Mucin gene expression in bile of patients with and without gallstone disease, collected by endoscopic retrograde cholangiography

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Abstract

AIM: To investigate the pattern of mucin expression and concentration in bile obtained during endoscopic retrograde cholangiography (ERC) in relation to gallstone disease.

METHODS: Bile samples obtained at ERC from 29 consecutive patients, 17 with and 12 without gallstone disease were evaluated for mucin content by gel filtration on a Sepharose CL-4B column. Dot blot analysis for bile mucin apoproteins was performed with antibodies to Mucin 1 (MUC1), MUC2, MUC3, MUC5AC, MUC5B and MUC6. Staining intensity score (0-3) was used as a measure of antigen expression.

RESULTS: MUC1, MUC2, MUC3, MUC5AC, MUC5B and MUC6 were demonstrated in 34.4%, 34.4%, 51.7%, 51.7%, 55.1% and 27.5% of bile samples, respectively. The staining intensity scores were 0.62 ± 0.94, 0.58 ± 0.90, 0.79 ± 0.97, 1.06 ± 1.22, 1.20 ± 1.26 and 0.41 ± 0.73, respectively. Mean mucin concentration measured in bile by the Sepharose CL-4B method was 22.8 ± 24.0 mg/mL (range 3.4-89.0 mg/mL). Mean protein concentration was 8.1 ± 4.8 mg/mL (range 1.7-23.2 mg/mL).

CONCLUSION: High levels of MUC3, MUC5AC and MUC5B are expressed in bile aspirated during ERC examination. A specific pattern of mucin gene expression or change in mucin concentration was not found in gallstone disease.

INTRODUCTION

Mucins are high-molecular-weight glycoproteins containing oligosaccharide side-chains attached to serine or threonine residues of the apomucin backbone by O-glycosidic linkages\[1-4\]. Several mucin (MUC) genes located on different chromosomes have been sequenced and cloned\[5-14\]. These genes encode apoproteins with specific tandem repeats of amino acids. Antibodies have been developed against the tandem repeats, enabling the identification of specific mucins by immunohistochemistry.

Mucins can be divided into two classes: gel-forming and membrane-associated. Bile mucin has two main domains: one rich in serine, threonine and proline, which contains the majority of the covalently-bound carbohydrates; and another, nonglycosylated domain, enriched in serine, glutamic acid, glutamine and glycine, which binds hydrophobic ligands such as bilirubin. An increased expression of gel-forming mucin, such as MUC5AC and MUC2, was found in patients with hepatolithiasis\[15\]. Although bile-duct mucin production has been extensively studied in malignant diseases\[16-22\], little is known about mucin synthesis and expression in cholelithiasis, choledochoolithiasis and cholangitis.

The aim of the present study was to examine mucin concentration and specific expression in bile samples of patients undergoing endoscopic retrograde cholangiography (ERC) for the evaluation...
of symptomatic bile duct disease, and to investigate the possible association between mucin expression and the clinical states of gallstone disease.

MATERIALS AND METHODS

Sampling
Twenty-nine patients who underwent ERC due to symptomatic bile duct disease were included in the study. Background data and results for ultrasound examinations and liver function tests were obtained from the files. Bile was collected by aspiration, as completely as possible, after the papilla was cannulated and before proceeding to any other procedures, such as papillotomy or choledochal stone removal. The Institutional Review Board (Ethical Committee) of Rabin Medical Center approved the study.

Bile analysis
To determine mucin concentration in the bile, we used the gel-filtration technique, as previously described[23,24]. Briefly, after centrifugation to remove debris, samples of bile were subjected to gel filtration on Sepharose CL-4B columns (1 × 40 cm). We used the closed-column system of Pharmacia Biotech (Cambridge, MA, USA): peristaltic pump, P-1; columns and adapters, C10; fraction collector, Redifrac and Ultraspec 1000; VV/visible spectrophotometer, and chart reader, 80-2109-03. Samples of 2 mL were applied to the columns and eluted with 10 mmol/L Tris-HCl buffer at pH 8.0. Fractions of 1 mL were collected, and optical density was determined at a wavelength of 280 nm. Findings were correlated with a standard curve of readings of mucin purified from porcine stomach (1% bound sialic acid), purchased from Sigma (St. Louis, MO, USA). The amount of protein was estimated by the Laury method.

Dot blot analysis
Samples were subjected to dot blot analysis on nitrocellulose membranes. Membranes were incubated with monoclonal antibodies to Mucin 1 (MUC1), MUC2, MUC3, MUC5AC, MUC5B and MUC6 (all mouse), followed by incubation with anti-mouse and IgG labeled with biotin. Antibody binding was detected with streptavidin-horseradish peroxide and chemiluminescent reagents (EZ-ECL, Beit-Haemek, Israel). Monoclonal antibodies were purchased from Neomarkers (Fremont, CA, USA). Staining intensity was scored (0-3) as a measure of antigen expression.

Statistical analysis
All results are expressed as mean ± SD. The analyses included descriptive statistics, χ² test, Student’s t-test, and linear regression analysis. P < 0.05 was considered significant.

RESULTS

Study population
The study group consisted of 13 men and 16 women aged 64.5 ± 16.8 years (Table 1). A gallstone disease was diagnosed in 17 patients and excluded in 12 patients. The indications for ERC, abdominal ultrasound results, and liver function test results before ERC are presented in Table 1.

| Clinical parameter | n (%) or mean ± SD |
|--------------------|--------------------|
| Age                | 64.5 ± 16.8        |
| Range              | 24-86              |
| Sex                |                    |
| Men                | 13 (44.8)          |
| Women              | 16 (55.2)          |
| Main indication for ERC |                |
| Cholelithiasis/choledocholithiasis/dilated CBD | 16 (55.2) |
| Cholangitis        | 5 (17.2)           |
| SOL of papilla     | 3 (10.3)           |
| Unresolved pancreatitis | 2 (6.9)  |
| Abdominal ultrasound results |            |
| Dilated CBD        | 15 (51.7)          |
| Cholelithiasis      | 12 (41.4)          |
| Dilated intrahepatic ducts | 10 (34.5) |
| Cholelithiasis      | 3 (10.3)           |
| Pancreatitis        | 1 (3.4)            |
| CBD width (mm)      |                    |
| mean ± SD          | 8.7 ± 3.7          |
| Range              | 6-18               |
| Liver function tests, mean ± SD (range) |        |
| Total bilirubin (mg/dL) | 4.5 ± 6.9 (0.3-32) |
| Direct bilirubin (mg/dL) | 3.0 ± 4.9 (0.1-23) |
| Alanine aminotransferase (U/L) | 206.5 ± 243.3 (12-895) |
| Aspartate aminotransferase (U/L) | 156.4 ± 185.6 (15-812) |
| Gamma glutamyl transpeptidase (U/L) | 369.4 ± 370.4 (13-1337) |
| Alkaline phosphatase (U/L) | 291.0 ± 371.4 (56-1893) |

ERC: Endoscopic retrograde cholangiography; CBD: Common bile duct; SOL: Space-occupying lesion.

ERC results
The ERC findings are shown in Table 2. Linear regression analysis revealed a positive correlation between the mean common bile duct (CBD) width measured on abdominal ultrasound and ERC. There was also a positive correlation between ultrasound findings of cholelithiasis and ERC findings of dilated CBD; between the presence of a clinical syndrome of cholangitis and ultrasound findings of pancreatitis; and between increased concentrations of serum direct bilirubin and ERC findings of CBD stricture. A wider CBD was demonstrated in patients with evidence of choledocholithiasis on ERC (10.90 ± 4.97 mm) than in patients without CBD stones (7.44 ± 1.98 mm). Information from the ultrasound studies and ERC results was used to stratify the patients into a group with gallstone related disease, and a group without gallstone disease.

Mucin concentration in bile
Mean ± SD mucin concentration in bile, measured by the Sepharose CL-4B method, was 22.8 ± 24.0 mg/mL (range 3.4-89.0 mg/mL). Mean protein concentration was 8.1 ± 4.8 mg/mL (range 1.7-23.2 mg/mL). Mucin concentration in bile was not significantly different between men and women (24.68 ± 27.29 mg/mL vs 21.38 ± 21.96 mg/mL), patients younger or older than 70
Mucin expression in bile

The expression of the mucin genes examined by dot blot analysis is shown in Table 3. Linear regression analysis revealed a positive correlation between MUC5AC and MUC5B expression [MUC5B = 0.273 + (0.874 × MUC5AC); R = 0.845]. There was also a positive correlation between MUC1 expression and papillary enlargement on ERC. The correlation between the expressions of the different MUC genes in bile is shown in Table 4.

Comparison of patients with and without gallstone disease

Summarizing the clinical and imaging data allowed the patients to be stratified into a group with diagnosed gallstone disease (n = 17), and a group with no evidence of gallstone disease (n = 12). There were no significant differences in gender, age, laboratory results, ultrasound finding, indication and results of ERC, except in the presence of gallstone disease (Table 5). Mucin concentration in bile was similar in both groups (21.68 ± 7.87 mg/mL vs 24.54 ± 24.10 mg/mL, P = 0.759), as was mucin gene expression (Table 5).

DISCUSSION

Different mucin genes are expressed in bile, and the role of each is unclear. Bile mucin is derived from pure hepatic bile, gallbladder-concentrated bile, and mucin secreted by the bile duct epithelium. Ko et al. found that in patients with biliary sludge, mucin concentration was higher in bile collected by ERC than in gallbladder bile. They concluded that the biochemical composition of hepatic bile is modified during residence in the gallbladder, contributing to sludge formation, and that hepatic bile samples are therefore inappropriate for microscopic detection of microlithiasis. However, although the mucin concentration in hepatic bile in the present study was similar to that reported by Ko et al. [22.8 ± 24.0 mg/mL vs 20 ± 30 mg/mL], the concentration of mucin in gallbladder bile in our previous study was 17.5 ± 16.4 mg/mL, close to that of hepatic bile and much lower than the 450 ± 290 mg/mL found by Ko et al. Thus, our studies do not support the assumption of Ko et al., and this controversy requires further investigation.

We demonstrated a higher expression of two secretory mucin proteins, MUC5AC and MUC5B, and the membrane-bound protein, MUC3. MUC5AC and MUC5B are both gel-forming mucins that may increase the viscosity of bile in cases of symptomatic bile duct disease. Since we could not find a change in mucin concentration or in these specific genes expressions in bile derived from patients with or without gallstone disease, our findings do not support a role for MUC5AC or MUC5B in the etiopathogenesis of gallstones.

Zen and coworkers described a lipopolysaccharide-induced increase in MUC2 and MUC5AC expression in cultured murine biliary epithelial cells, which was mediated by tumor necrosis factor alpha. They concluded that since lipopolysaccharide is a bacterial component, bacterial infection may be involved in the altered mucin secretion in the intrahepatic biliary tree and, thereby, in the lithogenesis of hepatolithiasis. Wandenhaute and coworkers noted a strong mRNA expression of MUC5B, MUC3, and MUC6, and a weak expression of MUC1, MUC2, and MUC5AC, in biliary epithelial cells. Lee and Liu found that MUC3 and MUC5B were the main mucin genes expressed in the biliary epithelium of stone-containing intrahepatic bile ducts and normal controls. Mucin gene expression

Table 2 Results of ERC (n = 29)

| Clinical parameter | n (%) or mean ± SD |
|--------------------|-------------------|
| Diagnosis          |                   |
| CBD width (mm)     | 9.4 ± 4.0         |
| Range              | 6-18              |
| Cholelithiasis     | 11 (37.9)         |
| Pigmented stones   | 4 (13.8)          |
| Cholecystitis      | 5 (17.2)          |
| Enlarged papilla   | 7 (24.1)          |
| Dilated CBD        | 15 (51.7)         |
| CBD stricture      | 4 (13.8)          |
| Intrahepatic ducts dilation & stricture | 3 (10.3) |
| Torn papilla       | 3 (10.3)          |
| Bile leakage       | 1 (3.4)           |
| Mirizzi syndrome   | 1 (3.4)           |
| Treatment          |                   |
| Sphincterotomy     | 16 (55.2)         |
| Biopsy of the papilla | 5 (17.2)   |
| Stent insertion    | 2 (6.9)           |
| Cholecystostomy    | 1 (3.4)           |

Table 3 Mucin gene expression in bile collected in ERC

| Mucin gene | Score mean ± SD (range) | Cases (%) |
|------------|-------------------------|-----------|
| MUC1       | 0.62 ± 0.94 (0-3)       | 34.4      |
| MUC2       | 0.58 ± 0.90 (0-3)       | 34.4      |
| MUC3       | 0.79 ± 0.97 (0-3)       | 51.7      |
| MUC5AC     | 1.06 ± 1.22 (0-3)       | 51.7      |
| MUC5B      | 1.20 ± 1.26 (0-3)       | 55.1      |
| MUC6       | 0.41 ± 0.73 (0-2)       | 27.5      |

Table 4 Correlation between the expression of the different mucin genes in bile collected by ERC

| Mucin gene | Correlation with mucin gene | P value |
|------------|-----------------------------|---------|
| MUC1       | MUC2                        | 0.0001  |
|            | MUC3                        | 0.0001  |
|            | MUC5AC                      | 0.00125 |
|            | MUC5B                       | 0.049   |
|            | MUC6                        | 0.0001  |
| MUC2       | MUC3                        | 0.0001  |
|            | MUC5AC                      | 0.0080  |
|            | MUC5B                       | 0.0001  |
| MUC3       | MUC5AC                      | 0.0003  |
|            | MUC5B                       | 0.0003  |

years (18.87 ± 15.72 mg/mL vs 26.59 ± 30.07 mg/mL), and patients with or without cholelithiasis (22.46 ± 24.94 mg/mL vs 23.11 ± 24.29 mg/mL).
was altered in dysplastic preneoplastic cells.

The main weakness of our study is the absence of healthy controls. We could not compare mucin concentration and gene expression in the cholestatic situation with that of normal bile collected in ERC, since ERC is usually performed with therapeutic intent in symptomatic patients.

In the present study, we observed a positive correlation between MUC1 expression in bile and the expression of all the other mucin genes examined. Wang and coworkers reported a similar result in mice. They described a positive correlation between MUC1 and MUC5AC expression, indicating a gene-gene interaction that might affect the accumulation of mucin gel and cholesterol gallstone formation.

In summary, we could not demonstrate a change in mucin secretion and expression between patients with and without gallstone disease, or support the role of mucin in the etiopathogenesis of biliary sludge or stone formation.

**Table 5** Comparison between patients with \((n = 17)\) and without \((n = 12)\) gallstones

| Gallstones disease \(n\) (%) | No evidence for gallstones \(n\) (%) | \(P\) value |
|-------------------------------|------------------------------------|------------|
| Age, mean ± SD (years)        | 61.35 ± 20.13                      | 69.00 ± 9.38 | 0.234 |
| Sex (men)                     | 8 (47.1)                           | 5 (41.7)    | 0.927 |
| Main indication for ERC       |                                    |            |
| Jaundice                      | 7 (41.1)                           | 9 (75)      | 0.153 |
| Dilated CBD                   | 4 (23.5)                           | 1 (8.0)     | 0.554 |
| Cholangitis                   | 3 (20.0)                           | 0           | 0.288 |
| SOL of papilla                | 1 (6.0)                            | 2 (17.0)    | 0.737 |
| Unresolved pancreatitis       | 2 (12.0)                           | 0           | 0.288 |
| Abdominal ultrasound results  |                                    |            |
| Dilated CBD                   | 10 (59.0)                          | 5 (42)      | 0.599 |
| Cholelithiasis                | 12 (70.6)                          | 0           | < 0.0001 |
| Dilated intrahepatic ducts    | 6 (40)                             | 4 (30)      | 0.873 |
| Choledocholithiasis           | 3 (17.6)                           | 0           | 0.360 |
| Pancreatitis                  | 1 (5.9)                            | 0           | 0.861 |
| CBD width (mm), mean ± SD     | 9.65 ± 4.39                        | 7.50 ± 2.24 | 0.132 |
| Liver function tests, mean ± SD |                                   |            |
| Total bilirubin (mg/dL)       | 5.12 ± 7.83                        | 3.68 ± 5.52 | 0.589 |
| Direct bilirubin (mg/dL)      | 3.38 ± 5.53                        | 2.55 ± 4.03 | 0.662 |
| Alanine aminotransferase (U/L)| 245.41 ± 270.76                    | 151.58 ± 196.14 | 0.315 |
| Aspartate aminotransferase (U/L)| 167.29 ± 150.54                | 141.08 ± 233.11 | 0.715 |
| Gamma glutamyl transpeptidase (U/L)| 443.47 ± 388.13        | 264.50 ± 331.14 | 0.206 |
| Alkaline phosphatase (U/L)    | 352.88 ± 449.95                    | 185.08 ± 174.44 | 0.323 |
| ERC diagnosis                 |                                    |            |
| CBD width (mm), mean ± SD     | 9.59 ± 3.99                        | 9.17 ± 4.37 | 0.790 |
| Cholelithiasis                | 11 (64.7)                          | 0           | 0.002 |
| Pigmented stones              | 4 (24.0)                           | 0           | 0.198 |
| Cholelithiasis                | 5 (29.0)                           | 0           | 0.122 |
| Enlarged papilla              | 3 (16.0)                           | 0           | 0.533 |
| Dilated CBD                   | 10 (60.0)                          | 5 (40.0)    | 0.494 |
| CBD stricture                 | 1 (10.0)                           | 3 (30.0)    | 0.376 |
| Torn papilla                  | 3 (17.6)                           | 0           | 0.360 |
| Treatment                     |                                    |            |
| Sphincterotomy                | 12 (71.0)                          | 4 (33.0)    | 0.099 |
| Biopsy of the papilla         | 1 (10.0)                           | 4 (33.0)    | 0.288 |
| Mucin gene score, mean ± SD   |                                    |            |
| Mucin concentration (mg/mL)   | 21.68 ± 7.87                       | 24.54 ± 24.1 | 0.759 |
| Protein concentration (mg/mL) | 7.87 ± 4.53                        | 8.61 ± 5.48 | 0.694 |
| MUC1                          | 0.59 ± 0.87                        | 0.66 ± 1.07 | 0.848 |
| MUC2                          | 0.53 ± 0.80                        | 0.66 ± 1.07 | 0.711 |
| MUC3                          | 0.88 ± 0.93                        | 0.66 ± 1.07 | 0.560 |
| MUC5AC                        | 0.47 ± 0.80                        | 0.33 ± 0.65 | 0.621 |
| MUC5B                         | 1.23 ± 1.25                        | 0.83 ± 1.19 | 0.394 |
| MUC6                          | 1.29 ± 1.26                        | 1.08 ± 1.31 | 0.667 |

**COMMENTS**

**Background**

Secretory mucins are gel-forming and may increase bile viscosity. The biochemical composition of hepatic bile is modified during residence in the gallbladder, contributing to sludge formation. An increased expression of gel-forming mucin, such as MUC5AC and MUC2, was found in patients with hepatolithiasis. Little is known about mucin synthesis and expression in cholelithiasis, choleodocholithiasis and cholangitis.

**Innovations and breakthroughs**

High levels of MUC3, MUC5AC and MUC5B are expressed in bile aspirated during endoscopic retrograde cholangiography examination. A specific pattern of mucin gene expression or change in mucin concentration was not found in gallstone disease.

**Applications**

Expression of other mucin genes or changes in concentration should be investigated in gallstone disease. The role of mucin synthesis and secretion in gallstone formation is still unknown.

**Peer review**

The manuscript by Vilkin et al describes the analysis of certain members of the Mucin gene family in the bile of patients with and without gallstone disease. The authors demonstrate the presence of Mucin 1 (MUC1), MUC2, MUC3, MUC5AC, MUC5B and MUC6 in the bile of all patients, but there was no correlation to
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