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7-Ketocholesterol stimulates differentiation of lens epithelial cells

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Purpose: To establish if oxysterols stimulate differentiation of lens epithelial cells (LEC).
Methods: Primary cultures of lens epithelial cells were incubated with 7-ketocholesterol (7-keto), 25-hydroxycholesterol (25-OH) or cholesterol at 10 μg/ml for 10 days. Cells incubated with 100 ng/ml basic fibroblast growth factor (b-FGF) were used as positive controls for differentiation. The expression of the differentiation marker $p57^{KIP2}$, proliferation marker PCNA (Proliferating Cell Nuclear Antigen) and fibers specific proteins γ-crystallin, CP49, MIP26 following treatment with oxysterols was determined by western blot. Differentiation into fiber cells was further confirmed by counting the number of lentoid bodies formed following incuabtion with 7-keto.
Results: LEC incubated with 7-keto presented higher levels of $p57^{KIP2}$ and showed expression of fiber specific proteins such as MIP26 and CP49, compared to cells incubated with 25-OH or cholesterol. The differentiation marker $p57^{KIP2}$ increased over time for cells incubated with 7-keto while there was a decline on the amount of the proliferation marker PCNA. The expression of the fiber specific proteins γ-crystallin, MIP26 and CP49 was detected after 5 days of incubation with 7-keto. Differentiation was accompanied by a seven-fold increase in the number of lentoid bodies formed.
Conclusions: Results show for the first time that 7-keto inhibits proliferation and stimulates differentiation of lens epithelial cells into fiber cells. The presence of 7-keto in the lens may disrupt the highly regulated differentiation program of LEC, compromising normal lens growth and transparency.

The eye lens contains two types of cells; the epithelial cells, which cover the anterior surface of the lens, and the fiber cells, that occupy almost the entire volume of the organ. Fiber cells differentiate form epithelial cells at the equator of the lens. During differentiation, lens epithelial cells exit from cell cycle and undergo significant morphological and biochemical changes that result in the formation of fully differentiated fiber cells, where virtually all organelles, including the nuclei, are absent [1-5]. This unique pattern of differentiation occurs only at the equatorial region of the lens, and fibers at increasingly advanced stages of differentiation accumulate concentrically at the interior of the lens [6]. Proper execution of the differentiation program seems to be required for lens transparency, since abnormalities that result in incomplete degradation of intracellular organelles are associated with various forms of cataract [7-10]. Lens plasma membrane is unique among eukaryotic cell membranes due to its extremely high content of cholesterol and deficit of polyunsaturated fatty acids. In fact, lens membranes contain the highest cholesterol concentration of any known biological membrane [11,12]. As the main unsaturated lipid present in lens membranes, cholesterol is prone to oxidation yielding a variety of oxidation products called oxysterols. Some of these were shown to be increased in human cataracts [13] and 7-ketocholesterol is the predominant oxysterol detected in cataractous lenses.

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Many oxysterols possess cytotoxic properties to a variety of cell types. In addition, oxysterols are implicated in a broad variety of cell mechanisms such as apoptosis [14], calcium uptake [15,16] and notably cell differentiation [17-19].

The physiological implications of accumulation of oxysterols in the lenses are still unknown. The aim of the present report is to determine whether oxysterols interfere with the normal differentiation program of lens epithelial cells by stimulating differentiation into fiber cells.

METHODS

Cell culture: For primary cultures of LEC eyeballs were removed from adult bovines and the anterior capsule of the lens, with the attached epithelium, was cut along the equator and cultured in a 24 well-plate containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco BRL Life Technologies, Inchinnan, UK), 100 iu/ ml penicillin, 100 μg/ml streptomycin, at 37 °C with 5% CO_2. The epithelial cells were then allowed to spread out from the capsule into the plate. At confluency, cells were trypsinised and cultured at a density of 1X 10^6 in 60 mm cell culture plates. The medium was changed every 3 days and bFGF or 7-keto were added back to the fresh medium.

Antibodies and reagents: The polyclonal antibody to $p57^{KIP2}$ was purchased from Sigma (Sigma, St Louis, MO). The polyclonal antibody to MIP26 was purchased from Alpha Diagnostic (San Antonio, TX). The polyconal antibodies to CP49 and γ-crystallin were a kind gift from Dr. Alan Prescott (University of Dundee, Scotland, UK). Monoclonal antibody to proliferating cell nuclear antigen (PCNA) was a kind gift

from Dr. David Lane (University of Dundee, Scotland, UK). Unless otherwise noted, all other reagents were from Sigma. Oxysterols were dissolved in ethanol.

Immunofluorescence: The cells seeded onto coverslips were fixed by incubation for 10 min in 4% paraformaldehyde (PFA) and permeabilised with 1% Triton X-100 in phosphate buffered saline solution (PBS) for 10 min at room temperature. The nuclei were stained with 4',6-diamidino-2-phenylndole (DAPI), diluted in PBS and incubated for 1 h at room temperature.

Western blotting: The cells were rinsed with PBS at 4 °C, ressuspended in lysis buffer (190 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 2.5% Triton X-100, 0.2% SDS, Roche protease inhibitor cocktail Complete Mini, 2 mM PMSF, 10 mM iodacetamide, 50 mM NaF, 500 μM $NaVO_4$), pH 8.3. Cells were lysed by sonication, denaturated with Laemmli buffer for 30 min, at 37 °C, and 40 μg of total protein were separated by SDS-PAGE. The proteins were then transferred to a PVDF membrane and probed with antibodies to γ-crystallin, MIP26, CP49, $p57^{KIP2}$ or PCNA. Entire bovine lenses were homogenized in lysis buffer and centrifuged at 12,000x g at 4 °C for 10 min. Supernatants were mixed with an equal volume of 2X Laemlli buffer, boiled for 5 min and 100 μg of total protein separated by SDS-PAGE. Lens extracts were then transferred to a PVDF membrane and probed as described above.

Lentoid scoring: The number of lentoid bodies was counted after 20 days of treatment with b-FGF or 7-keto. A lentoid body was considered as a spherical and highly refractive multicellular cluster, clearly distinguishable form non-differentiated cells. The scoring of lentoid bodies was done in a single blind manner, by four observers who do not know the identity of the cells. The coefficient of variation between observers on counting lentoids was typically lower than 8%.

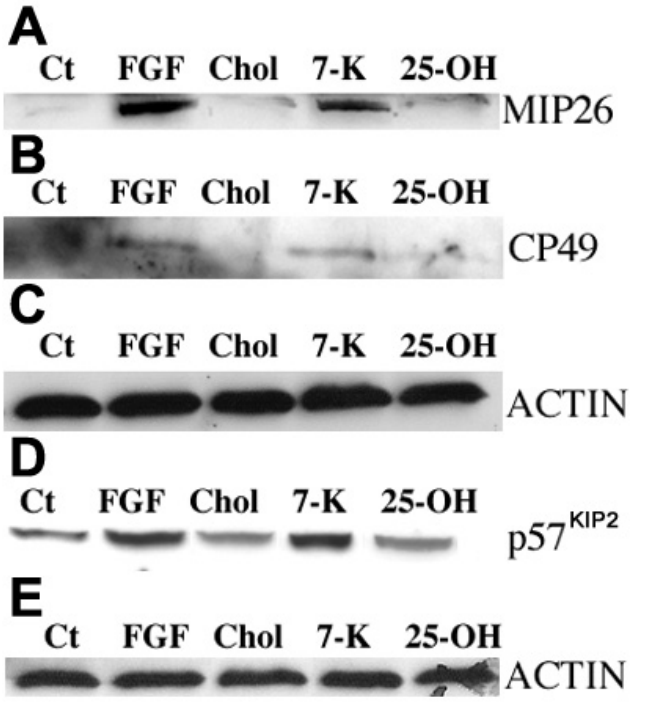

Figure 1. 7-keto increases expression of differentiation markers for fiber cells. Immunodetection of MIP26 (**A**), CP49 (**B**) and $p57^{KIP2}$ (**D**) in lens epithelial cells incubated with b-FGF or oxysterols, for 10 days. Lysates of LEC exposed to 100 ng/ml b-FGF (lane 2), or to 10 μg/ml cholesterol (lane 3), 7-keto (lane 4), or 25-OH (lane 5) for 10 days were western blotted and probed with **A**; polyclonal antibodies directed against MIP26, **B**; polyclonal antibodies directed against CP49, or **D**; polyclonal antibodies directed against $p57^{KIP2}$. Control cells were incubated in the presence of ethanol 0.1% ethanol (lane 1). Actin (Figure 1C and Figure 1E) was used as a loading control. Results are representative of at least three separate experiments.

RESULTS & DISCUSSION

It was recently shown that oxysterols stimulate differentiation of various types of cells, including keratinocytes [17], Leydig cells [19], and monocytes [18]. By analogy with these observations it is conceivable that oxysterols may induce differentiation of LEC into fibers, thus accounting for disruption of normal cell differentiation and growth, compromising lens transparency. To test this hypothesis, primary cultures of epithelial cells obtained from bovine lenses were incubated with 7-ketocholesterol (7-keto), 25-hydroxycholesterol (25-OH) or cholesterol at 10 μg/ml for 10 days. Cells incubated with 0.1% ethanol were used as control. Positive controls for differentiation were generated by incubating LEC with b-FGF. Indeed, fibroblast growth factor signaling is one of the most important regulators of differentiation of lens epithelial cells into fibers [20,21]. In concentrations higher than 50 ng/ml, FGFs effectively induces cell cycle withdrawal, loss of the characteristic cuboidal morphology, elongation of the cells [22,23], and expression of proteins characteristic of differentiated fibers, such as filensin, CP49, MIP26, connexin50 and crystallins [24-26]. Regulatory proteins involved in cell cycle, such as the cyclin-dependent kinase inhibitor $p57^{KIP2}$ [27-30] and PCNA [31-34] are good markers for lens cell differentiation and proliferation respectively. In this report the expression of general differentiation markers, such as $p57^{KIP2}$, and the expression of lens fiber specific proteins, including γ-crystallin, CP49 and MIP26 were all used as markers for differentiation of LEC into fibers. PCNA was used as a proliferation marker.

The levels of fiber specific proteins CP49 and MIP26 following treatment with two oxysterols was investigated by western blotting. Cells treated with 7-keto presented higher levels of MIP26 (lane 4 of Figure 1A) and CP49 (lane 4 of Figure 1B), as compared to controls (lane 1 of Figure 1A and Figure 1B), to cells incubated with 25-OH (lane 5 of Figure 1A and Figure 1B) or to cells incubated with cholesterol (lane 3 of Figure 1A and Figure 1B).

The content of $p57^{KIP2}$, a general marker of differentiation, was also determined. The results shown in Figure 1D indicate that cells incubated with 7-keto presented higher levels of $p57^{KIP2}$ (lane 4 of Figure 1D) as compared to controls (lane 1 of Figure 1D), to cells incubated with 25-OH (lane 5 of Figure 1D) or to cells incubated with cholesterol (lane 3 of Figure 1D). Cells incubated with b-FGF 100 ng/ml were used

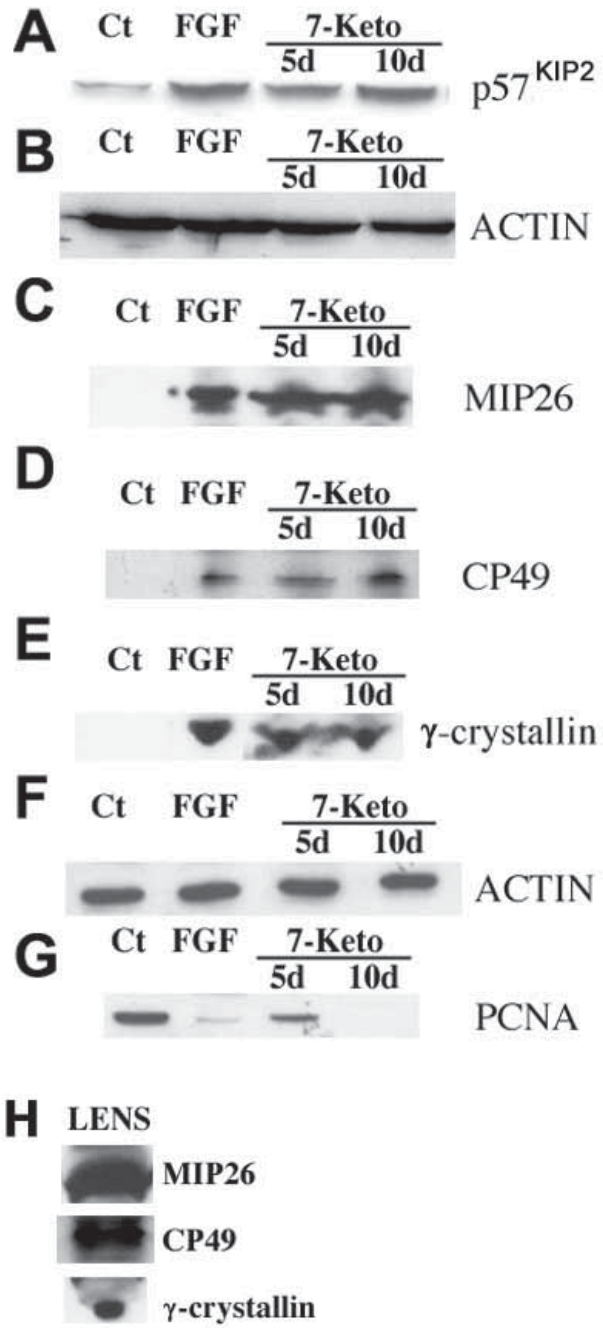

Figure 2. 7-keto stimulates differentiation and inhibits proliferation of LEC. Immunodetection of $p57^{KIP2}$ (**A**), MIP26 (**C**), CP49 (**D**), γ-crystallin (**E**), and PCNA (**G**) in lens epithelial cells incubated with 7-keto. LEC were incubated with 100 ng/ml b-FGF (lane 2) for 10 days or 10 μg/ml 7-keto for 5 (lane 3) or 10 days (lane 4). The controls were incubated in the presence of ethanol 0.1% ethanol for 10 days (lane 1). Cell lysates were western blotted and probed with polyclonal antibodies directed against $p57^{KIP2}$ (**A**), MIP26 (**C**), CP49 (**D**) or γ-crystallin (**E**), or probed with monoclonal antibodies directed against PCNA (**G**). Actin (**B** and **F**) was used as a loading control. Results are representative of at least three separate experiments. As positive controls for the presence of fiber specific proteins, lysates of entire bovine lenses (**H**) were western blotted and probed with polyclonal antibodies directed against MIP26 (line 1), CP49 (line 2) or γ-crystallin (line 3).

as positive controls for differentiation (lane 2 of Figure 1A, Figure 1B, and Figure 1D).

Figure 2 shows the levels of $p57^{KIP2}$ and expression of lens fiber specific proteins as a function of the time in culture. Data shows that the fiber specific proteins MIP26, CP49 and γ-crystallin were absent in non-treated controls but were highly expressed in cells incubated with 7-keto, as well as in positive controls incubated with b-FGF. The levels of these proteins reached a maximum after 5 days of incubation (lane 3 of Figure 2C through Figure 2E) and were not significantly altered at later times in culture. Moreover, when the concentration of

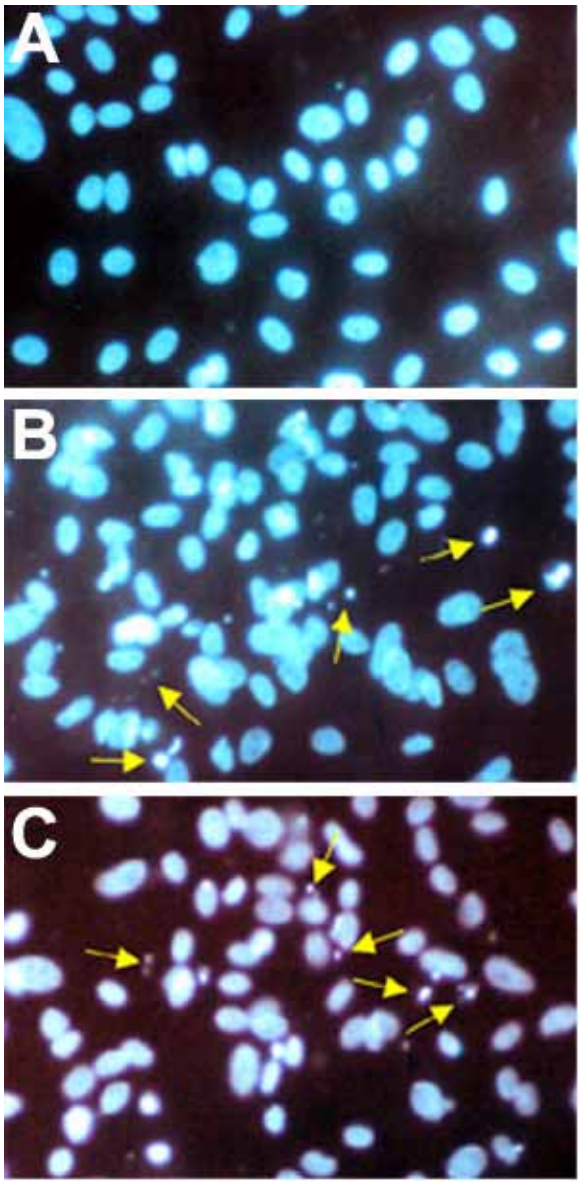

Figure 3. 7-keto induces nuclear pycnosis characteristic of differentiated LEC. Immunofluorescence detection of nuclei in LEC incubated with 100 ng/ml b-FGF or 10 μg/ml 7-keto for 10 days. Controls were incubated in the presence of ethanol 0.1% ethanol for 10 days **A**: The cells were stained with DAPI. The arrows point to pycnotic nuclei which are abundant in LEC treated with 7-keto (**C**) or b-FGF (**B**).

7-keto in the medium increased from 5 μg/ml to 10 μg/ml, the levels of differentiation markers did not vary (data not shown). On the other hand, the levels of the proliferation marker PCNA (Figure 2G) decreased with time of incubation (compare lane 3 with lane 4 of Figure 2G), whereas the levels of $p57^{KIP2}$ increased over time (compare lane 3 with lane 4 of Figure 2A), indicating that 7-keto consistently inhibited cell proliferation, while stimulating differentiation. LEC incubated with b-FGF 100 ng/ml for 10 days (positive controls) showed increased levels of $p57^{KIP2}$ (lane 2 of Figure 2A), MIP26 (lane 2 of Figure 2C), CP49 (lane 2 of Figure 2D) and γ-crystallin (lane 2 of Figure 2E). The levels of PCNA consistently decreased following incubation with b-FGF (lane 2 of Figure 2G). Fiber cells obtained from bovine lenses were used as a positive control for the presence of fiber specific proteins (Figure 2H).

A well established morphological change that accompanies differentiation of lens epithelial cells is nuclear pycnosis, eventually culminating in the entire disappearance of nuclei from mature fibers [35]. Changes in nuclear morphology associated with differentiation were evaluated by immunofluorescence, following staining with DAPI. Figure 3 shows that cells incubated with 7-keto (Figure 3C) presented an increased number of smaller and pycnotic nuclei (arrows). The same result was observed when cells were incubated with b-FGF (Figure 3B), while controls showed very few signs of pycnosis, with most nuclei presenting a normal and homogeneous morphology. Taken together, these results suggest that 7-keto stimulates differentiation of lens epithelial cells into fiber cells. Differentiation of primary cultures of LEC culminates in the formation of spherical and highly refractive multicellular clusters called lentoids. The number of lentoid bodies was counted and considered as an indicator of the extent of differentiation of LEC [24,36,37]. After 20 days of incubation the number of lentoid bodies increased about seven times in cells incubated with 7-keto, while in the presence of b-FGF the number of lentoids increased nine times, as compared to controls (Figure 4). The coefficient of variation associated with lentoid counting was found to be less than 8%.

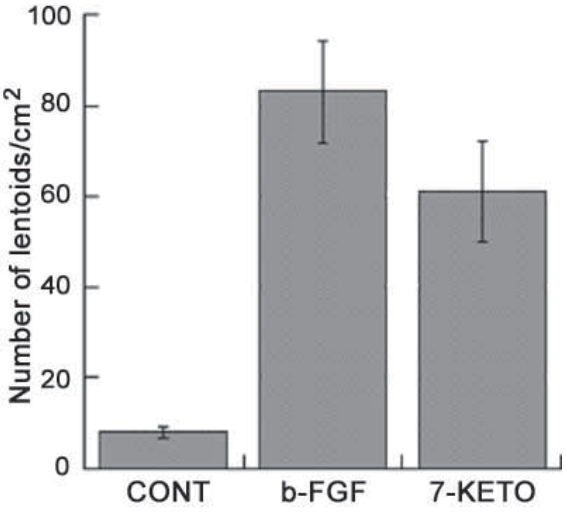

Figure 4. 7-keto induces formation of lentoid bodies. Lentoid bodies scoring. LEC were incubated with 100 ng/ml b-FGF or 10 μg/ml 7-keto for 20 days. Controls were incubated with 0.1% ethanol. The lentoid bodies formed were counted and plotted in a graph. Data represents the average±SD for counting of three separate plates. Coefficient of variation associated with lentoid scoring by different observers (n=4) was found to be less than 8%.

Lens epithelium consists of a single layer of cells containing three distinct regions: the quiescent central epithelium, the equatorial proliferative epithelium and the transition zone. Lens epithelial cells are formed in a peripheral germinative zone close to the equator, and then migrate towards the proliferating and transition epithelium at the equatorial region of the lens. At this point cells withdraw from cell cycle and begin to differentiate into fibers [2].

Proper execution of the differentiation program is vital for the formation of a normal transparent lens. Disregulation of the differentiation program may thus have a direct impact in degradation of intracellular organelles and formation of mature transparent fibers. Previous data obtained from our laboratory showed that oxysterols are present in human lens and that 7-keto accumulates in cataracts [13]. However, the physiological implications of the accumulation of such oxysterols remain unknown. The results presented in this report provide a new insight into the mechanisms whereby oxysterols may compromise normal lens growth and differentiation. Indeed data presented in this study shows, for the first time, that 7-keto stimulates differentiation of lens epithelial cells into fibers.

Differentiation of lens epithelial cell is a complex and tightly regulated biological process. At low levels oxysterols are suggested to participate in specific signaling pathways involved in cell differentiation [17-19]. This, however, does not preclude a cytotoxic role for oxysterols in the lens, but rather emphasises the importance of keeping the concentration of oxysterols present in the lens, at any given stage, under tight control. By interfering with the endogenous, highly regulated, differentiation program oxysterols may compromise normal lens growth and transparency and may be implicated in the cataratogenic process.

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