Pathogenesis of gallstones in Crohn's disease
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Chapter 7

GROWTH OF HUMAN CHOLESTEROL GALLSTONES IN GALLBLADDER BILE OF PATIENTS WITH INFLAMMATORY BOWEL DISEASE AND GALLSTONE PATIENTS; A PRELIMINARY REPORT
GROWTH OF HUMAN CHOLESTEROL GALLSTONES IN GALLBLADDER BILE OF PATIENTS WITH INFLAMMATORY BOWEL DISEASE AND GALLSTONE PATIENTS; A PRELIMINARY REPORT

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**Background & Aims:** Pigment and cholesterol crystallization promoting factors are increased in biles of Crohn's disease patients explaining their potential risk for both types of stones. The specific aim was to study whether presence of these factors would reveal differences in cholesterol stone growth. Therefore preexisting cholesterol gallstones were incubated in gallbladder biles from patients with Crohn's disease or with ulcerative colitis.

**Material and Methods:** Small human cholesterol gallstones were incubated in human gallbladder biles obtained from patients with inflammatory bowel disease and from cholesterol gallstone patients. Concentrations of biliary lipids, bile pigments and calcium were determined and accretion of material to the stone was measured during incubation. In addition the characteristics of the stone surface were analyzed by scanning electron microscopy.

**Results:** Similar increases in the mass of cholesterol gallstones were observed in patients with inflammatory bowel disease (IBD) and non-IBD patients. Biliary unconjugated bilirubin correlated with stone growth in biles from Crohn’s disease patients, but not in biles from ulcerative colitis patients. Microscopical analyses revealed cholesterol crystals adherent at the surface of the incubated stones and more intense pigmentation macroscopically.

**Conclusions:** Cholesterol gallstones accrete foremost cholesterol, whereas bile pigments were deposited to a lesser extent. Because patients with Crohn's disease and with ulcerative colitis showed the same growth potential in spite of a high gallstone prevalence, we speculated that stone nidation occurs more frequently in Crohn's disease bile samples.
Introduction

Humans suffering from impaired function of the distal ileum due to resection or disease show an increased incidence of gallstones in the gallbladder. The nature of the stones is still elusive. Recently, direct evidence from studies on bile composition of such patients and indirect evidence from animal studies have pointed to an important role for calcium bilirubinate precipitation in the overall etiology of gallstones in patients with ileal resection or ileal Crohn's disease. Excessive bile salt spilling into the colon because of decreased uptake of bile salts in the ileum facilitates the colonic uptake of bile pigments. This leads to enhanced cycling of pigments to the liver and thus to increased levels of re-secreted bilirubinates in bile. Unconjugated bilirubin has a high affinity for ionised calcium and pigment salt precipitation may occur. These precipitates are thought to function as a nidus from which mature gallstones can grow. The presence of calcium bilirubinate precipitates in sludge and in the centers of the majority of gallstones suggest an active role for these precipitates in gallstone formation. A stone nidus can grow subsequently into either a pigment gallstone or a cholesterol gallstone, in case the bile is either supersaturated with just pigments or with cholesterol, respectively.

Ex vivo, faster crystallization of cholesterol was observed in gallbladder biles from Crohn's disease patients compared to ulcerative colitis. A sub-selection of Crohn's disease patients with afflicted ileum showed increased levels of unconjugated bilirubin in bile. Therefore, both pigment and cholesterol gallstones have a growth potential in Crohn's disease patients. No increased incidence of gallstones has been reported in ulcerative colitis. The total lipid contents and cholesterol saturation indices of gallbladder biles from Crohn's disease and ulcerative colitis patients are very similar. We may assume that biles from Crohn's disease patients contain a factor that induces stone formation, or a fraction of proteins that favours crystallization of cholesterol. In the bile of ulcerative colitis patients no increased pigments levels are present, but biles frequently contained a glycoprotein fraction, which inhibits formation of cholesterol crystals. Thus, stone formation in ulcerative colitis patients may be less prevalent than in Crohn's disease patients either due to prevention of nidus formation, or prevention of growth of nidi formed into full stones.
Growth of a stone nidus into mature stones has not, to our knowledge, been studied directly. Recently, in our laboratory a method was developed to measure growth of small human gallstones in biles in vitro. We describe now some characteristics of growth of human cholesterol gallstones in human gallbladder biles from patients with inflammatory bowel disease (IBD) and as a control in biles from gallstone patients. Concentrations of lipids, pigments and calcium were measured in bile and accretion of precipitates to the stones was determined. Growth of stones was observed in all bile samples from IBD patients. Stones accreted both cholesterol and pigment. Unconjugated bilirubin levels in gallbladder bile correlated with stone growth in biles of Crohn's disease patients, but not in ulcerative colitis patients. We hypothesise that enhanced formation of stone nidi in Crohn's disease patients is responsible for the increased incidence of gallstones in these patients.

Materials and methods

Patients

After written informed consent and with approval of the medical ethics committee at the Academic Medical Center, Amsterdam, we studied 16 patients with radiologically or endoscopically suspected inflammatory bowel disease when they were admitted for an elective bowel resection. The diagnosis and localization of Crohn's disease or ulcerative colitis was confirmed by histological examination of the resected bowel segments.

Bile collection

Gallbladder bile from IBD patients was obtained by needle aspiration as described by Strasberg et al. and transported to the laboratory for immediate processing. Fresh bile was centrifuged (10 min, 1500xg) and stored at -20°C for later determinations of biliary lipid concentrations, calcium and bile pigments.

Biliary lipids and bile pigment analysis

Biliary bile salts, phospholipids and cholesterol were measured by standard enzymatic procedures. Total lipid concentration (TLC) was calculated from the sum of cholesterol, bile salt and phospholipid concentrations and expressed in g/dL. Cholesterol
saturation indices were calculated according to Carey’s critical tables. Bilirubin conjugates and unconjugated bilirubin were quantified by high-performance liquid chromatography, according to the procedure of Spivak and Yuey.

**Crystal Detection Time (CDT)**

Bile samples were passed through a 0.22μm Millipore (Millex-GP) filter into a sterile container and incubated at 37°C. Aliquots of bile were examined daily for cholesterol monohydrate crystals using polarized light microscopy. The crystal detection time (CDT) was defined as the time, at which the first cholesterol crystal in a bile sample was observed. Fast nucleating bile was defined as bile that formed crystals within 4 days after incubation as suggested by Jüngst et al.

**Measurement of gallstone growth**

Growth of cholesterol gallstones was determined as described elsewhere. Briefly, matched, small cholesterol gallstones (diameter 2-3 mm, dry weight 35-45 mg) were obtained from one patient who underwent cholecystectomy because of symptomatic gallstone disease. Stones were washed in demineralised water and dried under vacuum. Stones were placed individually in 2 ml of whole bile in sterile tubes and were incubated at 37°C. At several days of incubation, gallstone dry mass was determined. Mass increases were determined with an analytical balance with 0,1 mg accuracy, and were expressed as percentage increase of the initial mass. This was done to correct for the variation in the size of the various sets of incubated stones.

**Analysis of gallstone surface morphology and composition by light and scanning electron microscopy and analysis of energy dispersion of X-rays (EDAX)**

Representative specimens of gallstones from before and after incubation in human gallbladder bile were studied by light microscopy for gross morphology using a stereo microscope. Stones were mounted on a specimen table and sputter coated with gold. Micro-morphology of the stone surface was studied using a Philips SEM 525 electron microscope (Eindhoven, The Netherlands). Element quantification of selected deposits at the stone surface...
was performed by analysis of the energy of dispersed X-rays using a ECON IV P-510 (EDAX International).

**Results**

**Patient characteristics and composition of their gallbladder bile**

Table 1 shows the characteristics of the patients with inflammatory bowel disease. The groups were comparable in terms of mean age, gender distribution and immunosuppressive treatment, as exemplified by the use of corticosteroids, mesalazine and azathioprine, and inflammatory parameters.

<table>
<thead>
<tr>
<th></th>
<th>CD</th>
<th>UC</th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>39 ± 9</td>
<td>38 ± 5</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Sex (male/female)</strong></td>
<td>5/4</td>
<td>3/3</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Previous surgery (% of patients)</strong></td>
<td>4 (44)</td>
<td>4 (67)</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Immunosuppressive treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>2</td>
<td>3</td>
<td>N.S.</td>
</tr>
<tr>
<td>Mesalazine</td>
<td>6</td>
<td>2</td>
<td>N.S.</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>2</td>
<td>1</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>ESR (mm/hr)</strong></td>
<td>17 ± 12</td>
<td>35 ± 32</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>White blood cell count (10⁹/L)</strong></td>
<td>8.5 ± 2</td>
<td>11.7 ± 2</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

As shown in Table 2, the biliary lipid, bile pigment and total calcium compositions of gallbladder bile from IBD patients and gallstone patients were comparable. Almost all biles obtained from cholesterol gallstone patients were fast-nucleating, whereas the majority of bile samples obtained from patients with inflammatory bowel disease were slow-nucleating.
Table 2. Characteristics of gallbladder bile obtained from patients with Crohn's disease (CD), ulcerative colitis (UC) and gallstone patients (Gallstone).

<table>
<thead>
<tr>
<th></th>
<th>CD n = 9</th>
<th>UC n = 7</th>
<th>Gallstone n = 7</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipids (mM)</td>
<td>52.9 ± 14.7</td>
<td>57.0 ± 21.5</td>
<td>41.9 ± 14.4</td>
<td>N.S.</td>
</tr>
<tr>
<td>Cholesterol (mM)</td>
<td>20.9 ± 6.4</td>
<td>17.9 ± 8.2</td>
<td>14.2 ± 4.2</td>
<td>P = 0.03</td>
</tr>
<tr>
<td>Bile Salts (mM)</td>
<td>167.6 ± 48.7</td>
<td>178.6 ± 65.5</td>
<td>116.0 ± 25.6</td>
<td>P = 0.04</td>
</tr>
<tr>
<td>Total Lipid concentration (g/dL)</td>
<td>13.1 ± 2.4</td>
<td>12.9 ± 3.3</td>
<td>9.5 ± 3.9</td>
<td>P = 0.04</td>
</tr>
<tr>
<td>CSI</td>
<td>1.23 ± 0.49</td>
<td>0.97 ± 0.19</td>
<td>1.16 ± 0.26</td>
<td>N.S.</td>
</tr>
<tr>
<td>Crystal Detection Time (days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fast (&lt; 4 days)</td>
<td>1/7</td>
<td>1/7</td>
<td>6/7</td>
<td></td>
</tr>
<tr>
<td>slow (&gt; 21 days)</td>
<td>3/7</td>
<td>4/7</td>
<td>1/7</td>
<td></td>
</tr>
<tr>
<td>mean of crystallizers (days)</td>
<td>7 ± 2</td>
<td>5 ± 2</td>
<td>3 ± 2*</td>
<td>P ≤ 0.05</td>
</tr>
<tr>
<td>Total calcium (mM)</td>
<td>12.9 ± 6.0</td>
<td>11.2 ± 7.8</td>
<td>n.d.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Unconjugated Bilirubin (µM)</td>
<td>128 ± 45</td>
<td>117 ± 45</td>
<td>n.d.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute (mg)</td>
<td>1.6 ± 0.8</td>
<td>1.9 ± 1.7</td>
<td>1.6 ± 1.2</td>
<td>N.S.</td>
</tr>
<tr>
<td>%</td>
<td>5.1 ± 1.9</td>
<td>4.8 ± 4.1</td>
<td>6.6 ± 3.5</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

**Growth of small human gallstones incubated in human gallbladder bile**

Small human cholesterol gallstones were incubated in gallbladder biles from nine patients suffering from ulcerative colitis and seven patients suffering from Crohn's disease. In the biles of one ulcerative colitis and one Crohn's disease patient a decrease in the average mass, as well as in individual masses of three incubated gallstones was observed. This occurred in biles that were undersaturated for cholesterol (CSI's 0.62 and 0.72 respectively) in the presence of high mole percentages of bile salts. So, most probably bile salt dissolution of cholesterol crystals from the body of the gallstones was responsible for decreased stone weight. In five UC and eight CD patients growth of gallstone mass occurred with a mean increase in their dry masses of 0.5 mg to near 4 mg. Typical examples of kinetics of growth are given in Figure 1.
Figure 1. Representative kinetics of human cholesterol gallstones incubated in gallbladder bile obtained from Crohn's disease and ulcerative colitis patients.

The mean growth of gallstones, expressed as a percentage of their initial dry mass, was similar in biles from both IBD and non-IBD patients. In the biles, the mean concentrations of total calcium and lipids, and the cholesterol saturation indexes, were the same for IBD and non-IBD patients (Table 2). For all biles with CSI above 0.75 both for IBD patients and gallstone patients a positive relationship between increases in the mass of incubated stones and total lipid contents was observed (Figure 2).
For biles from cholesterol gallstone patients an inverse relationship between mass increases and the CSI (not shown) or mole percentage of cholesterol (Figure 3) was observed. No clear relationship was found for the mole percentage cholesterol and stone growth in Crohn’s diseased patients (Figure 3). In patients with ulcerative colitis however, stone growth increased with increasing mole percentages of cholesterol (Figure 3). No relationships were observed between stone growth and the concentration of total calcium or total bilirubinates, mono- or diconjugated bilirubin, in any of groups of IBD patients. However, as shown in Figure 4, unconjugated bilirubin correlated with stone growth in Crohn’s disease biles (r = 0.88; p = 0.005), but not in biles obtained from ulcerative colitis patients. Some stones turned from white to bright green after incubation in the biles, showing that some accretion of pigments to the stones had occurred (Plate 1). Scanning electron microscopy showed deposition of cholesterol crystals at the surface of the stones (Plate 2).

Figure 3. Relationship between mole percentage biliary cholesterol and growth of human cholesterol gallstones incubated in biles obtained from patients with cholesterol gallstone disease, Crohn’s disease and ulcerative colitis.
Figure 4. Relationship between biliary unconjugated bilirubin and growth of human cholesterol gallstones incubated in biles obtained from patients with Crohn's disease and ulcerative colitis.

![Graphs showing relationship between biliary unconjugated bilirubin (UCB) and growth of gallstones in patients with Morbus Crohn and colitis ulcerosa.](image)

Plate 1. Small human cholesterol gallstones before (left) and after (right) incubation in bile obtained from a patient with ileal Crohn's disease.

![Image of gallstones before and after incubation](image)

Discussion

We observed similar increases in the mass of small human cholesterol gallstones when incubated in gallbladder biles obtained from Crohn's disease, ulcerative colitis and cholesterol gallstone patients. The average composition of the gallbladder biles from nine Crohn's disease and seven ulcerative colitis patients was the same with respect to lipids, calcium and bile pigments. A previous study with larger numbers of patients showed similar lipid concentrations as we described here and also no differences between biles of Crohn's disease and ulcerative colitis patients were observed.\(^7,15\) For all the parameters studied, only the total
lipid contents of the biles showed a positive relationship with the mass increases of the incubated gallstones, in all these groups. The microscopical analyses showed new cholesterol crystals at the surface of incubated stones, and more intense pigmentation. These facts seem to indicate that in this system stones accrete cholesterol foremost, and to a lesser extent pigments. Binding of proteins or minerals to the stones possibly had contributed to the mass increases, but element analyses via energy dispersion of X-rays, indicated that depositions of these molecules had not occurred, at least not on the surface (results not shown). Because cholesterol gallstones are porous structures, some deposition in the interior cannot be ruled out. Measurements of stone composition before and after growth might provide an answer.

Plate 2. Scanning electron microscopical images of the surface of a cholesterol gallstone that was incubated in bile obtained from a patient with Crohn’s disease. The upper panel shows that new cholesterol plates have been deposited on the surface of the cholesterol gallstone (2000 x). The lower panel shows morphological details of the structure of newly accreted cholesterol crystal plates (8000 x).
The fact that growth of stones in biles obtained from Crohn's disease and ulcerative colitis patients was of the same magnitude shows that both groups carry the same potential for gallstone growth. Yet, gallstones are much more frequent in Crohn's disease than in ulcerative colitis, suggesting more frequent stone nidation in the former group. We hypothesize that increased levels of biliary pigments caused by the impairment of the function of the distal ileum, puts the Crohn's disease patients at risk for formation of calcium bilirubinate precipitates in the gallbladder. A role as a nidus for these precipitates is supported by their presence both in sludge, the pre-stone stadium12,13 and in the center of most gallstones.14

In this study, with small groups of ileal Crohn's disease (five) and ulcerative colitis patients, mean unconjugated bilirubin levels were comparable in biles from Crohn's disease patients and ulcerative colitis patients. Gallstones were observed in none. Biliary unconjugated bilirubin levels correlated with stone growth in biles from Crohn's disease patients, supporting the view that biliary pigments play a more prominent role in stone formation in ileal Crohn's disease. A study on a larger series of IBD patients could possibly show the nature of the stones that cause the increased incidence of gallstones in Crohn's disease and might reveal the true relevance of bile pigments in the formation and growth of the stones in the biles of these patients.

Remarkably, for both gallstone patients and Crohn's disease patients a slightly negative, but for ulcerative colitis patients a positive correlation with the mole percentage of cholesterol of the biles was found. Apparently, in the Crohn's disease and gallstone biles the excess cholesterol at higher relative concentrations does not readily accrete to the incubated stones. Studies with model biles have shown that stimulation of the formation of cholesterol crystals that float in the water phase, via addition of concanavalin A binding fraction (CABF) or biliary mucins and calcium phosphate precipitates, correlates with a decrease in the growth of stones.24 Extraction of CABF from Crohn's disease biles postpones the formation of the first cholesterol crystal detectable via polarized light microscopy.15 In contrast, in bile samples of ulcerative colitis patients, extraction of CABF speeds up the crystallization of cholesterol.15 One might reason that in Crohn's disease patients with high relative concentrations of cholesterol in their biles, a more active CABF is present that stimulates the formation of free cholesterol crystals and thereby reduces the growth of stones. In cholesterol gallstone patients a similar mechanism could be present, because the biles of these patients have more active
CABF compared to control gallbladder biles. Finally, in line with this speculation, in biles from ulcerative colitis patients, where we here showed a positive correlation of stone growth with the mole percentage of cholesterol, we previously found that extraction of CABF from bile resulted in faster crystallization of cholesterol. Of course other bile components that have an effect on crystal formation like mucins, might be equally or even more important in regulating stone growth. Furthermore, specific components that have a direct regulating effect on growth, on the accretion of solids from biles to stones, are possibly yet to be discovered.

References

24. van den Berg AA, van Buul JD, Tytgat GNN, Ostrow JD, Groen AK. Cholesterol gallstone growth in model biles is inversely related to cholesterol crystal formation. Gastroenterology 1998;G2237 (Abstract).

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