Oxysterols from human bile induce apoptosis of canine gallbladder epithelial cells in monolayer culture

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Abstract

Oxysterols have been detected in various mammalian organs and blood. Biliary epithelium is exposed to high concentrations of cholesterol and we have identified three keto-oxysterols (cholest-4-en-3-one, cholesta-4,6-dien-3-one, cholesta-3,5-dien-7-one) in human bile and gallstones. As the effects of oxysterols on biliary physiology are not well defined, we investigated their biological effects on dog gallbladder epithelial cells. Enriched medium (culture medium containing taurocholate and lecithin and cholesterol + various oxysterols) was applied to confluent monolayers of dog gallbladder epithelial cells in culture. Cytotoxicity and apoptosis were studied by morphologic analysis and flow cytometry. Oxysterols in the mitochondrial fraction were identified by GC/MS, while release of cytochrome c from mitochondria was assayed by spectrophotometry and Western blot. Compared to cells treated with culture medium or with enriched medium containing cholesterol, oxysterol-treated cells showed significantly increased apoptosis (p<0.05). Exogenously applied oxysterols were recovered from the mitochondrial fraction. Cytochrome c release from mitochondria was increased significantly by cholest-4-en-3-one, cholesta-4,6-dien-3-one and 5β-cholestan-3-one (all p<0.05). Thus oxysterols recovered from human bile and gallstones induce apoptosis of biliary epithelium via a mitochondrial-dependent pathway and may play a role in the pathogenesis of chronic inflammation and carcinogenesis in the gallbladder.

Key words: keto-oxysterols, mitochondria, cytochrome c, inflammation, carcinogenesis
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Cholesterol, the most abundant sterol in human and animal tissues, is essential for the formation and function of cellular membranes. Cholesterol may be oxidized in part to oxysterols, both during its conversion to bile acids and hormones in vivo and also when exposed to heat, air, light and oxidizing agents in vitro (52). The various oxysterols identified in animal and human tissues (51, 52) have diverse biological activities, including effects on cholesterol homeostasis, sphingolipid metabolism, platelet aggregation and cytotoxicity (47). In animal experiments (16, 33, 59), oxysterols were much more atherogenic than purified cholesterol. In human studies (7, 9, 37), oxysterols have been implicated in the initiation and progression of atherosclerosis, due to their toxicity to arterial endothelium and smooth muscle cells. Oxysterols also promote tissue inflammation and necrosis (3), produce immunosuppression (34) and enhance colon carcinogenesis (23). Oxysterols can act as ligands for certain nuclear receptors, through which they regulate cholesterol homeostasis (18). These studies suggest that oxysterols can play important roles in atherogenesis, cytotoxicity, mutagenesis and carcinogenesis.

Relatively little is known about the role of oxysterols in the biliary tract. The biliary epithelium is continuously exposed to bile, which is a complex mixture of lipids, including bile salts, phospholipids, cholesterol and bile pigments. These solutes become concentrated in the gallbladder due to its storage-concentration function. Recently, we reported that human gallbladder bile and gallstones contain the oxysterols cholesta-4,6-dien-3-one and cholest-4-en-3-one, with the highest concentrations observed in brown pigment stones (11). Although the biliary tract which contains stones often exhibits chronic inflammation, stricture formation and cancer, there have been no studies of the
biological effects on biliary epithelium of these unique oxysterols recovered from human bile and gallstones. Therefore we studied the effect of these and other oxysterols on biliary epithelium using an in vitro monolayer culture of dog gallbladder epithelial cells (36).

Materials and Methods

Materials. Eagle’s minimum essential medium, fetal bovine serum, trypsin/EDTA, penicillin/streptomycin, lecithin (L-α-phosphatidylcholine from frozen egg yolk, type V-E) and cholesterol were purchased from Sigma (St. Louis, MO). The cholesterol was purified by recrystallization three times from warm methanol and then was stored at –70°C under argon. Tissue culture plates were from Falcon (Lincoln Park, NJ). Vitrogen was purchased from Celtrix Laboratories (Palo Alto, CA). Oxysterols, including cholest-4-en-3-one, cholesta-4,6-dien-3-one, cholesta-3,5-dien-7-one and 5β-cholestan-3-one were from Steraloids (Newport, RI). Sodium taurocholate and fatty acid-free bovine serum albumin were from Calbiochem (La Jolla, CA). Hoechst 33342, CMX Rosamine and MitoTracker Green dyes were obtained from Molecular Probes (Eugene, OR). Diff-Quik staining kit was from Dade Behring (Dudingen, Switzerland). N-acetyl-L-cysteine was from Sigma (St. Louis, MO) and was dissolved in doubly distilled water (100 mM, pH 7.4), stored in aliquots at –20°C, and thawed immediately before use.

Cell culture. Cells were isolated from dog gallbladder epithelium by trypsinization, as previously described (36). Stock cultures were grown on 100 mm plates with 2 ml of a 1:1 mixture of Vitrogen and medium (Eagle’s minimum essential
medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 20 mM HEPES, 100 IU/ml penicillin and 100 µg/ml streptomycin, pH 7.4). Medium was changed twice per week and the cells were maintained in a 38°C incubator with 5% CO₂. Cells were passaged when confluent (every 7-10 days), using trypsin (2.5 g/L) and EDTA (1 g/L). Subsequent cultures were performed under the same conditions, except using 100 mm dishes, 60 mm dishes or 24 mm x 6-well plates according to the experimental purposes.

Preparation of model bile and oxysterols. Model bile with cholesterol saturation index of 1.5 was prepared as previously described (25). Sodium taurocholate was dissolved in methanol/water (85/15 v/v), mixed with lecithin (L-α-phosphatidylcholine) and purified cholesterol. The methanol then was evaporated under nitrogen and the residue was lyophilized. Oxysterol-containing model biles were prepared by the same method, except that 50% of the cholesterol was replaced by an oxysterol. We used oxysterols identified from human bile and gallstones: cholest-4-en-3-one, cholesta-4,6-dien-3-one (11) and cholesta-3,5-dien-7-one (58). In addition, for comparison, we studied another keto-oxysterol, 5β-cholestan-3-one, which is produced by bacterial action in the lower intestine and exhibits significantly increased fecal excretion in patients with adenomatous colon polyps, chronic ulcerative colitis or colon cancer (42, 43). Freeze-dried model bile and oxysterol-containing model biles were stored at –70°C until use.

For experiments, model biles containing cholesterol with or without oxysterol were dissolved in 5 ml phosphate buffered saline (PBS), pH 7.4, equilibrated overnight at 56°C, and then diluted 1:10 in serum-free culture medium and sterilized through a 0.45 µm filter. The resulting enriched medium, containing taurocholate and lecithin, plus
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cholesterol or equimolar cholesterol and oxysterol, was added to confluent epithelial cells to simulate the in vivo physiological scenario. Final concentrations in these enriched media were: taurocholate 20 mM, lecithin 4.8 mM and cholesterol 2.8 mM, or cholesterol + oxysterol, 1.4 mM each. Controls were cells treated with serum-free medium. For antioxidant experiments, N-acetylcysteine was added to confluent epithelial cells at a final concentration of 1mM, one hour before the application of the sterol-containing enriched medium.

Cell morphological analysis. Equilibrated enriched media were added to confluent cells in 24 mm diameter 6-well plates. Morphological changes were observed every 6 hrs using an Olympus IMT-2 inverted phase contrast microscope with an attached Sony CCD/RGB digital camera. Images were captured using MCID-M5 software (Imaging Research, St. Catherines, Ontario, Canada). Nuclear morphology of control and treated cells was studied by fluorescence microscopy after staining with 10 µg/ml 4,6-diamidino-2-phenylindole (DAPI) and 0.1% nonidet P-40 detergent in a Tris buffered saline. For transmission electron microscopy (TEM), the primary fixative (Karnovsky solution, 0.5x) was added directly to Transwell inserts and post-fixation was done with 1% osmium tetroxide in 0.1 M phosphate buffer (90 min at room temperature). The samples were dehydrated in a graded ethanol series (50-100%), and embedded in PolyBed 812 epoxy resin. The sections were cut with a diamond knife using a Reichert-Jung Ultracut E microtome, picked up on a 150 mesh copper/rhodamine grid, stained with uranyl acetate (6% uranyl acetate in 1% acetic acid) for 25 min and Reynold’s lead citrate for 7 min, and examined in a Philips 410 Electron microscope at 60 kV.
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*Flow cytometry.* Cell cycle status and number of apoptotic cells were quantitated by flow cytometry after simultaneous staining with Hoechst 33342, CMX Rosamine and MitoTracker Green dyes (40). Cells were plated onto 60 mm Petri dishes at 100,000 cells per dish, cultured until confluent and then enriched medium, with or without oxysterol, was added to the cells as described above. Twelve hours after treatment, floating cells, cell debris and medium were suctioned off. The attached cells were washed three times with PBS, harvested with trypsin/EDTA and then centrifuged at 500 x g for 10 min. Cell pellets were resuspended in 1.0 ml of culture medium (without added biliary lipids) and treated with 10 µl of 1 mM Hoechst 33342, 1 µl of 20 µM CMX Rosamine and 1 µl of 20 µM MitoTracker Green dyes. The treated cells were incubated at 37°C for 30 min in the dark. For each sample, 10,000 events were analyzed with a Coulter Epics-Elite Flow Cytometer (Coulter, Hialeah, FL). The Hoechst 33342 dye was excited with 365 nm UV laser and fluorescence was detected with a 424/22 nm bandpass filter. The CMX Rosamine and MitoTracker Green dyes were excited with 488 nm line of the argon laser and CMX Rosamine fluorescence and MitoTracker Green fluorescence were collected with a 645 nm longpass filter and a 525/40 nm bandpass filter, respectively.

*Sub-cellular fractionation.* Confluent cells, treated with enriched medium with or without oxysterol for 12 hours, were resuspended at 2 x 10^8 cells/ml in ice-cold homogenization buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 1mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5). Subsequent manipulations were performed at 4°C. Homogenization was accomplished by douncing 10-15 times with a Potter-Elvehjem homogenizer with a Teflon pestle. Lysates were re-centrifuged twice at 2500 x g for 5 min to remove nuclei.
and unbroken cells, then at 10,000 x g for 30 min to precipitate mitochondria. The supernatant after isolation of mitochondria was spun at 25,000 x g for 1 hr to yield a ‘cytosolic fraction’. Enrichment and purity of the mitochondrial fraction was assessed by EM. To exclude possible contamination by lysosomes, the activity of a lysosomal enzyme, acid phosphatase, was checked both in the mitochondrial fraction and the cytosolic fraction. The mitochondrial fraction showed acid phosphatase activity of 0.01-0.04 U/L, while the cytosolic fraction showed 0.27-0.45 U/L. Therefore we deduced that the mitochondrial fraction was markedly enriched, with little contamination by lysosomes.

*Analysis of oxysterols in mitochondrial fraction.* Mitochondrial pellets were re-suspended in 1 ml of distilled water in a glass tube and 5 ml of chloroform/methanol, 2:1 by volume, was added. The mixture was agitated thoroughly and centrifuged for 5 min at 1200 x g. The lower (chloroform) layer was removed and evaporated under nitrogen at 40°C. The residue was converted to trimethylsilyl derivatives with Sylon BFT (Supelco, Bellefonte, PA) in pyridine. The silylated samples were analyzed for sterols by gas chromatography/mass spectrometry (GC/MS). 1 µl was injected by autosampler (HP 7683) into a Hewlett Packard 6890 GC equipped with a 5973 quadrapole mass detector (Hewlett Packard, Palo Alto, CA). The column was an HP 30 m capillary with stationary phase of 5% phenyl/methylsilicone. Oven temperature was programmed from 225°C to 310°C at 3°C/min, with 10 min at final temperature. Sterols were identified by comparison of their mass spectra with standard spectra in the NIST Library of Spectra 1998, in combination with the Wiley Registry of Mass Spectra, 7th edition.
Cytochrome c release from mitochondria. One major step in the process of apoptosis is the release of cytochrome c from mitochondria into the cytosol, resulting in the activation of caspase-9 through the oligomerization of Apaf-1 (30, 61). Therefore, we measured cytochrome c release from mitochondria into the cytosol after 12 hrs of exposure of the cell monolayers to the 5 different enriched media. Cytochrome c content of the cytosolic fractions (isolated as described above) was quantitated by direct spectrophotometry, using two absorption maxima, 550 nm for $\alpha$-band and 522 nm for $\beta$-band (19). Cytochrome c release was checked also by Western blot analysis. After protein quantitation, cytosolic fractions were mixed with 2x sodium dodecyl sulfate loading buffer (125 mM Tris-HCl, pH 6.8, 10% $\beta$-mercapto-ethanol, 4.6% sodium dodecyl sulfate and 0.003% bromophenol blue) and 20 $\mu$g of cytosolic proteins were separated on a 15% sodium dodecyl sulfate-polyacrylamide gel for detection of cytochrome c. Following electrophoretic transfer onto polyvinylidene difluoride (PVDF) membrane (Micron Separations Inc., Westboro, MA), the immunoblots were incubated with blocking buffer containing 1% bovine serum albumin and 0.05% Tween-20 in phosphate buffered saline. After blocking nonspecific binding sites, the PVDF membranes were successively incubated for 1 hr with a sheep polyclonal antibody to cytochrome c (Novus, Littleton, CO) at a dilution of 1:10,000, and then for 45 min with secondary anti-sheep antibody conjugated with horseradish peroxidase (Abcam, Cambridge, UK). The membranes were processed for the detection of cytochrome c using SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL).

Statistical Analysis. Data are expressed as the mean ± standard deviation (SD). Student’s t-test was used to assess the significance of difference between two groups and
ANOVA test was used when comparing three or more groups. Significance levels were established at p<0.05 and p<0.01.

Results

Morphological changes after oxysterol treatment. Cells grown to confluency were treated with culture medium (controls) or with enriched media containing taurocholate and lecithin plus either cholesterol or cholesterol plus an oxysterol. Representative examples of cell morphology at 6 hr and 48 hr after treatment are shown in Figure 1. Cells treated with culture medium (Fig. 1 A) did not show any significant changes with time. However, cells treated with enriched medium containing cholesterol (Fig. 1 C) for 48 hours (but not for 6 or 24 hours), showed morphologic changes characterized by rounding of cell shape and intracellular vacuolization. As shown in Figure 1 D for 5β-cholestan-3-one, cells treated with enriched medium containing different oxysterols were more severely affected. At 48 hrs most cells had become shriveled with pyknotic nuclei, indicating widespread cell death (Fig. 1 D), with lesser damage at 24 h (not shown). Upon fluorescent microscopy after DAPI staining of nuclei, oxysterol treated cells revealed nuclear fragmentation, which is characteristic of apoptosis (Fig. 2 B). These changes were absent in cells exposed to serum free medium (Fig. 2 A) and in cells exposed to enriched medium containing cholesterol (Fig. 2 C). Ultrastructural changes are shown in Figure 3. Compared with cells exposed to serum free medium or to enriched medium containing cholesterol, those exposed to an oxysterol exhibited many of the characteristics of apoptotic processes, including the condensation and margination of
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nuclear chromatin, condensation of nucleoli and intra-cytoplasmic vacuoles. Apoptotic cells also showed highly condensed mitochondria with irregular shapes.

Quantitation of apoptosis by flow cytometry. To complement the morphological studies, we quantified apoptosis in different groups of cells using flow cytometry. Hoechst 33342 stains all live cells that contain DNA and resolves live cells from dead cells or cell debris. CMX Rosamine is sensitive to changes in mitochondrial membrane potential and MitoTracker Green reports the amount of mitochondria regardless of their membrane potential (17). As illustrated in Figure 4, this multiparameter approach allowed us to determine the number of live cells (Hoechst 33342 positive and CMX Rosamine/MitoTracker Green high) vs. early apoptotic cells (Hoechst 33342 positive and CMX Rosamine/MitoTracker Green low) and debris signals (40). The reason for using MitoTracker Green and CMX Rosamine simultaneously is that this combination gives a better resolution between normal cells and apoptotic cells whose mitochondria are already compromised.

In preliminary studies, we analyzed the relationship between apoptosis and time after treatment. The control group (cells treated with serum free medium) showed a relatively constant range of apoptosis (4-8 %) at different times over a 24 hr period. In oxysterol-treated cells, apoptotic changes were detectable 4 hr after treatment and thereafter increased continuously until 18-24 hrs after treatment. We selected a time point of 12 hr to compare the degree of apoptosis among the different treatment groups. We also performed a dose-response analysis of oxysterol effects in enriched medium (Fig. 5).
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Oxysterol concentrations higher than 1.4 mM increased the proportion of cells that were undergoing apoptosis, but those high concentrations also caused significantly increased debris signals and floating dead cells. Thus we selected the concentration of 1.4 mM to investigate the acute biological effect of oxysterols. Note that while the dose of oxysterols tested were higher than that reported in human gallbladder bile (11, 58), these oxysterols were presented to the cell in the context of the physiologically relevant physical-chemical milieu. The aggregate concentration of sterols carried within mixed micelles of bile salt and phospholipid may not, therefore, correspond to the sterol concentrations that interact directly with the plasma membrane.

Table 1 summarizes the proportion of apoptotic cells after treatment for 12 hrs with five different enriched media containing 2.8 mM cholesterol or 1.4 mM each of an oxysterol and cholesterol. When compared to cells treated with enriched medium containing taurocholate, lecithin and cholesterol, those containing any of the four oxysterols showed a significantly increased percentage of apoptotic cells. Cholesta-3,5-dien-7-one was the least toxic and 5β-cholestan-3-one the most toxic, with cholest-4-en-3-one and cholesta-4,6-dien-3-one showing intermediate effects. These values may be minimal, in that floating cells, already dead, were removed prior to analysis.

**Isolation of oxysterols from the mitochondrial fraction.** GC/MS revealed that, in addition to cholesterol, the mitochondrial fractions contained whichever oxysterol had been presented in the enriched incubation medium along with cholesterol. The GC/MS spectra also confirmed the structural assignments (e.g. Fig. 6 for cholesta-3,5-diene-7-one). The
molar ratio of recovered oxysterol to cholesterol varied between replicate samples for each oxysterol (cholest-4-en-3-one: 2.25 ± 1.7 (n=4); cholesta-4,6-dien-3-one: 0.96 ± 0.54 (n=4); cholesta-3,5-dien-7-one: 0.69 ± 0.6 (n=5); 5β-cholestan-3-one: 1.37 ± 0.65 (n=2)). A correlation between mitochondrial oxysterol levels and the degree of apoptosis as assessed by flow cytometry was apparent only for cholesta-3,5-dien-7-one (compare with data in Table 1 and Fig. 5).

Cytochrome c release from mitochondria. When compared to the replicated samples treated with enriched medium containing cholesterol, those treated with enriched medium containing cholest-4-en-3-one, cholesta-4,6-dien-3-one, or 5β-cholestan-3-one showed a significant increase in the cytosolic content of cytochrome c. The cells treated with cholest-3,5 dien-7-one did not show a statistically significant difference (Fig.7). Identity of the released hemoprotein as cytochrome c was confirmed by Western blot of the cytosolic fractions (Fig. 8). Densitometric analyses of the immunoblots showed increased cytochrome c release following exposure to each of the oxysterols tested with the exception of cholest-3,5 dien-7-one, consistent with the the results from the spectrophotometric assay.

Antioxidant effect on oxysterol-induced apoptosis. To check the effect of antioxidant (known to inhibit apoptosis by the mitochondrial pathway), N-acetylcysteine was added to confluent cells at a final concentration of 1mM, one hour prior to the addition of enriched medium. In the cells treated with enriched medium containing cholesterol, the addition of N-acetylcysteine did not cause any significant difference in the proportion of apoptotic cells. In the cells treated with enriched medium containing both cholesterol and
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cholest-4-ene-3-one, N-acetylcysteine decreased oxysterol-induced apoptosis by 30% (p<0.01) (Fig. 9).

Discussion

Oxysterols have been reported to show a wide variety of biological activities, including regulation of cholesterol synthesis (10), interference with lipid packaging in membranes (31, 53) and modulation of the activity of membrane-bound enzymes (41). They have been identified in human atherosclerotic plaques (5). They can cause inflammatory responses in endothelial cells (50) and in monocyte/macrophages (6) and foam cell formation in smooth muscle cells and macrophages (14). Oxysterols are cytotoxic to a variety of cell types (1, 4, 13,15, 38) and have been shown to trigger apoptosis (7,12, 37).

We have identified three different species of oxysterols in human gallstones and gallbladder bile: cholest-4-en-3-one, cholesta-4,6-dien-3-one and cholesta-3,5-dien-7-one (11, 58). These keto-sterols are unusual in that they contain no hydroxyl groups, but rather are oxidized by dehydrogenation. There is, however, a dearth of information on the interaction of these and other oxysterols with the biliary system. Although the biliary epithelium is a relatively resistant tissue which is chronically exposed to concentrated sterols, bile acids and other lipids, we speculate that toxic oxysterols might affect biliary epithelial cell physiology and might be involved in the pathogenesis of biliary tract diseases, such as cellular inflammation, formation of gallstones and carcinogenesis. Indeed, a positive correlation has been reported between oxysterol concentration and bacterial DNA content in pigment gallstones (11, 26). Hence, we studied the biologic
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effects of oxysterols, especially those we have previously identified from human
gallstones and gallbladder bile (11).

In using an *in vitro* cell culture model, we tried to simulate the *in vivo* situation realizing
the limitations inherent in such an approach. Oxysterol was delivered as a constituent of
model bile and applied to confluent, non-proliferating dog gallbladder epithelial cells
(36). Due to the extremely low aqueous solubility of cholesterol and oxysterols, *in vitro*
studies require dissolution of these sterols in model bile or organic solvents. We selected
the more physiological option, model bile, which was designed to mimic the lipid
composition and concentrations of dog gallbladder bile, in which taurocholate, the least
toxic primary bile acid in mammalian bile, constitutes approximately 75% of the bile
salts (55). Composition of the model bile approximated hepatic bile as to the
concentration of its components. The final concentration of an oxysterol in each culture
medium was 1.4 mM, in company with equimolar cholesterol. Although some of the
oxysterols produced high rates of apoptosis, these concentrations of oxysterols are high
compared with the micromolar levels found in hepatic and gallbladder bile (58). Local
concentrations in contact with the epithelium may be considerably higher than the
average bulk phase concentrations measured. Oxysterol concentrations were chosen
based on the effects on apoptosis found on the dose response experiments (Figure 5). We
included oxysterols that have been found in human gallbladder bile, and we applied these
oxysterols to cells in a physiologically relevant physico-chemical milieu comprised of
bile salt and phospholipid. These two aspects of our study design are physiologically
relevant, and have not been reported previously with respect to oxysterol-induced
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apoptotic or other biologic effects on gallbladder or bile duct epithelial cells. The aggregate concentration of sterols carried within mixed micelles of bile salt and phospholipid does not correspond to the monomeric sterol concentration that interacts directly with the plasma membrane. The latter sterol concentration, which is the most physiologically relevant, cannot be measured.

Cells exposed to culture medium enriched with taurocholate, lecithin and cholesterol (model bile) exhibited more apoptosis than cells exposed to culture medium alone (controls). This indicates that the biliary lipids themselves may induce a modest increase in apoptosis, and suggests that the toxic effects observed with the oxysterols were either additive with, or potentiated by, the concurrent presence of the bile salt and phospholipid. There is no way to actually evaluate the latter possibility, since organic solvents for dissolution of cholesterol would themselves damage cells and their membranes. Our system is relevant to the *in vivo* situation, in which gallbladder epithelium is, with rare exceptions, exposed to oxysterols only in the presence of bile salts and lecithin.

Another theoretical limitation is the species from which the gallbladder epithelial epithelial cells originated. There are significant differences among species in bile acid composition, and the dog has the lowest sterol saturation of nearly all animals examined (2, 20). Therefore, an argument can be made that dog gallbladder epithelial cells are not “equipped” to handle the sterol levels in the model biles used for these studies. In fact, our laboratory has accumulated extensive data to show that cholesterol saturation indices up to 1.5 are well tolerated by these cells in monolayer and in polarized culture.
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conditions (25, 27). Cell viability, cell polarity, and vectorial transport of cholesterol are well preserved in these cells despite treatment with model biles containing high levels of cholesterol. In this regard, the fact that oxysterols originally isolated in human gallbladder bile and gallstones induced apoptosis in the dog gallbladder epithelial cells suggests that similar processes may operate in the human gallbladder. Nevertheless, future studies in other model systems will be necessary before the results described can be extrapolated to human gallbladder epithelium.

We examined the effects of four oxysterols on apoptosis of gallbladder epithelial cells by analysis of nuclear morphologic changes and multiparameter flow cytometry. There are two major apoptotic pathways, commonly referred to as the extrinsic, or death receptor pathway; and the intrinsic, or mitochondrial pathway. While the involvement of the extrinsic death receptor pathway during oxysterol-induced apoptosis has been well studied in non-biliary cells (28, 29, 35, 44-46), relatively few reports describe the mitochondrial pathway and cytochrome c release (32, 54, 56). We focused on the mitochondrial pathway because the mitochondria showed significant ultrastructural damage during the early phase of oxysterol-induced apoptosis. These oxysterols, especially cholest-4-en-3-one and cholesta-4,6-dien-3-one, also caused a significant increase in the release of cytochrome c from mitochondria and they were directly identified in the mitochondrial fraction. The apoptosis results were somewhat unexpected, since dehydrogenation at position 3 has been reported to eliminate the apoptotic ability of 25- and 27-hydroxycholesterol (60). These findings suggest that the intrinsic mitochondrial pathway, causing cytochrome c release, mediates the apoptosis
caused by these oxysterols. Our results do not exclude the possibility of involvement of
the extrinsic death receptor pathway during oxysterol-induced apoptosis in the
gallbladder epithelium.

These results from acute/high dose studies may well be a useful indication of the long-
term effects of exposure to low concentrations of oxysterols. At least in diseased states,
the biliary tree appears to be chronically exposed to a wide variety of oxysterols (58),
including 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, and
cholestanetriol. In the gallbladder, however, we have found only cholest-4-en-3-one and
cholesta-4, 6-dien-3-one in significant concentrations (11), together with lesser amounts
of cholesta-3, 5-dien-7-one (58). Interestingly, these oxysterols were found in the
gallbladder, but not in hepatic bile (11), raising the possibility that they are generated in
situ in the gallbladder. Our results do show that oxysterols formed in the biliary tract
may interact with the biliary epithelium, can be absorbed into the cells and may have
profound effects on these cells. Indeed, gallstones are associated with increased
concentrations of oxysterols in bile (26) and epidemiological studies have shown that the
presence of gallstones is strongly correlated with gallbladder cancer (8) and intrahepatic
stones with cholangiocarcinoma (24). Considering the mutagenic (39, 48, 49) and
carcinogenic properties (21, 22) of other oxysterols, it may be hypothesized that chronic
exposure to biliary oxysterols are involved in the initiation and progression of cancers in
the biliary apparatus. This hypothesis has recently been advanced in the context of
cholangiocarcinoma (57).
In conclusion, we have demonstrated that unusual oxysterols that are found in human gallstones and gallbladder bile cause apoptosis in cultured gallbladder epithelial cells. The novelty of these studies lies in the demonstration that the gallbladder epithelial cell is susceptible to apoptosis when these sterols are present in a physical-chemical form appropriate for the biliary system. The apoptosis appears to be mediated by uptake of oxysterol from bile and subsequent incorporation into the mitochondria, causing release of cytochrome c into the cytosol. This may not be the exclusive apoptotic mechanism and other pathways (e.g. Fas-mediated) could be involved. The cytotoxic and apoptotic potential of biliary oxysterols might have important implications in the pathogenesis of biliary tract disorders, including cancers. Additional studies are clearly indicated.

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References


Figure Legends

Figure 1. Changes in morphology of a confluent monolayer of dog gall bladder epithelial cells after application of regular culture medium (A, B), culture medium enriched with taurocholate + lecithin + cholesterol (C), or culture medium enriched with taurocholate + lecithin + equimolar cholesterol and the oxysterol, 5β-cholestan-3-one (D). Phase contrast microscopy was performed at 6 hr intervals. All systems showed similar minimal changes after 6 h (A), but differed markedly after 48 h of incubation (B-D). At 48 h, the regular culture medium (B) engendered only occasional rounded cells, while the cholesterol-containing enriched medium (C) caused rounding of most cells (arrows). In contrast, the enriched medium containing 5β-cholestane-3-one (D) caused extensive cell shrinkage and nuclear pyknosis, characteristic of apoptosis. Less severe changes were seen at 24 h (not shown). In each case, the scale bar is 10 µm.

Figure 2. Fluorescent microscopy of dog gallbladder epithelial cells, treated with DAPI to stain nuclei, after 12 hr incubation in serum free culture medium (A), in enriched medium containing equimolar cholesterol and cholest-4-en-3-one (B) and in enriched medium containing cholesterol (C). The nuclei of cells treated with the oxysterol show fragmentation (arrows) characteristic of apoptosis. The scale bar is 5 µm.

Figure 3. Typical transmission electron microscopic images of dog gallbladder epithelial cells showing ultrastructural changes after sterol treatment. Cells were treated for 12 hours with serum free culture medium (A & C), enriched medium containing equimolar cholesterol and cholest-4-en-3-one (B & D), or enriched medium containing cholesterol...
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(E). Nuclei of oxysterol-treated cells (B) show condensation and margination of chromatin, condensation of nucleoli and intracytoplasmic vacuolizations, which were not seen in cells treated with serum free medium (A, C) or with enriched medium containing cholesterol (E). Normal mitochondria (arrows) are shown in control cells (C), which contrasts with swollen, irregular mitochondria with condensed cristae (arrows) in cholest-4-en-3-one treated cells (D). The scale bar is 1 μm.

**Figure 4.** Quantitation of apoptosis by flow cytometry. Confluent dog gallbladder epithelial cells in monolayer were treated for 12 hrs with serum free medium (left); or enriched medium containing taurocholate 20 mM and lecithin 4.8 mM plus cholesterol 2.8 mM (center); or enriched medium containing taurocholate 20 mM and lecithin 4.8 mM plus 1.4 mM each cholesterol + 5β-cholestan-3-one (right). Harvested cells were stained with Hoechst 33342, CMX Rosamine (CMXRos) and MitoTracker Green (MTG) and assayed with flow cytometry. Live cells (left upper ellipse) and apoptotic cells (right lower ellipse) were distinguished by fluorescent intensity distributions of CMXRos (ordinate) and MTG (abscissa), shown on logarithmic scales. Values for the % apoptotic cells in the figure are 4% (serum free medium), 11% (enriched medium containing cholesterol) and 36% (enriched medium containing equimolar cholesterol and 5β-cholestan-3-one).

**Figure 5.** Dose-response relationship of oxysterols on apoptosis of confluent dog gallbladder epithelial cells. Enriched medium, containing 20 mM taurocholate and 4.8 mM lecithin plus cholesterol + oxysterol (total sterol concentration 2.8 mM, with the proportion provided by the oxysterol component as indicated in the figure), was applied
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to the monolayer. Cells were harvested after 12 hours and the proportion of apoptotic
cells assessed by fluorescent staining and flow cytometry, as described in Fig. 4. Data
represent the mean ± S.D. of triplicate experiments. P values vs. cholesterol: * < 0.05, #
< 0.01.

**Figure 6.** Mass spectrum of cholesta-3,5-dien-7-one. Upper panel shows the spectrum of
cholesta-3,5-dien-7-one detected in the isolated mitochondrial fraction. Lower panel
shows virtually identical reference spectrum of an authentic sample.

**Figure 7.** Cytosolic content of cytochrome c, after treatment of confluent dog
gallbladder epithelial cells for 12 hrs with enriched medium containing taurocholate 20
mM and lecithin 4.8 mM + sterol (cholesterol 2.8 mM, or 1.4 mM each of cholesterol and
an oxysterol (cholesta-3,5-dien-7-one, cholest-4-en-3-one, cholesta-4,6-dien-3-one, or
5β-cholestan-3-one). The cells were harvested and the cytosolic fraction was isolated by
differential centrifugation. Cytosolic cytochrome c content was measured by direct
spectrophotometry at the absorption maxima of 550 nm and 522 nm. Data represent the
mean ± S.D. of six different experiments and each enriched medium containing an
oxysterol was compared with enriched medium containing cholesterol (* = p<0.05).

**Figure 8.** Western blot showing cytosolic content of cytochrome c, after treatment of
confluent dog gallbladder epithelial cells for 12 hrs with enriched medium containing
taurocholate and lecithin + cholesterol (lane 1), or equimolar cholesterol and an oxysterol
(lane 2: cholesta-3,5-dien-7-one; lane 3: cholest-4-en-3-one; lane 4: cholesta-4,6-dien-3-
Oxysterol-induced apoptosis in gallbladder epithelium

one; lane 5: 5β-cholestan-3-one). The graph depicts the densitometric analysis of immunoblots from two experiments.

**Figure 9.** Effect of N-acetylcysteine on oxysterol-induced apoptosis. Dog gallbladder epithelial cells were treated with serum free medium, enriched medium containing taurocholate 20 mM and lecithin 4.8 mM + cholesterol 2.8 mM, or enriched medium containing taurocholate 20 mM and lecithin 4.8 mM + 1.4 mM each of cholesterol and cholest-4-en-3-one. Data represent the mean ± S.D. of triplicate experiments and the proportion of apoptotic cells at 12 h was determined by flow cytometry, as in Fig. 4. Dark bars represent cells pretreated for 1 hr with 1 mM N-acetylcysteine. (* = p<0.01 vs. no N-acetylcysteine).
Table 1. Apoptosis of Dog Gallbladder Epithelial Cells Caused by Different Oxysterols

<table>
<thead>
<tr>
<th>Medium Applied</th>
<th>Apoptosis (% of control)(^a)</th>
<th>p-value(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched Medium (EM)(^b)</td>
<td>100 ± 2.91</td>
<td></td>
</tr>
<tr>
<td>EM + cholest-4-en-3-one(^c,e)</td>
<td>156 ± 8.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EM + 4, 6-cholestadien-3-one(^c,e)</td>
<td>152 ± 5.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EM + 3, 5-cholestadien-7-one(^c,e)</td>
<td>129 ± 16.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>EM + 5β-cholestan-3-one(^c)</td>
<td>184 ± 29.4</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

\(^a\) Apoptosis assessed by flow cytometry, as in Fig. 4 after 12 hours of incubation. Data expressed as mean ± SD from six individual experiments in triplicate wells (n=18).

\(^b\) Enriched medium is serum free medium containing taurocholate 20 mM, lecithin 4.8 mM and cholesterol 2.8 mM.

\(^c\) Oxysterols were added to a concentration of 1.4 mM and cholesterol content reduced to 1.4 mM.

\(^d\) p-value compared with enriched medium.

\(^e\) Oxysterols that are present in human bile and gallstones.
Figure 1
Oxysterol-induced apoptosis in gallbladder epithelium

Figure 2
Figure 3
Oxysterol-induced apoptosis in gallbladder epithelium

Figure 4
Oxysterol-induced apoptosis in gallbladder epithelium

Figure 5
Oxysterol-induced apoptosis in gallbladder epithelium

Figure 6
Oxysterol-induced apoptosis in gallbladder epithelium

Figure 7
Figure 8
Figure 9