Cholestan-3β,5α,6β-triol, but not 7-ketocholesterol, suppresses taurocholate-induced mucin secretion by cultured dog gallbladder epithelial cells

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Abstract In order to investigate oxysterol-mediated effects on the biliary system, we studied the effects of cholestan-3β,5α,6β-triol (TriolC) and 7-ketocholesterol (7KC) on gallbladder epithelial cells. We compared their cell proliferation effects in cultured dog gallbladder epithelial cells (DGBE) to their effects in cultured human pulmonary artery endothelial cells (HPAE). Oxysterols inhibited cell proliferation in a dose-dependent fashion. Oxysterols inhibited cell growth to 50% of control at a higher dose for DGBE cells than for HPAE cells. TriolC was more cytotoxic than 7KC. We also investigated the effect of oxysterols on bile salt-induced mucin secretion by DGBE cells. TriolC suppressed mucin secretion by DGBE cells, whereas 7KC did not. These findings support the hypothesis that biliary oxysterols affect gallbladder mucosal function.

Key words: Epithelial cell; Gallbladder; Cytotoxicity; Mucin secretion; Oxysterol; Cholesterol

1. Introduction

Cholesterol is essential for the formation and function of cell membranes. Oxysterols are derived in vitro as autoxidation products of cholesterol [1]. Cholesterol is also metabolized to various cholesterol oxides (oxysterols) in vivo during the production of bile acids and hormones. Many oxysterol species have been identified in animal and human tissues [1,2] and in plasma [3]. Oxysterols are also found in high concentrations in atheromatous plaques [4]. In animal experiments oxysterols are much more atherogenic than cholesterol itself [5]. In humans, oxysterol involvement in the initiation and progression of atherosclerosis has been postulated.

Cholesterol and its derivatives such as bile salts are found in high concentrations in bile. Oxysterols are postulated to be present in high concentrations in bile, but the study of biliary oxysterols is in its infancy. Indeed, little is known currently about the composition and concentrations of oxysterol species in bile. There have been no studies on the effects of oxysterols on the biliary tract. Moreover, there have been no scientific publications on oxysterols in bile in the last two decades. The gallbladder is often regarded as a passive reservoir that stores and concentrates bile secreted by the liver. To achieve this function, during the storage-concentration period, the gallbladder epithelium is exposed to high concentrations of bile acids, phospholipids, and cholesterol. We hypothesize that oxysterols in bile may be involved in the pathogenesis of biliary tract disease such as cholelithiasis and biliary tract cancers. This is analogous to oxysterols in blood, which are believed to be involved in the pathogenesis of atherosclerosis.

We have reported the long-term culturing and passaging of normal, well-differentiated gallbladder epithelial cells from the dog [6]. These cells form electrically leak-proof monolayers and synthesize protein and mucus glycoprotein. Mucin secretion in these cells is stimulated by several secretagogues that caused an increase in intracellular cAMP [7]. Mucin secretion by these cells is stimulated by model bile solutions [8–10]. Bile salts were responsible for the stimulatory effect of model bile on mucin secretion and caused a dose-dependent stimulation of mucin secretion. In the present study, we have used dog gallbladder epithelial (DGBE) cells as a model system to investigate the cytotoxic effects of oxysterols on biliary cells, as well as the effects of oxysterols on bile salt-induced mucin secretion. Because the composition and concentrations of biliary oxysterols are currently not well defined, we chose to use the commercially available oxysterols cholestan-3β,5α,6β-triol (TriolC) and 7-ketocholesterol (7KC) for these studies.

2. Materials and methods

2.1. Chemicals and reagents

Vitrogen was purchased from Celtrix Laboratories (Palo Alto, CA, USA). Tissue-culture plates were from Falcon (Lincoln Park, NJ, USA). Transwell inserts (24.5 mm diameter, 3.0 μm pore size) were obtained from Costar (Cambridge, MA, USA). [^{3}H]*N*-acetyl-D-glucosamine and $Na_2[^{51}Cr]O_4$ were purchased from ICN (Irvine, CA, USA). TriolC, >99% purity and 7-ketocholesterol 7KC, >99% purity were obtained from Steraloids (Wilton, NH, USA). Lecithin (L-α-phosphatidylcholine from frozen egg yolk, type V-E, approximately 99% pure) was from Sigma Chemical Co (St. Louis, MO, USA). Cholesterol was obtained from Sigma, and was >99% pure. Taurocholate was from Calbiochem (La Jolla, CA, USA), and was >90% pure. Cell culture medium and other reagents were obtained from Sigma.

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Abbreviations: 7KC, 7-ketocholesterol; TriolC, cholestan-3β,5α,6β-triol; DGBE, dog gallbladder epithelial; HPAE, human pulmonary artery endothelial

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2.2. Isolation and culture of DGBE cells

Epithelial cells were isolated from dog gallbladder by trypsinization, as previously described [6]. Stock cultures were grown on 60-mm Petri dishes coated with 1 ml Vitrogen gel (1:1 mixture of Vitrogen and medium) in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Medium was changed twice a week and the cells were maintained in a 37°C incubator with 5% CO_2. The cells were passaged when confluent (every 7–10 days), using trypsin (2.5 g/l) and EDTA (1 g/l) treatment.

2.3. Culture of normal human pulmonary artery endothelial (HPAE) cells

HPAE cells were obtained from Clonetics (San Diego, CA, USA). Stock cultures were grown on 60-mm Petri dishes in endothelial cell growth medium obtained from Clonetics. Medium was changed three times a week and the cells were maintained in a 37°C incubator with 5% CO_2. The cells were passaged when 80–90% confluent (every 7–10 days), using trypsin (2.5 g/l) and EDTA (1 g/l) treatment.

2.4. Growth inhibition experiments

For the growth experiments, either DGBE cells or HPAE cells were plated at a density of 30 000 cells per well on 24-well plates, and incubated for 24 h. Subsequently, solutions of either TriolC or 7KC in 0.5% ethanol/cell culture medium were added to triplicate wells. 48 h after adding the oxysterols, cell proliferation was measured by a non-radioactive colorimetric method [11] using tetrazolium salts (CellTiter 96, Promega, Madison, WI, USA), according to the manufacturer's instructions with the following modifications. Tetrazolium dye in phosphate-buffered saline (PBS, 150 μl) was added to each well. After incubation for 4 h, 1 ml of solubilization solution was added to each well, and the plates were incubated for another hour. The solutions were then transferred to a cuvette and the optical density was read at 570 nm, using a spectrophotometer. Cell proliferation was expressed as a percentage of control, measured in the absence of oxysterols.

2.5. ^{51}Cr release assay

Viability of confluent cells was quantified by measuring ^{51}Cr release from prelabeled cells [12]. DGBE cells were grown to confluence on Transwell inserts in 6-well tissue-culture plates. This allowed separate and independent access to the apical (luminal) and basolateral (serosal) compartments, and enabled simulation of luminal events mediated by biliary components. Culture medium containing 1 μCi/ml of ^{51}Cr was added to the basolateral compartments of the wells. After labeling overnight, cells were washed once with sterile PBS (pH 7.4) for 30 min, followed by washing for another 30 min with serum-free medium. Then, test components in 2 ml of serum-free medium were added to either the apical or the basolateral compartments, and 2-ml aliquots of serum-free medium were added to the other compartments. The plates were returned to the incubator for 24 h. Cell-free supernatant medium (500 μl) was removed from the apical compartments for determination of ^{51}Cr release. ^{51}Cr release (% maximum) was calculated as follows: (tested ^{51}Cr counts/minute (cpm) released)/(the mean maximum ^{51}Cr cpm released)×100%. Maximum ^{51}Cr release was determined by incubating the cells in 2.5 mM Triton X-100. We also calculated specific ^{51}Cr release as caused by oxysterols as follows: specific ^{51}Cr release (%) = (tested ^{51}Cr cpm released−the mean spontaneous ^{51}Cr cpm released)/(the mean maximum ^{51}Cr cpm released−the mean spontaneous ^{51}Cr cpm released)×100%. Spontaneous ^{51}Cr release was determined by incubating the cells in serum-free medium.

2.6. Mucin secretion assay

The mucin secretion assay was performed as described [8] with slight modifications. DGBE cells were grown to confluence on Transwell inserts in 6-well tissue-culture plates. Cells were labeled overnight (16–24 h) with 2 μCi/well [^{3}H]*N*-acetyl-D-glucosamine, in medium containing 10% (v/v) fetal calf serum. The precursor sugar was added to the bottom compartment of the wells, exposing only the basolateral side of the cells to the label. To remove unincorporated label, cells were then washed with sterile PBS (pH 7.4) for 30 min, followed by washing for another 30 min with serum-free medium. Next, 2-ml aliquots of serum-free medium containing the test components were added to the apical compartments and 2-ml aliquots of serum-free medium to the basolateral compartments of the wells. The plates were returned to the incubator. After the incubation, 1 ml of medium was harvested from each Transwell and spun at 500×*g* for 10 min to pellet released cells. A sample (500 μl) of the supernatant was then mixed with 8 ml of 10% trichloroacetic acid/1% phosphotungstic acid. This mixture was vortexed and incubated overnight at 4°C. The cells in the Transwells were washed once with PBS and harvested with trypsin/EDTA. After collection, they were again washed with PBS and spun at 500×*g* for 10 min. Aliquots of the cell pellets were sampled for protein content as described by Smith et al. [13]. The remaining cells were treated with 10% trichloroacetic acid/1% phosphotungstic acid, similar to the processing of the medium samples. After precipitation overnight, the samples were spun at 1500×*g* for 15 min. The resulting protein pellets were washed twice, first with 5 ml of 10% trichloroacetic acid/1% phosphotungstic acid, then with 2 ml of 90% ethanol. Finally the pellets were dissolved in 0.5 ml of water and counted in 10 ml of scintillation fluid. Results were expressed as percentage of control in cpm/mg cell protein.

2.7. Preparation of model biles

Model biles were prepared according to Kibe et al. [14]. Taurocholic acid was dissolved in methanol and water (85/15 v/v), and mixed with lecithin and cholesterol (or oxysterols) in chloroform. The solvent was then evaporated under nitrogen gas, and the residue was lyophilized. Model biles were stored at −70°C until further use. For the ^{51}Cr release assay and the mucin secretion assays, the model biles were dissolved in 5 ml of PBS (pH 7.4), and equilibrated overnight at 56°C. The model biles were diluted 1:10 in serum-free culture medium and sterile filtered through a 0.45-μm filter. The final concentration of each lipid was 20 mM for taurocholic acid, 4.6 mM for lecithin, and 1.2 mM for cholesterol or oxysterols, respectively. Their cholesterol saturation index was 1.1. The model biles containing cholesterol and those containing oxysterol were mixed in a suitable ratio before applying these model biles to the cell culture system. The resulting model bile solutions were added to the apical compartment of the Transwell system. The treated cells were incubated for 24 h in the incubator before harvesting of the medium and cells as described above.

2.8. Calculation and statistics

Data are expressed as mean ± S.D. of triplicate wells in at least three individual experiments. Analysis of variance and Student's *t*-test were used to assess the significance of differences; $P < 0.05$ was considered significant.

3. Results

3.1. Effects of oxysterols on DGBE and HPAE cell proliferation

The effects of TriolC and 7KC on cell proliferation of two different cell lines, DGBE cells and HPAE cells, were examined. Cells were plated at equal density and cultured for 24 h before treatment with the oxysterols. Oxysterols were added to the cells in concentrations ranging from 1 to 100 μM. After treatment with oxysterols for 48 h, the cell numbers were measured by a non-radioactive colorimetric method using tetrazolium salts [11]. Fig. 1 shows the inhibitory effects of TriolC and 7KC on DGBE cells (A) and HPAE cells (B), respectively. The proliferation of both cell types was inhibited by the oxysterols in a dose-dependent fashion. The concentrations at which TriolC and 7KC inhibited cell growth to 50% of control (IC_{50}) were estimated at 24.2 and 60.5 μM, respectively, for the DGBE cells. In contrast, much stronger effects of both oxysterols on the HPAE cells were observed. The IC_{50}s of TriolC and 7KC for the HPAE cells were estimated at 5.7 and 16.4 μM, respectively. The IC_{50}s of both oxysterols were about four times higher for DGBE cells than for HPAE cells. This suggests that, when proliferating, DGBE cells are more resistant to oxysterols than HPAE cells. Furthermore, TriolC had a greater growth inhibitory effect than 7KC.

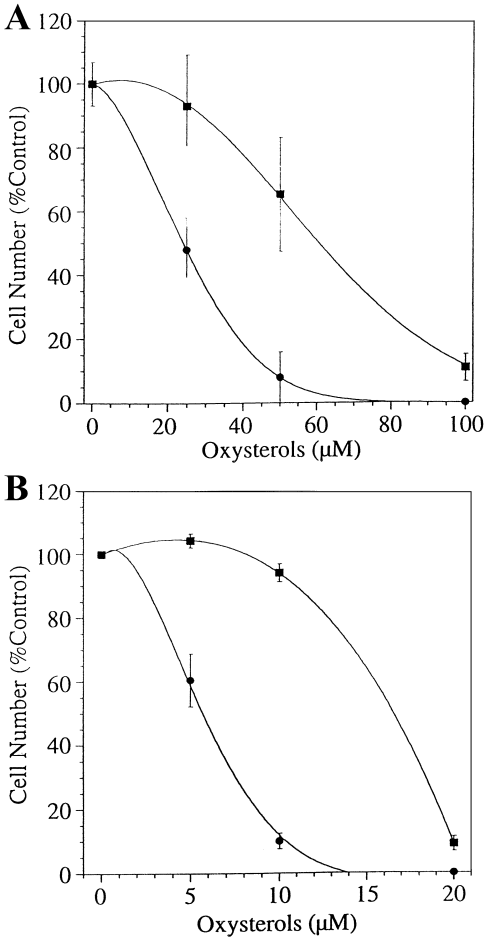

Fig. 1. The effect of oxysterols on cell growth of DGBE cells (A) and HPAE cells (B). DGBE and HPAE cells were plated at equal density and cultured for 24 h. Oxysterols dissolved in 0.5% ethanol/cell culture medium were then added to the cells in concentrations ranging from 1 to 100 μM. After 48 h of incubation cell proliferation was measured. Values are expressed as a percentage of controls incubated without oxysterols. Circles: TriolC, squares: 7KC. Data expressed as mean ± S.D. for three individual experiments in triplicate wells ($n = 9$).

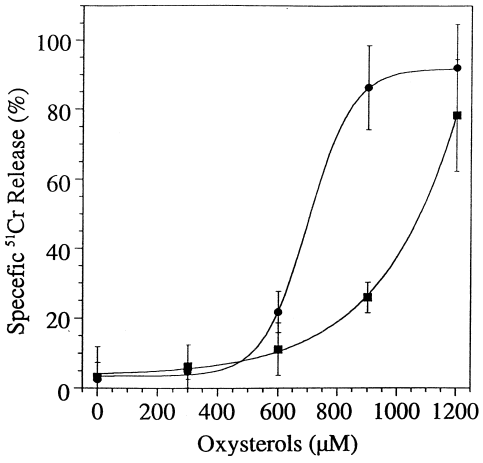

Fig. 2. Specific ^{51}Cr release by confluent DGBE cells treated with oxysterol-containing model biles. DGBE cells were grown to confluence on Transwell inserts and labeled with $Na_2[^{51}Cr]O_4$. Cells were either incubated in cell culture medium (control) or in model biles containing 300–1200 μM TriolC or 7KC. Model biles were added to the apical compartments of the wells only. Maximum ^{51}Cr release from the cells was measured by treatment with 2.5 mM Triton X-100. ^{51}Cr release from the cells was measured after 24 h of incubation. Specific ^{51}Cr release was calculated as follows; (observed ^{51}Cr release−spontaneous release)/(maximum release−spontaneous release)×100 (%). Circles: TriolC, squares: 7KC. Data expressed as mean ± S.D. for three individual experiments in triplicate wells ($n = 9$).

3.2. Cytotoxic effects of oxysterols on confluent cultured DGBE cells

We next investigated the cytotoxic effects of oxysterols on confluent DGBE cells grown on Transwell inserts. This culture system allowed separate and independent access to the apical (luminal) and basolateral (serosal) compartments. TriolC (120 or 240 μM) was dissolved in a solution of 1% ethanol in serum-free medium. TriolC solutions were added to either the apical compartments or the basolateral compartments of Transwells containing confluent monolayers of DGBE cells labeled with ^{51}Cr. Release of ^{51}Cr from the cells was measured after 24 h of incubation (Table 1). No increase in ^{51}Cr release was observed after application of TriolC to either the apical or basolateral compartments. At a concentration of 240 μM, TriolC was oversaturated. Therefore, to dissolve higher concentrations of oxysterols, we made model

Table 1
^{51}Cr release by confluent DGBE cells treated with TriolC

Treatment	^{51}Cr Release (% of maximum)
Control	34.1 ± 1.8
Triton X-100	100 ± 1.9
EtOH	39.0 ± 3.2
120 μM TriolC (apical)	35.6 ± 1.9
120 μM TriolC (basolateral)	33.9 ± 4.0
240 μM TriolC (apical)	35.2 ± 4.1
240 μM TriolC (basolateral)	35.3 ± 2.0

DGBE cells were grown to confluence on Transwell inserts and labeled with $Na_2[^{51}Cr]O_4$. Cells were either incubated in cell culture medium (control), 1% ethanol in cell culture medium (EtOH) or with TriolC dissolved in 1% ethanol/cell culture medium (120 or 240 μM). TriolC was added to either the apical or the basolateral compartments. Maximum ^{51}Cr release from the cells was measured by treatment with 2.5 mM of Triton X-100. ^{51}Cr release from the cells was measured after 24 h of incubation. Data expressed as mean ± S.D.

Table 2
^{51}Cr release by confluent DGBE cells treated with oxysterol-containing model biles

Oxysterol concentration (μM)	TriolC (% of maximum)	7KC (% of maximum)
0	28.5 ± 5.4	23.0 ± 9.8
300	30.1 ± 2.9	25.5 ± 7.4
600	42.6 ± 4.7	29.4 ± 8.3
900	90.1 ± 9.5	41.1 ± 5.1
1200	94.3 ± 9.8	82.5 ± 13.7
Control	26.6 ± 2.8	20.6 ± 4.0
Triton X-100	100 ± 6.7	100 ± 6.4

DGBE cells were grown to confluence on Transwell inserts and labeled with $Na_2[^{51}Cr]O_4$. Cells were either incubated in cell culture medium (control) or in model biles containing 300–1200 μM TriolC. Model biles were added to the apical compartments of the wells only. Maximum ^{51}Cr release from the cells was measured by treatment with 2.5 mM Triton X-100. ^{51}Cr release from the cells was measured after 24 h of incubation. Data expressed as mean ± S.D.

biles containing the oxysterols. Oxysterol concentrations in model bile ranged from 300 μM (25% oxysterols, 75% cholesterol) to 1200 μM (100% oxysterols, 0% cholesterol). These model bile solutions were added to the apical compartments of the Transwell system and incubated for 24 h. Table 2 shows the ^{51}Cr release from DGBE cells after treatment with the model biles. Model biles containing less than 300 μM TriolC or those containing less than 600 μM 7KC did not cause an increase of ^{51}Cr release from the cells. At higher concentrations of oxysterols, ^{51}Cr release increased in a dose-dependent fashion. Furthermore, the specific ^{51}Cr release as caused by oxysterols was evaluated (Fig. 2). The concentrations at which oxysterols caused 50% of specific ^{51}Cr release were estimated at 709 μM (TriolC) and 1079 μM (7KC), respectively.

3.3. The effects of oxysterol-containing model biles on mucin secretion by cultured DGBE cells

Our previous studies [9–11] have consistently shown that model biles induce mucin secretion by DGBE cells. This stimulatory effect was caused by bile salts. Therefore, we examined the effects of oxysterol-containing model biles on mucin secretion by cultured DGBE cells. The concentrations of oxysterols in model biles ranged from 6 to 360 μM. Cytotoxic effects were not observed at these concentrations as previously assessed by the ^{51}Cr release assay. Model bile containing cholesterol alone was used as a control. The basal mucin secretion by DGBE cells (treated with serum-free medium) was 60.5 ± 10.6% of control. Table 3 shows the mucin secretion by DGBE cells treated with model biles containing TriolC and 7KC. At low concentrations of TriolC (< 12 μM), mucin secretion was slightly increased, but this effect was not statistically significant. However, TriolC at concentrations above 60 μM suppressed taurocholate-induced mucin secretion in a dose-dependent fashion. At concentrations of 240 μM and higher, mucin secretion was maximally inhibited, but still remained higher than basal secretion. In contrast, mucin secretion remained unaltered after treatment with model biles containing 7KC.

Table 3
Mucin secretion by DGBE cells treated with oxysterol-containing model biles

Concentration (μM)	TriolC (% of control)	7KC (% of control)
6	113.8 ± 22.8	112.2 ± 21.7
12	115.6 ± 28.9	98.2 ± 13.4
60	98.1 ± 25.7	97.6 ± 17.3
120	77.6 ± 6.5**	93.6 ± 10.5
240	81.6 ± 7.9**	95.4 ± 10.6
360	83.8 ± 8.1**	93.4 ± 6.6
Cholesterol	100 ± 10.6	100 ± 11.2
Medium	60.5 ± 10.6	62.9 ± 12.0

DGBE cells were grown to confluence on Transwell inserts and labeled with [^{3}H]*N*-acetyl-D-glucosamine. Cells were either incubated in cell culture medium (Medium), model bile containing cholesterol only (Cholesterol) or in model biles containing 6–360 μM TriolC or 7KC. Model biles were added to the apical compartments of the wells only. Mucin secretion was measured after 24 h of incubation. Data expressed as mean ± S.D. **; Significant difference between cholesterol model bile and oxysterol model bile by Student's *t*-test ($P < 0.01$).

4. Discussion

Oxysterols have multiple biological activities including inhibiting cholesterol synthesis [15], membrane insertion [16,17], immunosuppression [18–21], modifying arachidonic acid metabolism [22,23], augmenting cell permeability [24], inhibiting intercellular communications [25] and modulating the activity of membrane-bound enzymes [26]. Cytotoxic effects of oxysterols on different cell types have also been described [27–31]. Oxysterols induce apoptosis in different types of cells [32–34]. Some oxysterols, such as 27-hydroxycholesterol, 7KC, 7β-hydroxycholesterol and 7α-hydroxycholesterol have been identified in human atherosclerotic plaques [4]. Since these oxysterols are synthesized in human liver [35–37], they might be secreted into the biliary tract. Recently, we have found 4,6-cholestadiene-3-one and 4-cholestene-3-one in human gallbladder bile [38]. Other undefined oxysterols were also detected. Moreover, oxysterol contents as high as 70% of total sterol were found in pigment gallstones.

The effects of oxysterols on biliary epithelial cells have not been studied. Gallbladder epithelial cells, especially on the luminal surface, are exposed to a high concentration of lipids, and therefore probably oxysterols, in bile. We hypothesize that oxysterols may affect biliary epithelial cell physiology and may be involved in the pathogenesis of biliary tract disease, such as gallstone disease and biliary tract cancer. In the absence of any further physiological information, we have begun mechanistic studies on the effects of oxysterols on biliary epithelial cells. We have investigated the cytotoxicity of two commercially available oxysterols, TriolC and 7KC, on gallbladder epithelial cells and their effects on mucin secretion, one of the most important functions of gallbladder epithelial cells.

We studied the effects of oxysterols on DGBE cells and HPAE cells during cell proliferation. The concentrations at which TriolC and 7KC inhibited cell growth to 50% of control were approximately four times higher for DGBE cells than for HPAE cells. The sensitivity of the cells to oxysterols might vary in both cell types, as described by other investigators [27–34]. Since gallbladder epithelial cells are exposed to high concentrations of lipids in vivo, this might partially explain the increased relative resistance of these cells to the effects of oxysterols. Furthermore, the IC_{50} for 7KC was 2.5–2.9 times higher than that for TriolC for both cell lines. Zhou et al. reported that TriolC has a greater growth inhibitory effect than 7KC on human arterial smooth muscle cells [39]. Therefore, in addition to the dose-dependency, a structure–function relationship may also affect the oxysterol-induced effects on cell proliferation.

We also examined the cytotoxic effect of oxysterols on confluent, non-proliferating DGBE cells. Confluent DGBE cells are highly polarized by scanning electron microscopy [6]. Growing these cells on the Transwell insert system allows separate and independent access to the apical (luminal) and basolateral (serosal) compartments, due to the formation of a leak-tight monolayer [8]. We used a ^{51}Cr release assay for evaluating the cytotoxicity of oxysterols on this culture system. No cytotoxic effects were observed with concentrations up to 240 μM TriolC in aqueous solution. Subsequently, DGBE cells were treated with model biles containing oxysterols. An increase in ^{51}Cr release was observed at concentrations > 300 μM TriolC and > 600 μM 7KC. The concentra-

tions of oxysterols at which 50% of specific ^{51}Cr release occurred were more than 15 times higher than the IC_{50} of oxysterols for proliferating DGBE cells. Although the methods for assessment of cytotoxicity were different between proliferating cells and confluent cells, it is likely that the cytotoxic susceptibility to oxysterols depends on the proliferation status of the cell. Proliferation of biliary epithelial cells in vivo might be slow, just like the confluent cells in the cell culture system. Therefore, these results suggest that gallbladder epithelial cells are very resistant to oxidized lipids in vivo.

Mucin secretion is an important function of epithelial cells. Secreted mucin protects epithelial cell membranes from harmful agents [40]. Mucin hypersecretion has long been implicated in the formation of cholesterol gallstones [41]. We have previously shown that model biles and bile salts induce mucin secretion by DGBE cells [8–10] without any associated cytotoxicity. One explanation for increased mucin secretion by the gallbladder in vivo may be mediation by oxysterols. However, in the present studies TriolC actually suppressed taurocholate-induced mucin secretion, whereas 7KC did not affect it. TriolC, at concentrations between 120 and 360 μM, suppressed mucin secretion dose-dependently. At higher concentrations of TriolC (>240 μM) the inhibition of mucin secretion was maximal, although the level of mucin secretion was still higher than basal secretion. These results could indicate that TriolC did not directly inhibit the mucin secretion as stimulated by taurocholate. If TriolC inhibits the mucin secretion pathway stimulated by taurocholate, mucin secretion would be expected to decrease to basal secretion in a dose-dependent fashion. Therefore, TriolC may suppress mucin secretion via an alternative mechanism. Oxysterols insert into cell membranes and decrease membrane fluidity [16]. The decrease of membrane fluidity causes tightening of membrane packing. This may inhibit mucin granule exocytosis. We have tested the effect of oxysterols on mucin secretion in only one model bile system. The stimulatory mechanism of mucin secretion by taurocholate also applies to other bile salts, and likewise the suppressive effects on bile salt-induced mucin secretion by TriolC. Currently little information is available on the species and concentrations of oxysterols in bile. The present study consists of short-term experiments with relatively high concentrations of oxysterols. The kinetics of oxysterol–cell membrane interactions remain unexplored. However, considering that in vivo the gallbladder epithelium is in continuous contact with cholesterol (and oxysterols), we think that even at lower concentrations of oxysterols in bile, it is possible that long-term exposure to oxysterols alters cell membrane structure and cellular function.

The choice of oxysterols and their concentrations are justified for the following reasons. TriolC and 7KC are present in human hepatic bile. TriolC concentration ranged from 18 to 387 nM; 7KC concentration ranged from 4 to 1800 nM (Yoshida, T., unpublished observations). As these measurements were performed in hepatic bile, and as lipid concentrations in gallbladder bile can be 10-fold or more higher, we estimate that gallbladder bile concentrations of TriolC and 7KC could be 4 and 20 μM, respectively. While these concentrations are at the lower end of the range used for our studies, numerous other species of oxysterols are present in human bile samples. For example, 7α-hydroxycholesterol, 7-β-hydroxycholesterol, 7-keto-cholesterol, TriolC, and other oxysterol species are present (Yoshida, T, unpublished observations). Therefore, the total concentration of oxysterols is likely to be present in the range that we studied. Finally, local concentrations of oxysterols in the vicinity of the apical membrane of gallbladder epithelial cells may be much higher than what can be measured in gallbladder and hepatic bile due to gallbladder stasis. Hence, specific interactions at the cellular level likely occur in the presence of oxysterols at higher concentrations than can be measured. Due to these considerations, concentrations of TriolC and 7KC in the micromolar range were used.

These results support a model of wide-ranging oxysterol effects on gallbladder epithelial cells in the setting of inflammation. The high concentrations of cholesterol in bile are subject to oxidative damage leading to the formation of oxysterols. Such oxidative damage may occur when inflammatory cells are activated in the biliary system, as would occur with infection. This would lead to bacterial–neutrophil–cholesterol interactions, leading to the formation of oxysterols. Oxysterols, in turn, would have far reaching effects on gallbladder epithelial cell function, including effects on cell proliferation, cytotoxicity and mucin secretion. The suppression of mucin secretion by certain oxysterols may be a further route to gallbladder epithelial cell injury. The effects of oxysterols on cell proliferation and cytotoxicity may have implications for the development of gallbladder cancer.

In conclusion, we have for the first time studied the effects of oxysterols on gallbladder epithelial cells. We found that oxysterols have cytotoxic effects on DGBE cells and that these effects were dependent on the proliferation status of the cells. Moreover, oxysterols with different structures had varying effects on the DGBE cells. TriolC was more cytotoxic than 7KC. TriolC also suppressed taurocholate-induced mucin secretion by the cells, whereas 7KC did not. We suggest that the cytotoxic effects of oxysterols in general and the specific decrease in mucin secretion in particular, as caused by TriolC, might have important implications in the pathophysiology of biliary tract diseases. Further research into the role of oxysterols in the biliary tract therefore seems warranted.

References

[1] Smith, L.L. (1987) Chem. Phys. Lipids 44, 87–125.
[2] Smith, L.L. and John, B.H. (1989) Free Radic. Biol. Med. 7, 285–332.
[3] Brooks, C.J.W., McKenna, R.M., Cole, W.J., MacLachlan, J. and Lawrie, T.D.V. (1983) Biochem. Soc. Trans. 11, 700–701.
[4] Brown, A.J., Leong, S., Dean, R.T. and Jessup, W. (1997) J. Lipid Res. 38, 1730–1745.
[5] Yuan, Y.V., Kitts, D.D. and Godin, D.V. (1997) Br. J. Nutr. 78, 993–1014.
[6] Oda, D., Lee, S.P. and Hayashi, A. (1991) Lab. Invest. 64, 682–692.
[7] Kuver, R., Savard, C.E., Oda, D. and Lee, S.P. (1994) Am. J. Physiol. 267, G998–G1003.
[8] Klinkspoor, J.H., Kuver, R., Savard, C.E., Oda, D., Azzouz, H., Tytgat, G.N.J., Groen, A.K. and Lee, S.P. (1995) Gastroenterology 109, 264–274.
[9] Klinkspoor, J.H., Tytgat, G.N.J., Lee, S.P. and Groen, A.K. (1996) Biochem. J. 316, 873–877.
[10] Klinkspoor, J.H., Yoshida, T. and Lee, S.P. (1998) Biochem. J. 332, 257–262.
[11] Mosmann, T. (1983) J. Immunol. Methods 65, 55–63.
[12] Hiraishi, H., Terano, A., Ota, S., Mutoh, H., Razangi, M., Sugimono, T. and Ivey, K.J. (1991) Am. J. Physiol. 260, G556–G563.
[13] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gart-

ner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[14] Kibe, A., Dudley, M.A., Halpern, Z., Lynn, M.P., Breuer, A.C. and Holzbach, R.T. (1985) J. Lipid Res. 26, 1102–1111.
[15] Goldstein, J.L. and Brown, M.S. (1990) Nature 343, 425–430.
[16] Szostek, R., Kucuk, O., Lis, L.J., Tracy, D., Mata, R., Dey, T., Kauffman, J.W., Yachnin, S. and Westerman, M.P. (1991) Biochem. Biophys. Res. Commun. 180, 730–734.
[17] Li, Q.-T. and Das, N.P. (1994) Arch. Biochem. Biophys. 315, 473–478.
[18] Richert, L., Beck, J.P. and Shinitzky, M. (1986) Immunol. Lett. 13, 329–334.
[19] Moog, C., Luu, B., Beck, J.P., Italliano, L. and Bischoff, P. (1988) Int. J. Immunopharmacol. 10, 511–518.
[20] Moog, C., Luu, B., Altmeyer, A. and Bischoff, P. (1989) Int. J. Immunopharmacol. 11, 559–565.
[21] Kucuk, O., Stoner-Picking, J., Yachnin, S., Gordon, L.I., Williams, R.M., Lis, L.J. and Westerman, M.P. (1992) Cell Immunol. 139, 541–549.
[22] Lahoua, Z., Vial, H., Michel, F., Crastes de Paulet, A. and Astruc, M.E. (1991) Cell Signal. 3, 559–567.
[23] Astruc, M.E. and Lahoua, Z. (1994) Cell Signal. 6, 763–775.
[24] Boissonneault, G.A., Hennig, B. and Ouang, C-M. (1991) Proc. Soc. Exp. Biol. Med. 196, 338–343.
[25] Zwijsen, R.M.L., Oudenhoven, I.M.J. and de Haan, L.H.J. (1992) Eur. J. Pharmacol. 228, 115–120.
[26] Ramasamy, S., Boissonneault, G.A. and Hennig, B. (1992) J. Am. Coll. Nutr. 11, 532–538.
[27] Hennig, B. and Boissonneault, G.A. (1987) Atherosclerosis 68, 255–261.
[28] Peng, S.K., Tham, P., Taylor, C.B. and Mikkelson, B. (1979) Am. J. Clin. Nutr. 32, 1033–1042.
[29] Highley, N.A. and Taylor, S.L. (1984) Food Chem. Toxicol. 22, 983–992.
[30] Boissonneault, G.A. and Heiniger, H.J. (1985) J. Cell Physiol. 125, 471–475.
[31] Bakos, J.T., Johnson, B.H. and Thompson, E.B. (1993) J. Steroid Biochem. Mol. Biol. 46, 415–426.
[32] Lizard, G., Deckert, V., Dubrez, L., Moisant, M., Gambert, P. and Lagrost, L. (1996) Am. J. Pathol. 148, 1625–1638.
[33] Aupeix, K., Weltin, D., Mejia, J.E., Christ, M., Marchal, J., Freyssinet, J.-M. and Bischoff, P. (1995) Immunobiology 194, 415–428.
[34] Nishino, E. and Watanabe, Y. (1996) Biochem. Biophys. Res. Commun. 226, 928–934.
[35] Myant, N.B. and Mitropoulos, K.A. (1977) J. Lipid Res. 18, 135–153.
[36] Song, W., Pierce, W.M., Saeki, Y., Redinger, R.N. and Prough, R.A. (1996) Arch. Biochem. Biophys. 328, 272–282.
[37] Shoda, J., Toll, A., Axelson, M., Pieper, F., Wikvall, K. and Sjövall, J. (1993) Hepatology 17, 395–403.
[38] Haigh, G. and Lee, S.P. (1999) Hepatology 30, 429A.
[39] Zhou, Q., Wasowicz, E. and Kummerow, F.A. (1995) J. Am. Coll. Nutr. 14, 169–175.
[40] Van Klinken, B.J., Dekker, J., Buller, H.A. and Einerhand, A.W. (1995) Am. J. Physiol. 269, G613–G627.
[41] Carey, M.C. (1993) Am. J. Surg. 165, 410–419.