Cholestanol Induces Apoptosis of Corneal Endothelial and Lens Epithelial Cells

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PURPOSE. To determine whether cholestanol induces corneal endothelial and lens epithelial cell death in vitro.

METHODS. Cornea endothelial and lens epithelial cells were cultured in minimum essential media with 10% fetal bovine serum containing 10 µg/ml cholesterol in ethanol, 10 µg/ml cholestanol in ethanol, or 1% ethanol. These cells, stained using the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) method, and a commercially available quantitative apoptosis of corneal endothelial cells and lens epithelial cells could explain the mechanism of corneal opacities observed in hypercholestanolemic mice.

In the present study we found that cholestanol induced apoptosis of corneal endothelial cells and lens epithelial cells, determined by using the TdT-mediated dUTP nick-end labeling (TUNEL) method, and a commercially available quantitative method (Apoptiser A: Takara, Shiga, Japan; SYBR Green I Nucleic Acid Stain; Molecular Probes, Rockland, ME). We also observed the concomitant induction of interleukin-1β-converting enzyme (ICE) and CPP32 protease activities. These results indicate that cholestanol-induced apoptosis of corneal endothelial and lens epithelial cells could explain the mechanism of corneal opacities observed in hypercholestanolemic mice and of cataract in patients with CTX.

RESULTS. Both cornea endothelial and lens epithelial cells cultured with 10 µg/ml cholestanol showed a significant loss of viability. The nuclei of these cells cultured with 10 µg/ml cholestanol were more frequently stained than those exposed to 10 µg/ml cholesterol or 1% ethanol. Quantitative analysis of apoptotic DNA fragmentation confirmed that the cholestanol induced apoptosis of these cells in a time-dependent manner. The activities of interleukin-1β-converting enzyme (ICE) and CPP32 proteases for cells cultured with 10 µg/ml cholestanol were significantly higher than those observed in control cells.

CONCLUSIONS. In vitro, cholestanol was taken up by corneal endothelial cells and lens epithelial cells, an event that led to apoptosis of these cells. (Invest Ophthalmol Vis Sci. 2000;41:991–997)

Cerebrotendinous xanthomatosis (CTX) is an hereditary lipid storage disease characterized by hypercholestanolemia, Achilles tendon xanthomas, cerebellar ataxia, dementia, and cataract.1–3 The cause of cerebellar ataxia, dementia, and cataract is poorly understood. We previously reported that corneal opacities were observed in 20% of hypercholestanolemic mice.4 More recently, we found that the level of cholestanol in the serum, cerebellum, lens, and aqueous humor was high in hypercholestanolemic rats.5 We hypothesized that cholestanol may induce apoptosis of cells, and we found that cholestanol induces cerebrospinal neuronal cells in vitro.6 Next, we asked whether cholestanol induced apoptosis of corneal endothelial cells and lens epithelial cells in vitro.

In the present study we found that cholestanol induced apoptosis of corneal endothelial cells and lens epithelial cells, determined by using the TdT-mediated dUTP nick-end labeling (TUNEL) method, and a commercially available quantitative method (Apoptiser A: Takara, Shiga, Japan; SYBR Green I Nucleic Acid Stain; Molecular Probes, Rockland, ME). We also observed the concomitant induction of interleukin-1β-converting enzyme (ICE) and CPP32 protease activities. These results indicate that cholestanol-induced apoptosis of corneal endothelial and lens epithelial cells could explain the mechanism of corneal opacities observed in hypercholestanolemic mice and of cataract in patients with CTX.

METHODS

Cell Cultures and Treatment of Bovine Cornea Endothelial Cells and Lens Epithelial Cells

Bovine cornea endothelial cells6 and lens epithelial cells7 were prepared and kept in culture. Bovine eyeballs were collected from a local abattoir. The culture dishes were first coated with poly-L-ornithine (100 µg/ml). Corneas from the eyes were excised together with the scleral rims. Under a light microscope, endothelium and Descemet’s membrane were removed from the corneal stroma. Explants of endothelium and Descemet’s membrane were incubated in a solution of trypsin (0.05%) and EDTA Na (0.53 mM) in Ca2+- and Mg2+-free Hanks’ balanced salt solution for 5 minutes at 37°C. The cells were placed in tissue culture dishes (60 mm; Falcon Labware, Oxnard, CA). Lenses were removed, and the lens capsule was removed at the anterior pole and separated from the lens fibers, by using two pairs of forceps. The explants were pinned down with the epithelial cells facing downward. Cultures were maintained in culture medium consisting of minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Gibco, Grand Island, NY) in a humidified atmosphere of 5% CO2 at 37°C. The exponentially growing cells were collected after trypsin-EDTA treatment and subcultured in dishes at a split ratio of 1:3. Half the medium was replaced with
fresh medium after 3 days. The medium was replaced with four different media (MEM with 10% FBS, MEM with 10% FBS containing 10 μg/ml cholesterol in ethanol, 10 μg/ml cholestanol in ethanol, or 1% ethanol) after 6 days. Media were replaced with fresh media every 3 days.

**Cell Viability**

At various time points, cells cultured with medium containing 10 μg/ml cholesterol, 10 μg/ml cholestanol, or 1% ethanol were collected and suspended in phosphate-buffered saline (PBS). Aliquots of the preparation were mixed with an equal volume of 0.4% trypan blue stain (Gibco). The nuclear area of the cells stained with trypan blue stain was counted by light microscope (×400). The experiment in triplicate was performed three times.

**Biochemical Analysis**

Cholesterol (5-cholesten-3β-ol), cholestanol (5α-cholestan-3β-ol), and epicoprostanol (5β-cholestan-3α-ol) as an internal standard were purchased from Sigma Chemical (St. Louis, MO). All other chemicals and solvents used were of the highest grade available, unless otherwise stated. Sample preparation and analysis of sterols by high-performance liquid chromatography (HPLC) were performed as described.10 Cultured bovine cornea endothelial cells (1.3–2.0 × 10^4 cells) and lens epithelial cells (0.4–2.0 × 10^4 cells) were diluted with 10 volumes of 1 M ethanolic KOH and hydrolyzed at 80°C for 1 hour, followed by extraction twice with n-hexane. The solvent was evaporated under a stream of nitrogen, derivatized with a benzoyl chloride reagent, and analyzed by HPLC using 5β-cholestan-3α-ol as an internal standard. The column was packed with SBC-ODS (2.5-mm inside diameter × 15 cm; Shimadzu, Tokyo, Japan) and maintained at 47°C during analysis.

**Detection of Apoptosis**

DNA breaks were detected in situ by the Tdt-UTP nick-end labeling (TUNEL) method,9 an approach based on specific binding of terminal deoxyxynucleotidyl transferase (TdT) to 3'-OH ends of DNA, thus ensuring synthesis of a polydeoxynucleotide polymer. Cells were trypsinized, collected by centrifugation, and rinsed with PBS. Protein concentration was determined from a standard curve. The reader at 365-nm excitation and 450-nm emission wavelengths. The experiment in triplicate was performed three times. The ratio of fluorescence level of samples derived from cells cultured with medium containing 1% ethanol, 10 μg/ml cholesterol, or 10 μg/ml cholestanol versus the fluorescence level of samples derived from cells cultured with MEM containing 10% FBS was calculated.

**Activities of ICE and CPP32 Proteases**

Cysteine protease activity was measured using a modified procedure of Walker et al.10 Cells were trypsinized and collected by centrifugation and rinsed with PBS. Protein concentration was measured by the Bradford method.11 Cells were suspended in lysis buffer (50 mM Tris-HCl [pH 7.5] and 0.2% Triton, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and incubated at 37°C for 10 minutes. Lysates were mixed with reaction buffer (50 mM Tris-HCl [pH 7.5], 2 mM dithiothreitol, 1 mM EDTA, and 40% glycerol) and incubated with 10 mM cysteine substrate Ac-Tyr-Val-Ala-Asp-MCA (Ac-YVAD-MCA) for 1 hour. Amino-4-methylcoumarin release was measured spectrophotometrically using the plate reader at 365-nm excitation and 450-nm emission wavelengths. Its concentration was determined from a standard curve. The activities of ICE and CPP32 proteases in colon cancer cells (Colo 201; Japan Health Sciences Foundation, Osaka, Japan) treated with 10 mM or 100 mM 1-β-D-arabinofuranosylcytosine (ara-C) for 12 hours were measured as a positive control.12-14

**Statistical Analysis**

Data from two independent experiments performed in triplicate are presented as mean ± SD unless otherwise indicated. Statistical analysis was made using analysis of variance, with comparison of different groups by Fisher’s partial least-squares difference (PLSD) test, and Scheffe’s F test (Statview II; Abacus Concepts, Berkeley, CA).

**RESULTS**

**HPLC Determination of Sterols in Cultured Cells**

Cornea endothelial cells and lens epithelial cells were cultured with three kinds of media. Figure 1A shows contents of sterols in cornea endothelial cells cultured for 12 days. The density of cholesterol in cells cultured with cholesterol (2.01 ± 1.53 μg/10^5 cells) was significantly higher than that in cells cultured with cholesterol (0.10 ± 0.20 μg/10^5 cells) and 1% ethanol (0.19 ± 0.45 μg/10^5 cells; P < 0.01; Fig. 1A, right panel). On the contrary, the level of cholesterol did not significantly differ among these three groups (Fig. 1A, left). Figure 1B shows the contents of sterols in lens epithelial cells cultured for 24 days. The density of cholesterol in cells cultured with cholestanol...
was significantly higher than that in cells cultured with cholesterol (0.27 ± 0.63 mg/10^5 cells) and 1% ethanol (0.13 ± 0.33 mg/10^5 cells; P, 0.05; Fig. 1B, right). The level of cholesterol was not significantly different among these three groups (Fig. 1B, left).

Cell Viability of Cultured Cells

The viability of cornea endothelial cells and lens epithelial cells was measured using the trypan blue method. Figure 2 shows the viability of cornea endothelial cells and lens epithelial cells. The viability of cornea endothelial cells cultured with cholestanol for 9 and 18 days was 65.6% ± 1.1% and 54.0% ± 0.8%, respectively (Fig. 2A). The viability was significantly lower than that of control cells cultured for 9 (89.2% ± 1.2%; P < 0.01) and 18 days (80.5% ± 2.4%; P < 0.01; Fig. 2A). There was no significant difference in cell viability between cells cultured with cholesterol and control cells (Fig. 2A). The doubling times of corneal endothelial control cells, of cells cultured with cholesterol, and of cells cultured with cholestanol were 10.9 ± 2.5 hours, 8.4 ± 0.6 hours, and 9.4 ± 0.9 hours, respectively. The differences among these groups were not statistically significant.

As shown in Figure 2B, the viability of lens epithelial cells cultured with cholestanol for 14 and 28 days was 72.4% ± 0.9% and 65.2% ± 2.3%, respectively. The viability was significantly lower than that of control cells cultured for 14 (80.2% ± 1.2%; P < 0.01) and 28 days (74.8% ± 0.9%; P < 0.05). There was no significant difference in cell viability between cells cultured with cholesterol and control cells (Fig. 2B). The doubling times of lens epithelial control cells, of cells cultured with cholesterol, and of cells cultured with cholestanol were 13.8 ± 1.8 hours, 13.6 ± 1.6 hours, and 13.2 ± 1.4 hours, respectively. The differences between these groups were not statistically significant.

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932907/)

**Figure 1.** Concentration of sterols in cultured bovine cornea endothelial and lens epithelial cells. Cornea endothelial cells (A) were cultured for 12 days and lens epithelial cells (B) were cultured for 24 days in medium containing 1% ethanol (control), 10 µg/ml cholesterol, or 10 µg/ml cholestanol, and the contents of cholesterol and cholestanol were determined. Values are means ± SD from triplicate assays. Significantly different: *P < 0.01, **P < 0.05, by Scheffe’s F test.

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932907/)

**Figure 2.** Viability of cornea endothelial cells and lens epithelial cells. Cell viability of cornea endothelial cells cultured for 9 and 18 days (A) and lens epithelial cells cultured for 14 and 28 days (B) was measured by trypan blue staining. Cells cultured with 1% ethanol (control), 10 µg/ml cholesterol, or 10 µg/ml cholestanol were stained with trypan blue solution. Values are means ± SD from triplicate assays. Significantly different from control values: *P < 0.01, **P < 0.05, by Fisher’s PLSD test.
Because cholestanol decreased cell viability, we next asked whether cholestanol would induce apoptosis of cornea endothelial and lens epithelial cells. The cells were stained using the TUNEL method and analyzed by the cytometer every 3 and 10 days, respectively. Figure 3 shows a typical distinct pattern of staining in cornea endothelial after 9 days (Fig. 3A) and lens epithelial cells after 20 days (Fig. 3B) culture with cholestanol. On the contrary, in the control and cholesterol groups, no significant nuclear staining was evident (Figs. 3A, 3B). Figure 4 shows the time course of apoptosis induced in cornea endothelial and lens epithelial cells. In cornea endothelial cells (Fig. 4A) for up to 6 days, the percentages of positive cells were not significantly different between any of the three groups: cells cultured with cholestanol, with cholesterol, and with 1% ethanol. However, after 9 days the percentage of positive cells treated with cholestanol was 34%, a value significantly higher than that in the control cells (8%; \( P < 0.01 \)). In lens epithelial cells (Fig. 4B) for up to 10 days, the percentages of positive cells did not differ significantly between any of the three groups. The percentages of positive cells after 20 and 30 days were 27% and 42%, respectively—values significantly higher than values for the control cells (5% and 7% after 20 and 30 days, respectively; \( P < 0.01 \)).

**Apoptotic DNA Fragmentation of Cultured Cells**

The quantity of apoptotic DNA fragments was measured using the ApopLadder Ex/SYBR Green I Nucleic Acid Stain method. Figure 5 shows the ratio of fluorescence level for each sample, compared with that observed in normal cells cultured in MEM containing 10% FBS. The ratio of apoptotic DNA fragmentation of cells cultured with cholestanol was significantly higher than that of the control group in both cornea endothelial cells after

**Figure 3.** Apoptosis induction in cornea endothelial cells and lens epithelial cells. Cornea endothelial cells (A) and lens epithelial cells (B) were stained using the TUNEL method and analyzed by laser cytometry (magnification \(< 100\)).
6 days (P = 0.01; Fig. 5A) and lens epithelial cells after 18 days (P = 0.01; Fig. 5B).

**Activities of ICE and CPP32 Proteases of Cultured Cells**

Because caspases such as ICE and CPP32 are induced in apoptosis, we next performed experiments to determine whether cholestanol induces ICE and CPP32 protease activities. Neither ICE nor CPP32 activity was induced in cornea endothelial cells cultured for 4 days in any medium (Figs. 6A, 6B). However, the ICE activities in cornea endothelial cells cultured with cholestanol for 9 days were 11.6 ± 0.62 U/mg protein, a value significantly higher than that for control cells (5.13 ± 0.35 U/mg protein; P < 0.01; Fig. 6A). The CPP32 protease activity of cornea endothelial cells cultured with cholestanol for 9 days was 12.3 ± 1.34 U/mg protein, a value significantly higher than that for control cells (7.10 ± 0.78 U/mg protein; P < 0.01; Fig. 6B). On the contrary, neither ICE nor CPP32 activity was induced in cells cultured with cholesterol or control cells.

Thus, both ICE and CPP32 protease activities of cornea endothelial cells cultured with cholestanol were significantly induced by cholestanol in a time-dependent manner.

The ICE protease activities of lens epithelial cells cultured with cholestanol for 9 days (7.16 ± 0.6 U/mg protein) were not significantly different from those of control cells (6.60 ± 0.28 U/mg protein) but were significantly different after 20 days (13.2 ± 0.94 U/mg protein) compared with controls (8.51 ± 0.53 U/mg protein; Fig. 6C; P < 0.01). On the contrary, the ICE protease activities of lens epithelial cells cultured with cholesterol for 9 days (7.16 ± 0.6 U/mg protein) and for 20 days (9.66 ± 0.58 U/mg protein) were not significantly different compared with those of control cells for 9 days (6.60 ± 0.28 U/mg protein) and 20 days (8.51 ± 0.53 U/mg protein; Fig. 6C). After 9 days, the ICE activities (4.39 ± 0.39 U/mg protein) of lens epithelial cells cultured in MEM containing 10% FBS were significantly different from those of control cells cultured in MEM containing 1% ethanol. The data may suggest that 1% ethanol induces ICE activity. However, after 20 days the ICE activity in lens epithelial cells cultured in MEM containing 10% FBS (8.57 ± 0.52 U/mg protein) was not significantly different from that in control cells (8.51 ± 0.53 U/mg protein). The CPP32 protease activities of lens epithelial cells cultured with cholestanol for 9 days (10.8 ± 0.79 U/mg protein) were not significantly different compared with control cells (9.47 ± 1.07 U/mg protein) but were significantly different after 20 days (16.0 ± 3.74 U/mg protein) compared with control cells.
with controls (10.1 ± 0.67 U/mg protein; Fig. 6D; P < 0.01). These observations suggest that cholestanol induced both ICE and CPP32 protease activities and induced apoptosis of both cornea endothelial and lens epithelial cells. The magnitude of induction of both ICE and CPP32 protease activities was comparable to that observed in Colo 201 cells treated with 10 mM ara-C for 12 hours. The ICE protease activity of Colo 201 cells treated with 10 mM or 100 mM ara-C for 12 hours was 13.5 ± 0.98 and 15.2 ± 1.50 U/mg protein, respectively. The CPP32 protease activity of Colo 201 cells treated with 10 mM or 100 mM ara-C for 12 hours was 19.4 ± 1.96 and 27.2 ± 2.72 U/mg protein, respectively.

**DISCUSSION**

CTX is characterized by hypercholestanolemia, Achilles tendon xanthomas, cerebellar ataxia, dementia, and cataract. Although cholestanol deposit can be present in various tissues, such as xanthoma and neural tissues in patients with CTX, the cause of cerebellar ataxia, dementia, and cataract in CTX is poorly understood. We previously reported corneal dystrophy in mice fed a diet containing 1% cholestanol, which histologically resembles calcific band keratopathy and Schnyder’s crystalline dystrophy. More recently, we found a higher level of cholestanol in the serum, cerebellum, lens, and aqueous humor in cholestanol-fed rats, and cholestanol-induced apoptosis of cerebellar neuronal cells, especially in Purkinje cells. Although corneal dystrophy or cataract was not observed in hypercholestanolemic rats, we hypothesized that cholestanol may induce apoptosis of lens epithelial and cornea endothelial cells. In the present study, we clearly demonstrated that cholestanol induced apoptosis of lens epithelial cells and cornea endothelial cells in vitro. The reason cornea dystrophy or cataract was not observed in hypercholestanolemic rats is not clear, but it may relate to differences in species.

Apoptosis plays an important role in lens development. The rapid apoptotic death of the lens epithelial cells, as induced by UVB, initiates cataract development. Calcimycin also induces apoptosis of lens epithelial cells and contributes to cataract formation. In the present study, we obtained the first evidence that cholestanol induces apoptosis of lens epithelial cells. Because the vertebrate lens contains only a single layer of epithelial cells, apoptotic death of lens epithelial cells could lead to a rapid loss of epithelial control of lens homeostasis, and opacification could occur.

Because the corneal cuboidal endothelium forms a single layer on the posterior corneal surface, the corneal endothelial cell plays an important role in maintaining corneal integrity and transparency. When endothelial cell functions deteriorate, the corneal stroma swells, and the transparency is damaged, a condition known as endothelial dysfunction or bullous keratopathy. Our evidence shows that cholestanol induces apoptosis of cornea endothelial cells. The finding that cholestanol induces apoptosis of cornea endothelial cells could explain the mechanism involved in corneal opacities in hypercholestanolemic mice.
Apoptosis is a type of cell death in which cells actively commit suicide. The process of apoptosis usually requires transcription of messenger RNA and protein synthesis to occur and is thought to underlie cell death in a variety of tissues and organisms. Apoptosis has been observed in the superficial epithelium of normal rabbits. After photorefractive keratectomy, apoptosis was detected in kerocytes and endothelial cells of rabbits. Apoptosis was also induced in kerocytes by herpes simplex virus type-1 infection and interleukin-1.

In the present study we demonstrated that cholestanol induced both ICE and CPP32 protease activities with a concomitant induction of apoptosis. ICE-like proteases are induced at the onset of apoptosis. The results found in the present study are consistent with the hypothesis that ICE and CPP32 proteases play an important role in apoptosis.

In summary, cholestanol induced apoptosis of cornea endothelial cells and lens epithelial cells. The induction of the apoptosis seen in cornea endothelial cells and lens epithelial cells suggests that cholestanol may eventually induce cataract and corneal opacity and could explain the mechanism of corneal opacities observed in hypercholestanolemic mice and of cataract, a characteristic symptom seen in patients with CTX.

References