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

Dong Wan Seo, Ho-Soon Choi, Sum P. Lee, and Rahul Kuver

Division of Gastroenterology, Department of Medicine, University of Washington, and the Puget Sound Veterans Affairs Health Care System, Seattle Division, Seattle, Washington 98195

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Seo, Dong Wan, Ho-Soon Choi, Sum P. Lee, and Rahul Kuver. Oxysterols from human bile induce apoptosis of canine gallbladder epithelial cells in monolayer culture. Am J Physiol Gastrointest Liver Physiol 287: G1247–G1256, 2004. First published July 8, 2004; doi:10.1152/ajpgi.00013.2004.—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. Because 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 ≥2 ± various oxysterols) was applied to confluent monolayers of dog gallbladder epithelial cells in culture. Cytotoxicity and apoptosis were studied by morphological analysis and flow cytometry. Oxysterols in the mitochondrial fraction were identified by gas chromatography/mass spectrometry, whereas release of cytochrome c from mitochondria was assayed by spectrophotometry and Western blot analysis. Compared with 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.

keto-oxysterols; mitochondria; cytochrome c; inflammation; carcinogenesis

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 (t-α-phosphatidyl-choline 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 was then 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 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 37°C incubator with 5% CO2. Cells were passaged when confluent (every

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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- or 60-mm dishes or 24-mm × 6-well plates according to the experimental purposes.

Preparation of model bile and oxysterols. Model bile with a cholesterol saturation index of 1.5 was prepared as previously described (25). Sodium taurocholate was dissolved in methanol/water (85:15, vol/vol) mixed with lecithin (t-α-phosphatidylcholine) and purified cholesterol. The methanol was then 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, cholest-4,6-dien-3-one (11), and cholest-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 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 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 (in mM): 20 taurocholate, 4.8 lecithin, and 2.8 cholesterol or 1.4 each of cholesterol plus oxysterol. Controls were cells treated with serum-free medium. For antioxidant experiments, N-acetylcysteine was added to confluent epithelial cells at a final concentration of 1 mM 1 h 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 h 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. Catharines, 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, the primary fixative ( Karnovsky solution; 0.5×) was added directly to Transwell inserts, and postfixation 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 Epon. Sections were deparaffinized, dehydrated in a graded ethanol series, and embedded in paraffin. The 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 ultraviolet laser, and fluorescence was detected with a 424/22-nm bandpass filter. The CMX Rosamine and MitoTracker Green dyes were excited with a 488-nm line of the argon laser, and CMX Rosamine fluorescence and MitoTracker Green fluorescence were collected with a 645-nm long-pass filter and a 525/40-nm band-pass filter, respectively.

Subcellular fractionation. Confluent cells, treated with enriched medium with or without oxysterol for 12 h, were suspended at 2 × 10⁶ cells/ml in ice-cold homogenization buffer (in mM: 250 sucrose, 20 HEPES, 10 KCl, 1.5 MgCl₂, 0.1 EDTA, 1 EGTA, 1 dithiothreitol, and 0.1 phenylmethylsulfonyl fluoride, pH 7.5). Subsequent manipulations were performed at 4°C. Homogenization was accomplished by bouncing 10–15 times with a Potter-Elvehjem homogenizer with a Teflon pestle. Lysates were recentrifuged twice at 2,500 g for 5 min to remove nuclei and unbroken cells, then at 10,000 g for 30 min to precipitate mitochondria. The supernatant after isolation of mitochondria was spun at 25,000 g for 1 h to yield a “cytosolic fraction.” Enrichment and purity of the mitochondrial fraction were assessed by electron microscopy. 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, whereas 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 resuspended 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 1,200 g. The lower (chloroform) layer was removed and evaporated under nitrogen at 40°C. The residue was converted to trimethylsilyl derivatives with Sylon BPT (Supelco, Bellefonte, PA) in pyridine. The silylated samples were analyzed for sterols by gas chromatography/mass spectrometry (GC/MS). One microliter was injected by autosampler (HP 7683) into a model 6890 GC equipped with a 5973 quadrupole 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 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 National Institute of Standards and Technology 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 h of exposure of the cell monolayers to the five different enriched media. Cytochrome c content of the cytosolic fractions (isolated as described in Subcellular fractionation) was quantitated by direct spectrophotometry, using two absorption maxima: 550 nm for the α-band and 522 nm for the β-band (19). Cytochrome c release was checked also by Western blot analysis. After protein quantitation, cytosolic fractions were mixed with 2% sodium dodecyl sulfate loading buffer (125 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 4.6% sodium dodecyl sulfate, and 0.003% bromophenol blue) and 20 μg of cytosolic proteins were separated on a 15% sodium dodecyl sulfate-polyacrylamide gel for detection of cytochrome c. After electrophoretic transfer onto polyvinylidene difluoride (PVDF) membrane (Micron Separations, Westboro, MA), the immunoblots were incubated with blocking buffer containing 1% bovine serum albumin and 0.05% Tween-20 in PBS. After nonspecific binding sites were blocked, the PVDF membranes were successively incubated for 1 h 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 means ± SD. Student’s t-test was used to assess the significance of difference between two groups, and ANOVA 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 and 48 h after treatment are shown in Fig. 1. Cells treated with culture medium (Fig. 1A) did not show any significant changes with time. However, cells treated with enriched medium containing cholesterol (Fig. 1C) for 48 h (but not for 6 or 24 h) showed morphological changes characterized by rounding of cell shape and intracellular vacuolization. As shown in Fig. 1D for 5β-cholestan-3-one, cells treated with enriched medium containing different oxysterols were more severely affected. At 48 h, most cells had become shrunken with pyknotic nuclei, indicating widespread cell death (Fig. 1D), with lesser damage at 24 h (not shown). On fluorescent microscopy after DAPI staining of nuclei, oxysterol-treated cells revealed nuclear fragmentation, which is characteristic of apoptosis (Fig. 2B). These changes were absent in cells exposed to serum-free medium (Fig. 2A) and in cells exposed to enriched medium containing cholesterol (Fig. 2C). Ultrastructural changes are shown in Fig. 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 nuclear chromatin, condensation of nucleoli, and intracytoplasmic 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 Fig. 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) vs. 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-h period. In oxysterol-treated cells, apoptotic changes were detectable 4 h after treatment and thereafter increased continu-
ously until 18–24 h after treatment. We selected a time point of 12 h 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). Oxysterol concentrations >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 whereas the dose of oxysterols tested was 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 h with 5 different enriched media containing 2.8 mM cholesterol or 1.4 mM each of an oxysterol and cholesterol. Compared with 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 was 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 before 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.** Compared with 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 analysis of the cytosolic fractions (Fig. 8).
sitometric 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 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 1 mM, 1 h before 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 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 that 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 biological 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, nonproliferating 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 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...

Table 1. Apoptosis of dog gallbladder epithelial cells caused by different oxysterols

<table>
<thead>
<tr>
<th>Medium Applied</th>
<th>Apoptosis, % of control</th>
<th>P value</th>
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<tbody>
<tr>
<td>EMb</td>
<td>100 ± 2.91</td>
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</tr>
<tr>
<td>EM + cholest-4-en-3-onecd</td>
<td>156 ± 8.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EM + 4, 6-cholestadien-3-onecd</td>
<td>152 ± 2.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EM + 5, 5-cholestadien-7-onecd</td>
<td>129 ± 16.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>EM + 5β-cholestan-3-one</td>
<td>184 ± 29.4</td>
<td>&lt;0.05</td>
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Values are means ± SD. *Apoptosis assessed by flow cytometry, as in Fig. 4 after 12 h of incubation from 6 individual experiments in triplicate wells (n = 18). *Enriched medium (EM) is serum-free medium containing 20 mM taurocholate, 4.8 mM lecithin, and 2.8 mM cholesterol. †Oxysterols were added to a concentration of 1.4 mM and cholesterol content was reduced to 1.4 mM. ‡Oxysterols that are present in human bile and gallstones. *P value compared with EM.
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 (Fig. 5). We included oxysterols that have been found in human gallbladder bile, and we applied these oxysterols to cells in a physiologically relevant physicochemical 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 apoptotic or other biological 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 with 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...
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, because 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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Fig. 9. Effect of N-acetylcysteine on oxysterol-induced apoptosis. Dog gallbladder epithelial cells were treated with serum-free medium, enriched medium containing 20 mM taurocholate and 4.8 mM lecithin + 2.8 mM cholesterol, or enriched medium containing 20 mM taurocholate and 4.8 mM lecithin + 1.4 mM each of cholesterol and cholest-4-en-3-one. Data represent the means ± SD of triplicate experiments, and the proportion of apoptotic cells at 12 h was determined by flow cytometry, as in Fig. 4. Filled bars represent cells pretreated for 1 h with 1 mM N-acetylcysteine (*P < 0.01 vs. no N-acetylcysteine).
OXYSTEROL-INDUCED APOPTOSIS IN GALLBLADDER EPITHELIAL CELLS

GRANTS

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