Quantitative and qualitative comparison of gall bladder mucus glycoprotein from patients with and without gall stones

P R C Harvey, C A Rupar, S Gallinger, C N Petrunka, and S M Strasberg

From the Department of Surgery, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada and Department of Biochemistry, The Children’s Psychiatric Research Institute, University of Western Ontario, London, Ontario, Canada

Summary  Human gall bladder mucus glycoprotein was isolated by Sepharose 4B gel filtration followed by caesium chloride density gradient ultracentrifugation from four groups: patients with cholesterol gall stones, patients with pigmented stones, patients with complete obstruction of the cystic duct and patients with no biliary tract abnormalities (controls). Mucus glycoprotein concentrations in cholesterol gall stone bile (203 μg/ml±199 SD, n=17), pigment gall stone bile (110 μg/ml±77 SD, n=6) and control gall bladder bile (96 μg/ml±98 SD, n=11) were not significantly different. While bile from patients with complete obstruction of the cystic duct contained significantly higher concentrations of mucus glycoprotein (6220 μg/ml±4130, n=4). In vitro cholesterol nucleation time was not correlated to gall bladder mucus glycoprotein concentrations. Qualitative analysis of the carbohydrate and amino acid composition showed a basic structure typical of mucus glycoproteins in general. It is unlikely that either quantitative or qualitative differences in mucus glycoproteins are responsible for the rapid in vitro nucleation time characteristic of cholesterol gall stone patients.

Supersaturated bile is necessary for stone formation but cholesterol supersaturation is a frequent finding in normal gall bladder bile. Evidence that a nucleating defect is also present in the gall bladder bile of patients with cholelithiasis has recently been obtained by several investigators. Mucus glycoprotein might be the nucleating factor. Gall stones have a mucopolysaccharide matrix and inhibition of gall bladder mucus glycoprotein secretion by aspirin prevents stones. Human gall bladder mucus glycoprotein accelerates nucleation in model bile and bile obtained from prairie dogs.

The purpose of the present study is to determine whether there is a quantitative or qualitative difference in the mucus glycoprotein secreted by the gall bladder of patients with cholesterol cholelithiasis, which might support the preceding observations. Earlier studies measuring mucus glycoprotein used indirect techniques and produced conflicting results. One of the difficulties in such studies has been to obtain representative and comparable bile samples. Studies to characterise gall bladder mucus glycoprotein have been limited because of the difficulty in obtaining human bile samples. Often bile has to be obtained at necropsy or by T-tube drainage. Once bile is obtained, the isolation of pure mucus glycoprotein has been difficult. Proteolytic digestion and organic extractions have been used which might cause degradation of native mucus glycoprotein into glycoprotein subunits. Techniques to isolate intact mucus glycoprotein have been developed and applied to purifying degraded gall bladder mucus glycoprotein. Only three patients with mixed type gall stones were studied. Few studies have compared the composition of gall bladder mucus glycoprotein from normal controls with that of patients with cholelithiasis.

Address for correspondence: Professor S M Strasberg, Department of Surgery, Mount Sinai Hospital, Suite 1142, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada.

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The present investigation, using non-degradative procedures, quantifies, and characterises native biliary mucus glycoprotein isolated from four different patient groups; patients with cholesterol gall stones, patients with pigmented stones, patients with complete obstruction of the cystic duct whose gall bladder contained white bile, and patients without biliary tract disease.

Methods

Patients

Gall bladder bile was obtained from 27 patients undergoing elective cholecystectomy for cholelithiasis. Bile collection methods were approved by the Human Experimentation Committee of the University of Toronto. Patients were given detailed information regarding technique before consenting to take part in the study. All patients had normal liver function tests. The gall bladder was functioning as defined by the presence of typical dark bile and a total lipid concentration greater than 5 g/dl in 23 of the 27 patients with cholelithiasis. Four patients had obstructed gall bladders. Gall bladder bile from these four patients was characteristic ‘white bile’ which is a colourless bilirubin-free fluid with a low total lipid content.

Patients with functioning gall bladders were divided into two groups: those with cholesterol stones (n=17) and those with pigmented stones (n=6). The type of stone was determined by inspection and by chemical analysis for cholesterol.25 All stones designated cholesterol gall stones were greater than 70% cholesterol by weight. Pigment stones were black, amorphous, friable, and less than 10% cholesterol by weight. The patients with obstructed gall bladders all had cholesterol gall stones.

Gall bladder bile was also collected from 11 patients requiring a laparotomy for problems not related to the biliary tract. This group of patients defined as normal controls were having surgery for obesity, duodenal ulcer, Crohn’s disease, or colonic cancer. Ultrasound studies of the gall bladder and bile ducts were normal in these patients and the gall bladder appeared normal at surgery.

Bile collection and extraction of mucus

The gall bladder was completely aspirated of bile by syringe and needle before any surgical manipulation. Each sample was processed separately, immediately after collection and mucus glycoprotein isolated using the method of Pearson et al24 with Sepharose 4B gel chromatography followed by caesium chloride density gradient ultracentrifugation. Briefly, phenylmethylsulfonyl fluoride (PMSF) 1.0 mg was added immediately to bile samples (5.0–7.0 ml) to give a final concentration of 0.82 mM–1.15 mM of PMSF. Bile was dialysed at 4°C against distilled water containing 0.02% sodium azide for 72 hours. Dialysed bile was concentrated by lyophilisation and reconstituted with phosphate buffered saline containing 0.02% sodium azide (10 mM phosphate buffer/0.2 M NaCl/0.02% NaN3; pH 7.4). The reconstituted bile solutions were homogenised for 1.0 minute (Stirrer type RzRL–64, Canlab), centrifuged at 4°C for 30 minutes at 100 000 g to remove undissolved lipids and the lipid pellet was discarded. The supernatant was chromatographed on a Sepharose 4B column (85×2.5 cm) eluted with the phosphate buffered saline at a flow rate of 120 ml/hour. The void volume fractions as determined by dextran blue were pooled and concentrated by lyophilisation.

The excluded glycoprotein peak from Sepharose 4B chromatography was further purified by caesium chloride density gradient ultracentrifugation23 in a Beckman L5–65 preparative ultracentrifuge with a SW 41 Ti rotor. Dry caesium chloride was added to the reconstituted void volume fractions to give a final concentration of 60%. Centrifugation was carried out at 4°C at 150 000 g for 48 hours. One millilitre fractions aspirated from the top of the gradient were analysed for protein and carbohydrate as described under analytical methods. Densities were determined by weighing a 500 µl aliquot of each fraction.

Analytical methods

Sepharose 4B chromatographic column effluents were screened for protein using the Coomassie Blue binding reaction (Bio-Rad protein assay).26 After caesium chloride density gradient protein was estimated by measuring absorbance at 280 nm. Glycoprotein was measured by the method of Mantle and Allen27 using pig gastric mucin as standard. This is a modified periodic acid/Schiff method which is practical for estimating glycoproteins in a large number of samples. Mucus glycoprotein in bile was quantified following caesium chloride density gradients correcting for losses as estimated by the addition of purified gall bladder mucus to separate aliquots of bile.

Bile pigments were measured by absorbance at 420 nm. Slab sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done in 10% gels with a 3% stacking gel according to the method of Laemmli.28 After electrophoresis gels were stained for glycoproteins by the periodate/Schiff method29 and for protein by the Bio-Rad silver staining procedure.30
The nucleation time defined as the time required for microscopic cholesterol crystals to appear in a bile sample which has been initially cleared of crystals was determined using a standard technique, which is a modification of the method Holan and Holzbach. Cholesterol was measured by GLC phospholipid by the method of Bartlett and bile salts by the 3-hydroxysteroid dehydrogenase technique. Cholesterol saturation of bile samples was expressed as the cholesterol saturation index. Mucus glycoprotein amino acids were quantified on a Durrum amino acid analyser after hydrolysis for 16 hours in 6 N constant boiling HCl. The Beckman lithium citrate three buffer elution system was used with ninhydrin detection and norleucine as an internal standard. In this system glucosamine elutes between alanine and valine and galactosamine elutes between valine and leucine. The large galactosamine peak interfered with the quantification of valine, cystine, methionine, and isoleucine. Separation and quantitation of these amino acids from galactosamine was accomplished in separate analyses by extending the length of buffer one elution to include the neutral amino acids. Carbohydrates were analysed on a Hewlett-Packard 5710 A gas liquid chromatograph using a 3% SE-30 column as trimethyl silyl ethers of methyl glycosides obtained by hydrolysis at 80°C for 16 hours in 1-0 N methanolic HCl essentially as described by Laine et al. Mannitol was used as an internal standard for quantification.

**Results**

Sepharose 4B chromatography of white bile separated biliary proteins into an excluded strongly positive PAS glycoprotein fraction and a heterogeneous included protein fraction of lower molecular weight (Fig. 1). White bile has the unique feature...
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...of being free of bile pigments and lipids that would interfere with the protein analysis. Sepharose 4B chromatography of bile from an unobstructed gall bladder resulted in a pigment peak which overlapped the included protein peak. This bile pigment peak interferes very strongly with the periodic acid/Schiff assay for glycoproteins. Previous investigators have also encountered this difficulty with the included protein peak. Minor interference was also encountered for the Coomassie blue binding assay for proteins.

The pooled void volume glycoprotein peak from Sepharose 4B chromatography of unobstructed gall bladder bile contained contaminants of lower molecular weight proteins as shown by SDS-PAGE. Traces of biliary pigment as observed by visual inspection, phospholipids and cholesterol as determined by lipid analysis were also present in Sepharose 4B pooled void volume fractions. These contaminants were removed from mucus glycoprotein by CsCl density gradient ultracentrifugation (Fig. 2). Visual inspection revealed a greenish low density fraction that floated on top of the CsCl density gradient. Lipid analysis and SDS-PAGE of a clear colourless glycoprotein peak of high density following CsCl density gradient confirmed that low molecular weight contaminants were removed.

Mucus glycoprotein concentrations in the three bile sample groups: normal bile, cholesterol gall stone bile and pigment gall stone bile are shown in Figure 3. Recovery of added mucus glycoprotein was 83%±9.5 (n=4). Mucus glycoprotein concentrations in normal gall bladder bile (96 μg/ml±98 SD, n=11) cholesterol gall stone bile (203 μg/ml±199 SD, n=17) and pigment gall stone bile (110 μg/ml±77 SD, n=6) were not significantly different (Students t test) and there was a large overlap among the values in the three groups. White bile samples contained significantly higher concentrations of mucus glycoproteins (6220 μg/ml±4130, n=4). Gall bladder mucus glycoprotein concentration, total lipid concentration, cholesterol saturation index and nucleation time of individual gall bladder bile are given in Table 1.

Human biliary mucus glycoprotein has a carbohydrate and amino acid composition typical of mucus glycoprotein in general (Table 2). Carbohydrates accounted for 79% (molar basis) whereas amino acids accounted for 21% of the glycoprotein. The major sugar moieties fucose, galactose, N-acetylglucosamine and N-acetylgalactosamine characteristic of other mucus glycoproteins, together made up 95% of the carbohydrates. Minor
quantities of sialic acid were detected in all mucus glycoprotein preparations. No mannose could be detected in any preparation indicating that serum glycoprotein contamination has not interfered with the analysis.

Amino acid analysis of human biliary mucus glycoprotein revealed that threonine, serine, and proline which are characteristically high in mucus glycoproteins constituted 43% by weight of the protein content. Amino acid analysis also confirmed previous reports that gall bladder mucus glycoprotein is relatively rich in the acidic amino acids, aspartic and glutamic acids, as well as in glycine and alanine. Relatively little of the basic and aromatic amino acids could be detected.

Carbohydrate and amino acid analyses were done in four patients with cholesterol gall stones and three controls (Table 2). There were no significant differences in constituent carbohydrates. The large mean difference in sialic acid was because of a single high value in a control patient. The amino acid pattern in the control and cholesterol gall stone patients was also quite similar. The mean results of analysis in two patients with obstructed gall bladders and cholesterol gall stones and two patients with pigment stones are also given.

Table 1 Mucus glycoprotein concentration, total lipid concentration, cholesterol saturation index and nucleation time of gall bladder bile

<table>
<thead>
<tr>
<th>Patients</th>
<th>Cholesterol stone bile</th>
<th>Pigment stone bile</th>
<th>Control bile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mucus glycoprotein concentration (μg/ml)</td>
<td>Total lipids (g/dl)</td>
<td>Cholesterol saturation index</td>
</tr>
<tr>
<td>KO</td>
<td>195</td>
<td>10.74</td>
<td>1.19</td>
</tr>
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<td>BE</td>
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<td>1.02</td>
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<tr>
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<td>132</td>
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<td>0.91</td>
</tr>
<tr>
<td>CH</td>
<td>89</td>
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<td>1.26</td>
</tr>
<tr>
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<td>71</td>
<td>15.55</td>
<td>1.32</td>
</tr>
<tr>
<td>WI</td>
<td>123</td>
<td>16.39</td>
<td>1.18</td>
</tr>
<tr>
<td>PH</td>
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<tr>
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<td>345</td>
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<td>178</td>
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</tr>
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<td>851</td>
<td>12.83</td>
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<tr>
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<td>77</td>
<td>13.07</td>
<td>1.45</td>
</tr>
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<td>1.13</td>
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<td>1.00</td>
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<tr>
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<td>28</td>
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<td>0.97</td>
</tr>
<tr>
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<td>15.03</td>
<td>0.92</td>
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<td>SH</td>
<td>275</td>
<td>6.43</td>
<td>1.50</td>
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<td>BO</td>
<td>201</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>Mean±SD</td>
<td>203±199</td>
<td>11.78±3.17</td>
<td>1.12±0.19</td>
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<tr>
<td>TH</td>
<td>146</td>
<td>19.4</td>
<td>0.95</td>
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<tr>
<td>WA</td>
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<tr>
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<td>nd</td>
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<td>0.77</td>
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<td>Mean±SD</td>
<td>110±77</td>
<td>13.88±4.60</td>
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<td>1.17</td>
</tr>
<tr>
<td>PR</td>
<td>101</td>
<td>15.24</td>
<td>1.30</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>96±98</td>
<td>12.09±3.24</td>
<td>0.86±0.24</td>
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</tbody>
</table>
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Table 2 Carbohydrate and amino acid analyses of human bile mucus glycoproteins (mol/100 mol)

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Normal (3)*</th>
<th>Cholesterol stone (4)</th>
<th>White bile (2)</th>
<th>Pigmented (2)</th>
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<tr>
<td>Fucose</td>
<td>9.1±3.9</td>
<td>5.2±4.2</td>
<td>2.8</td>
<td>8.3</td>
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<tr>
<td>Galactose</td>
<td>30.4±2.0</td>
<td>35.1±2.6</td>
<td>33.8</td>
<td>40.3</td>
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<tr>
<td>N-Ac-Galactosamine</td>
<td>8.9±3.8</td>
<td>9.4±2.0</td>
<td>10.0</td>
<td>4.0</td>
</tr>
<tr>
<td>N-Ac-Glucosamine</td>
<td>37.1±7.5</td>
<td>46.0±5.7</td>
<td>47.7</td>
<td>44.3</td>
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<tr>
<td>Sialic acid</td>
<td>14.6±14.9</td>
<td>4.4±0.2</td>
<td>5.8</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Amino acids
- Asp: 4.4±0.9
- Thr: 13.8±3.0
- Ser: 10.5±1.3
- Glu: 8.5±1.2
- Gly: 14.2±5.1
- Ala: 7.9±2.4
- Val: 4.4
- Cys: —
- Met: —
- Ile: 1.9
- Leu: 9.8±4.6
- Tyr: —
- Phe: 1.8±0.9
- His: 2.8±0.8
- Lys: 3.0±0.6
- Arg: 2.6±0.2
- Pro: 20±7.1

*Number of patients in each group.
*Mean±SD.
*p<0.05 when compared with cholesterol gall stone values (Student's t test).
Not detected or less than 1%.
Detected in two samples only.

Discussion

Quantitative Analysis

Gall bladder mucus glycoprotein concentrations have been determined indirectly by assay of hexosamine content. The results have been conflicting. Indirect determination by hexosamine content may lead to uncertainties because of the presence of other glycoproteins containing hexosamines such as plasma glycoproteins. Erroneous results may also occur owing to incomplete removal of monosaccharides from bile or by incomplete hydrolysis of mucus to hexosamines before analysis. Direct measurement of undegraded native mucin is a more reliable measurement.

Pearson et al recently described methods for isolation of mucus glycoprotein from other biliary constituents, particularly pigment. This represented a certain advance as it permitted direct analysis of mucus glycoprotein concentrations. They measured concentrations of mucus glycoprotein in gall bladder bile of three patients with mixed type cholesterol gall stones and found concentrations to be 200–500 μg/ml. In the present study, we have used the methods of Pearson et al to isolate and quantify mucus glycoprotein concentrations in several patient groups.

No difference between mucus glycoprotein concentrations among patient groups with functioning gall bladders was found. Nor was there any correlation between nucleation time and mucus glycoprotein concentration. This indicates that an increased concentration of mucus glycoprotein in bile is not responsible for the characteristic rapid nucleation time found in cholesterol gall stone patients in vitro. Although unlikely, the possibility exists that the onset of nucleation in vivo may be associated with a temporary increase in glycoprotein concentration which then returns to normal levels once nucleation has occurred.

A few patients with cholesterol gall stones did have high concentrations of mucus glycoprotein. This may have been due to partial obstruction of the cystic duct, a response to inflammation or these patients may represent a small subpopulation in which mucus glycoprotein concentrations in bile are truly raised. Obstruction of the gall bladder results in an approximate 10-fold increase in mucus glycoprotein concentrations, as seen in the white bile group.
white bile provides an excellent source of gall bladder mucus glycoprotein.

QUALITATIVE ANALYSIS
A change in mucus glycoprotein composition has been suggested in inflammatory bowel disease and it seemed possible that a qualitative alteration of mucus glycoprotein might explain nucleation time differences in patients with and without gall stones.

Previous qualitative investigations had been limited to animal models, patients with functional liver disturbances, or gall bladder bile obtained at necropsy. Isolation procedures usually used harsh extraction procedures and proteolytic digestion or failed to remove contaminating material. Some studies have also limited their analysis to only the carbohydrate component of the mucus glycoprotein. Other studies have used hepatic bile obtained by T-tube drainage as their source of mucus glycoprotein. Therefore, direct comparison of the qualitative analysis of mucus glycoprotein with earlier studies are difficult.

In general, the carbohydrate composition of our biliary mucus is compatible with previous preparations of biliary glycoprotein. Our data can probably be compared with that of Pearson et al because the isolation methods were the same. There was less fucose and more glucosamine in our preparations. Pearson and colleagues found proportionately more galactosamine and less glucosamine in the mucus glycoproteins isolated from a blood group A individual. Schrager et al also found proportionately more galactosamine in the biliary mucus associated with blood group A and increased galactose residues associated with blood group B. Individual blood group type was not obtained in the present study and could account for the differences.

The amino acid analyses in this study are generally quite similar to those reported by Pearson et al with the major entities being threonine, serine, and proline. These amino acids constituted about 43% by weight of the protein content compared with 39% by Pearson et al.

There were no significant differences in carbohydrate composition between patients with cholesterol gall stones and control patients. The amino acid composition in the two groups was also very similar although there was a significant difference in per cent aspartic acid. The significance of this finding must be interpreted with caution because of the small sample size. Our qualitative analysis has given only ‘crude’ compositional data of mucus glycoprotein in patients with and without gall stones. Obviously glycoproteins whose amino acid and carbohydrate sequences are very different can contain similar amino acid and carbohydrate compositions. Our qualitative analysis of mucus glycoprotein has not included sulphate groups. Gall bladder mucins are sulphated and a change in the sulphate content might be important. Whether structurally different mucus glycoproteins exist in patients with cholesterol gall stones remains to be determined. Functional studies may determine whether this is so. Levy et al have reported that mucus glycoprotein from stone patients increases the number of cholesterol crystals nucleating from model biles. Control mucus glycoprotein isolated from cadaver bile had a lesser effect. We have been unable to substantiate this difference using mucus glycoprotein obtained at surgery from control and cholesterol gall stone patients using nucleation time as the end point. Furthermore, complete removal of mucus glycoprotein from bile does not slow nucleation time of abnormal bile.

A few samples of pigment stone bile and white bile from cholesterol gall stone patients were available for analysis. The results indicate that there are probably no major differences in composition from controls in these groups.

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References


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