following day, explants were incubated with appropriate secondary antibodies (conjugated with Alexa-488 or Alexa-594) (diluted 1:1000 with 0.1% BSA/PBS) for 120 minutes at room temperature. Primary and secondary antibodies used were as follows: mouse anti-E-cadherin and mouse anti- β -catenin (both from BD Biosciences-Pharmingen, San Diego, CA, USA), rabbit anti- β -catenin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), rabbit anti- β -crystallin (prepared as previously described (McAvoy, 1978a), mouse anti-acetylated tubulin (Sigma, St Louis, USA), donkey anti-rabbit IgG conjugated with Alexa-488/594 and donkey anti-mouse IgG conjugated with Alexa-488/594 (all from Molecular probes, Invitrogen, OR, USA). Labelled explants were visualized using a laser-scanning confocal microscopy (Axioskop 2 – LSM 5 Pascal, Carl Zeiss, Jena, Germany) and accompanying software.

2.4 Assessment of cell proliferation by EdU incorporation

Standard explants from rats of P19–21 were used for assessment of cell proliferation. To identify proliferating cells, 5-ethynyl-2'-deoxyuridine (EdU, 10 μ M, EdU HCS Assays Click-iT, Molecular probes, Invitrogen OR, USA), a nucleoside analog of thymidine which is incorporated into DNA during active DNA synthesis, was added to the medium after 24 hours or 48 hours culture and the explants incubated for a further 12 hours. Only explants with virtually complete cell coverage were included in the study. Explants were fixed in 2% paraformaldehyde for 30 min and then permeabilized using 0.5% Triton X-100 for 20 min. After blocking and washing, explants were incubated with Alexa 488-conjugated EdU mixture reagents for 60 min, prepared according to the manufacturer's instruction. Propidium iodide (PI, 100ng/ml) (Molecular Probes, Invitrogen, OR, USA) (1:10000) was applied for 10 min for visualization of the cell nuclei. Images were viewed by inverted fluorescence microscopy and laser-scanning confocal microscopy. Lens cell proliferation was determined by assessing the proportion of cells that were EdU-labeled. Two representative regions from each of the 12 explants in every treatment group were employed for counting. Cells within an area of 200 μ m² in each region were counted. The experiment was performed in triplicate. EdU-labelled cells were expressed as a percentage of total cells.

2.5 Assessment of lens cell elongation

Cell elongation is an early event of cell differentiation and is clearly evident in explants after 5 days culture with FGF2. Cell lengths in representative regions of standard explants cultured with FGF2 alone and FGF2/DEX were measured in order to compare the effects of these different treatments on cell elongation. In addition, immunofluorescent analysis using antibodies against β -crystallin, acetylated tubulin and β -catenin was employed for the assessment of cell differentiation on day 18 of culture.

2.6 Statistical analysis

Statistical analysis was performed with the SPSS software for Microsoft Windows (version 17.0; SPSS Inc., Chicago, IL, USA). Values were reported as Mean $\pm$ SD. One-way ANOVA followed by a Tukey's post-hoc test was used for comparison of cell proliferation and spreading in four treatment groups. The Mann-Whitney test was used for semi-quantitative

assessment of cell spreading. Independent T-test was used for comparison of cell length in FGF2- and FGF2/DEX-treated explants. A p value of 0.05 was considered statistically significant.

3. Results

3.1 Effects of DEX on lens cell coverage in explants cultured with FGF2

In the first set of experiments, reverse explants from rats of P6–7 were used to study the effects of DEX on cell coverage. After 1 day in all explants, epithelial cells near the lens equator showed prominent cell processes and began to spread out onto the posterior capsule (Fig.1 A–C). Note that whilst cell processes are indicative of migratory activity, it is acknowledged that changes in cell shape (notably flattening or spreading) are likely to contribute to the increased coverage of the capsule. Similarly increased cell proliferation (see later) is also likely to contribute to this process. For convenience we have opted to refer to the results of these phenomena collectively as cell coverage. At this early stage, no distinguishable difference in cell morphology was observed in the four treatment groups. Differences were observed as the culture progressed and after 3–5 days culture, cell processes were rarely detected in explants cultured without FGF2 (Control and DEX-treated groups). In these explants cells showed cobblestone-like packing (Fig.1D). Capsule wrinkling and cell-free areas were also detected in these explants after 5 days of culture (Fig.1G). By contrast, in FGF2 and FGF2/DEX-treated explants, cell processes were sustained for 5 days or more and the leading cells became elongated (Fig.1E, F). In some, but not all, explants, cell coverage increased until the posterior capsule was completely populated (Fig.1H, I); this occurred in 3 out of 10 FGF2-treated explants and in 4 out of 10 FGF2/DEX-treated explants after 5–7 days. Interestingly, suture-like structures formed where groups of cells from different segments of the lens met (Fig.1H, I, white dotted lines). In this region the growth factor-treated explants were approximately twice as thick as controls, indicating some multilayering of the elongated cells. For some explants, a small exposed region on the capsule remained even with an extended culture period of up to 15 days.

To quantify these cellular responses, the area of bare capsule was measured in each explant and the increase in cell coverage was calculated daily (Fig. 2). Over a five-day culture period, compared with controls (no FGF2), adding FGF2 to explants enhanced cell coverage significantly, consistent with earlier reports (see McAvoy and Chamberlain, 1989). In the presence of DEX, FGF2-treated explants showed significant enhancement of cell coverage compared with FGF2 alone from the second day ($p < 0.05$). DEX on its own had no obvious effects and cell coverage was similar to that of explants cultured with no additives (controls) ($p > 1.00$). The quantitative analysis showed that the biggest increase in cell coverage occurred between 1–2 days of culture in all four treatment groups. After 2 days of culture there was little, if any, increase in cell coverage in controls. In the FGF2 and FGF2/DEX-treated explants the daily increases in cell coverage continued up to 5 days but they were relatively smaller than the increases recorded over the first 2 days of culture.

An additional experiment was conducted with P19–21 day old rats to ensure that the influence of DEX on cell coverage was not restricted to the peri-natal stage. In general, epithelial cells from these older rats did not exhibit the same capacity to cover the capsule as cells from peri-natal rats. For this reason it was considered more appropriate to attribute each explant with a ‘grade of coverage’ after the 5 day culture period. The results presented in Table 1 show that in controls (both with DEX and with no additives), about 80% of explants showed no evidence of increased cell coverage. In explants treated with FGF2 alone about 66% showed no increased cell coverage; however, the presence of DEX reversed this trend and in FGF2/DEX a similar proportion showed evidence of an enhanced

response. Moreover, the extent of cell coverage was greatest in the FGF2/DEX treatment with 30% of explants having one-fifth to two thirds of the posterior capsule populated with cells. Taken together, these results show that for juvenile, as well as for peri-natal rats, DEX enhances cell coverage in FGF2-treated explants.

3.2 Phenotypic characteristics of the cells migrating on the posterior capsule

To assess the phenotypic characteristics of migrating cells, explants were immunostained for β -crystallin, E-cadherin and Acetylated-tubulin after 3 days culture. β -catenin immunolocalization was also used to delineate cellular boundaries. This showed that cells in control and DEX-treated groups retained their epithelial-shape and characteristic cobblestone-like packing arrangement (Fig.3A, B). In explants treated with FGF2 and FGF2/DEX, migrating cells in the leading edge became elongated, whereas cells in the rear were more similar morphologically to those in control explants (Fig. 3C, D).

Beta-crystallin synthesis is regarded as an early marker for fiber cell differentiation. The present study showed that in the absence of FGF2, weak reactivity for β -crystallin was present in some cells scattered throughout the explant (Fig.3E). By contrast, in the presence of FGF2, the elongated cells at the leading edge showed a strong reactivity for β -crystallin, indicative of an initiation of fiber cell differentiation (Fig.3G). Similarly, as a marker of epithelial cells, E-cadherin was strongly localized to the margins of most cells in the control and DEX groups but was only prominent in the cells behind the leading edge in FGF2-treated explants (Fig.3I, J, K). The addition of DEX to FGF2-treated explants appeared to have no effects on both β -crystallin (Fig.3H) and E-cadherin (Fig.3L) expression over and above those observed for FGF2 alone.

Acetylated-tubulin tended to localize in a radial array in the cytoplasm of lens epithelial cells in control and DEX-treated explants (Fig.3M, N). In FGF2- and FGF2/DEX-treated explants, acetylated tubulin was organized along the length of the elongated cells at the leading edge (Fig.3O, P). Also in explants-treated with FGF2 only, acetylated tubulin tended to be localized around the margins of the epithelial-like cells behind the leading edge. This contrasted with the more radially arranged localization of acetylated tubulin seen in controls and DEX-treated explants (compare insets in 3O and 3M).

3.3 Effects of DEX on lens cell proliferation

To determine the effect of DEX on cell proliferation in explants after 24 and 48 hours we added EdU after these time periods and harvested 12 hours later. EdU-labelled cells were present in all of the treatment groups; however, it was clear that substantially more EdU-labelled cells were present in the FGF2- and FGF2/DEX-treated groups (Fig. 4 C, D) compared with the controls (Fig 4. A, B). Quantitative analysis confirmed this and showed that FGF2 significantly promoted epithelial cell DNA synthesis at both time points ($p<0.001$) (Fig. 5). The combination of FGF2 and DEX promoted more EdU labeling than FGF2 alone, but this difference only reached significance after 48 hours ($p<0.001$). Promotion of cell proliferation by FGF2 and FGF2/DEX occurred mostly in central region of standard explants as illustrated in Fig.4C and D. There was no significant difference in EdU labeling between controls and DEX-treated explants at either time point ($p>0.3$).

3.4 Assessment of lens cell elongation

For the assessment of FGF2-induced cell elongation, standard explants from rats of P19–21 were cultured up to 18 days. Only explants that had virtually complete cell coverage after 2 days culture were included in this part of the study. After 5 days culture, cell elongation was evident in peripheral regions in all FGF2-treated explants. The elongating cells were usually radially aligned and oriented towards the explant center. Cells in the central regions tended

to remain epithelial-like, interspersed with irregularly enlarged cells. The length of elongated cells progressively increased over the culture period and the explant appeared to become multilayered. This was confirmed by confocal microscopy and optical sectioning of explants immunostained for acetylated-tubulin (Fig.6A). Evidence that this was part of the differentiation response came from strong reactivity for β -crystallin in these elongated cells (Fig.6A, insert). In the presence of DEX, FGF2-induced cell elongation was not as extensive and average cell length (0.211 ± 0.095 mm) was significantly less than that in FGF2-treated explants (0.482 ± 0.204 mm; $p=0.001$). Similar results were observed in reverse explants after 13 days culture and in these explants the elongated cells induced by the FGF2/DEX treatment were usually present in small groups with differing orientations (Fig.6C, D). Small groups of epithelial-like cells were also more prominent in FGF2/DEX treated explants compared to explants treated with FGF2 alone (Fig.6D).

4. Discussion

Tight regulation of the processes of proliferation, migration and differentiation are required for the lens to maintain its highly ordered cellular architecture and functional capacity. When these processes are disturbed cataract often results. Such is the case in PSC when groups of cells that result from aberrant migration/differentiation of epithelial cells become located in the posterior cortex and towards lens posterior pole. This form of cataract often accompanies the steroid administration that is commonly used to treat a range of conditions (Gupta and Wagner, 2009). Given the role of FGF in regulating proliferation, migration and differentiation of lens cells, we investigated GC effects on these FGF-induced processes. Here we showed that DEX significantly enhanced FGF2-induced lens cell proliferation and cell coverage of the exposed capsule. In contrast, DEX was observed to retard FGF2-induced elongation of lens fibers. These results provide insights into the mechanism for the disturbed cell behaviour below the lens equator that leads to the presence of abnormal lens cell phenotypes associated with the posterior capsule.

As already noted, a defining characteristic of PSC is the presence of swollen fiber-like cells with retained nuclei that are located in the posterior cortex and towards lens posterior pole. In addition globular and amorphous material and smaller necrotic migrating cells have also been described (Eshaghian and Streeten, 1980; Greiner and Chylack, 1979). In the current study we did not detect such abnormal phenotypes, however, our studies were restricted to relatively younger age groups so it may be informative to conduct similar experiments with older animals. Nevertheless, this study did show that DEX disturbed growth factor-induced responses and as this is likely to be a common feature of all age groups it opens the way for further studies on mechanisms of its action.

As mammalian lens cells have been shown to express functional GC receptors (GR) it has been suggested that PSC is a direct consequence of their activation (Gupta and Wagner, 2009). The activation of the cytoplasmically located GR is initiated by the binding of the GC ligand that can readily cross the cell membrane through passive diffusion. The GC-GR complex thereafter translocates into the nucleus and binds to the glucocorticoid response element (GRE) in the promoter region of specific genes and thereby can influence patterns of gene expression. The GC-induced response can also include secondary effects by virtue of the product of the primary response modifying the expression of another gene as well as the GC itself binding and interfering with the effectiveness of a transcription factor (Gupta and Wagner, 2009). Evidence that lens cells can respond to DEX comes from earlier studies with rat epithelial explants. In these, DEX was shown to modify aberrant lens cell behaviour induced by TGF β in a posterior capsular opacification (PCO) model (Mansfield et al., 2004; Symonds et al., 2006). In the presence of FGF or TGFs, DEX was also shown to induce the formation of 'arrays of needle-like cells' in explants after 5 days of culture (Symonds et al.,

2006). Evidence was presented that these ‘needle-like cells did not express the cataract marker, alpha-smooth muscle actin, or the epithelial marker pax-6. In the absence of any fiber differentiation makers, it is not clear if the distinctive cell elongation of these needle-like cells is part of a fiber differentiation response; however, given similarities with the FGF/DEX treatment used in the current study they may have some features in common with the elongated β -crystallin-expressing cells described in Figure 6. Other studies on primary cultures of human lens epithelial cells and on a human lens cell line have also described changes in global gene expression induced by DEX (Gupta et al., 2007; James et al., 2005). The analysis indicated that a broad range of transcripts were altered and that a proportion of these were known regulators of cell proliferation, migration, differentiation and apoptosis. Moreover, amongst these it was noted that components of MAPK/ERK and PI3-K/Akt signaling pathways were markedly changed by activation of GR. This is highly relevant to the current study because these signaling pathways, particularly ERK1/2, have been shown to play key roles in regulating FGF-induced lens cell proliferation and differentiation (Le and Musil, 2001; Lovicu and McAvoy, 2001; Zatechka and Lou, 2002).

In ocular lens, different cell behaviours are controlled by various growth factor families in the surrounding medium; however, up to now, FGF is the only factor that has been shown to initiate lens fiber cell differentiation (Lovicu et al., 2011). As already noted, a number of studies employing the lens epithelial explant system have reported that FGF-activated ERK1/2 is required for the fiber differentiation process to proceed. Interestingly, the duration of ERK1/2 activation determines the nature of the response; specifically, a low dose of FGF gives a transient activation of ERK1/2 (about 6 hours) and this results in proliferation, whereas a high dose of FGF gives sustained ERK1/2 activation (about 18 hours), which is required for fibre differentiation (Iyengar et al, 2007). In addition, other growth factors, such as PDGF, EGF and IGF, potentiate FGF-induced lens cell proliferation and differentiation by fine-tuning activation of ERK1/2 (Wang et al., 2010). Therefore, given the reports that DEX can influence ERK1/2 activity, one possible mechanism whereby DEX has its effects on FGF2-induced responses is by modulating ERK1/2 activity. Imbalance of the relationship between proliferation and differentiation would likely lead to aberrant cell behaviour and subsequent lens opacities. For example, if GC signaling reduced the duration of FGF2-induced ERK1/2 activation from sustained to transient then the cells would not progress from proliferation to differentiation compartments as required. In fact, our results showed that the presence of DEX enhanced FGF2-induced cell proliferation but retarded aspects of fibre differentiation. Based on this, assessment of the effects of DEX on FGF2-induced ERK1/2 activation and how this relates to lens cell proliferation and differentiation will be important areas for future study.

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Highlights

1. Aberrant lens cell behaviour is the basis for development of glucocorticoid (GC)-induced cataract;
2. Rat lens epithelial explants were used to assess the effects of dexametasone (DEX) on FGF2-induced lens cell behaviour;
3. The results show DEX promotes cell proliferation and cell coverage but inhibits cell elongation in the presence of FGF2;
4. The results provide insights into the molecular mechanism underlying GC-induced cataract.

Fig. 1. Morphology of posteriorly migrating cells in epithelial explants

Each image shows a representative segment of a reverse explant prepared from a P6–7 rat lens (inset in A shows whole explant) treated as indicated. Explants with nothing added (controls) or DEX gave a similar result over the culture period so representative images are combined (A, D, G). Epithelial cells began to spread out on day 1 in all treatment groups, with prominent cell processes evident in the leading cells (A, B, C, arrows and insets). By 5 days, cell processes were no longer apparent in control and DEX-treated explants and after this time there was little, if any, increase in cell coverage (D, G). Capsule wrinkling (arrowhead) and cell-free areas (arrow) were also observed after 5 days culture (G). For explants treated with FGF2 (B, E, H) and FGF2+DEX (C, F, I), there were indications of cell migration up to and beyond 5 days. Where cells from different segments met, suture-like structures formed (H, I, white dotted lines). Scale bar: 500 μ m. Insets, A: 1.5mm; B, C: 200 μ m; D: 100 μ m.

Fig. 2. Quantitative analysis of cell coverage in explants from 1–5 days of culture

Reverse explants from P6–7 rats were cultured in different conditions for up to 5 days. For explants with no additives (control) or DEX, cell coverage increased daily from 1–3 days but showed no increase at day 4 or day 5 of culture. There was no significant difference in cell coverage on any day between control and DEX-treated explants ($p > 0.3$). In the presence of FGF2, cell coverage increased significantly every day ($p < 0.003$). When DEX was included with FGF2 cell coverage also increased daily and in each case the increase was significantly different from the increase in explants cultured in FGF alone ($p < 0.05$).

Fig. 3. Phenotypic characteristics of cells migrating on posterior capsule

β -catenin (A–D), β -crystallin (E–H), E-cadherin (I–L) and Acetylated tubulin (M–P) were immunolocalized in reverse epithelial explants of P6–7 rats after 3 days culture. Images were taken just behind the leading edge of migrating cells in all treatment groups. Cells in explants without any drug treatments (control) remained epithelial-like, with characteristic cobblestone packing as delineated by membrane-associated β -catenin localization (A). Weak reactivity for β -crystallin was evident in the cytoplasm of some cells (E) but positive reactivity for E-cadherin at cell margins confirmed a predominantly epithelial phenotype (I). Explants treated with DEX showed similar localization patterns to controls (B, F, J). In FGF2- and FGF2/DEX-treated explants, elongated cells were evident at the leading edge (C, D, arrows). These elongated cells showed strong reactivity for β -crystallin (G, H) and diminished E-cadherin reactivity (K, L). Reactivity for acetylated-tubulin was prominent in the elongated cells near the leading edge in both FGF2- and FGF2/DEX-treated explants (O, P). It was also noted that in control and DEX-treated explants the acetylated tubulin tended

to be radially arranged throughout the cytoplasm (inset, M), whereas in FGF2-treated explants, the acetylated tubulin in the epithelial-like cells situated behind the elongated cells at the leading edge, tended to be located at cell margins (inset, O). Similar marginal localization of acetylated tubulin was not obvious in FGF2+DEX-treated explants (P). Scale bar: 50 μ m, insets: 25 μ m.

Fig. 4. Cell proliferation in response to DEX

EdU-labelling was used to identify cells undergoing DNA synthesis in standard explants from P19–21 rats after 48 hours culture. In control (A) and DEX-treated (B) explants EdU-labelled cells were found in patches and often these predominated towards the explant periphery. In FGF2 (C) and FGF2+DEX (D) treatment groups EdU-labelled cells were abundant throughout the explants; however, a higher density of labelled cells was evident in the FGF/DEX treatment group compared with FGF alone. Scale bar: 1.0mm

Fig. 5. Quantification of cell proliferation in response to DEX

Quantitative analysis of EdU-labelled proliferating cells after 24 hours (A) and 48 (B) hours standard explant culture. Compared with control and DEX-treated explants, EdU labelling in FGF2-treated explants was significantly promoted at both time points ($p < 0.001$). More EdU labelled cells were present in explants treated with FGF2/DEX compared with FGF2 alone. This difference was statistically significant after 48 hours ($p = 0.024$), but not after 24 hours ($p = 0.108$). Compared with controls, DEX had no significant effect on EdU labelling after either 24 hours ($p = 0.582$) or 48 hours ($p = 0.647$).

Fig. 6. Cell elongation in explants

Localization of Acetylated-tubulin immunoreactivity was used to assess cell elongation in standard explants after 18 days culture (A, B) and reverse explants after 13 days culture (C, D). Peripheral cells in FGF2-treated explants showed extensive elongation after 18 days culture. Cell elongation and differentiation were confirmed by localization of Acetylated tubulin (A) and synthesis of β -crystallin (insert, A). In FGF2+DEX-treated explants (B), elongated cells were mostly located at the explant periphery and cells were generally shorter in length than cells in explants treated with FGF alone. Reactivity for β -crystallin also tended to be weaker in FGF2/DEX-treated explants compared to explants treated with FGF2 alone (Compare insets in A and B that show elongated cells at explant periphery). Essentially similar differences between FGF2 and FGF2/DEX treatments were observed in reverse explants after 13 days culture (C, D). It was also noted that with FGF2/DEX treatment the elongated cells tended to be in small groups and often with different orientations. This was in contrast to the elongated cells in FGF2-treated explants that tended to have radial alignment and be similarly oriented. Scale bar: 1.0mm.

Table 1

Grades of cell coverage in epithelial explants from P19–21 rats

After 5 days culture, adding FGF2 appeared to increase cell coverage but this did not reach statistical significance (Mann-Whitney, $p=0.524$). In the presence of FGF2, DEX significantly promoted cell coverage (Mann-Whitney, $p=0.036$), whereas in the absence of FGF2, no significant difference was observed with addition of DEX (Mann-Whitney, $p=0.731$).

Grades of cell coverage	Control	DEX	FGF2	FGF2+DEX	Total
^a 1 (0)	16	16	14	7	52
2 (0+)	3	3	5	7	19
3 (1/5-1/3)	1	2	1	4	8
4 (1/3-2/3)	0	0	1	2	3
Total	20	21	21	20	82

^aCell spreading was categorized into 4 grades: 1) 0, no cells spreading out; 2) 0+, a few cells spreading out; 3) 1/5-1/3 cell coverage of the posterior capsule; 4) 1/3-2/3 cell coverage of the posterior capsule.