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ORIGINAL RESEARCH

A comparative study of changing patterns of concanavalin A-binding proteins in early stage of cholesterol gallstone formation

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Abstract

AIM: To elucidate the importance and the changing patterns of biliary concanavalin A-binding proteins (CPs) in the early stage of cholesterol gallstone formation.

METHODS: CP concentration and nucleation activity were measured by lectin affinity chromatography in bile samples of patients with cholesterol gallstones, pigment gallstones, gallbladder cholesterosis and non-biliary diseases.

RESULTS: The concentrations of CPs were much higher in patients with cholesterol gallstones (0.39 ± 0.11 g/L, n = 36, P < 0.01) or gallbladder cholesterosis (0.40 ± 0.09 g/L, n = 9, P < 0.01) than in those with pigment gallstones (0.2 ± 0.12 g/L, n = 7) and/or non-biliary diseases (0.27 ± 0.09 g/L, n = 10). Pronucleating activities were much stronger in patients with cholesterol gallstones (nucleation time ratio: 0.57 ± 0.21, n = 5, P < 0.01 *vs* pigment gallstones and/or non biliary diseases) and gallbladder cholesterosis (nucleation time ratio: 0.44 ± 0.23, n = 5, P < 0.01 *vs* pigment gallstones or non-biliary diseases). The binding percentages of CPs to model biliary vesicles were also higher for patients with cholesterol gallstones (n = 6) than those with pigment gallstones (n = 6) (2.4% ± 0.9% *vs* 0.9% ± 0.5%, P < 0.01).

CONCLUSION: Hypersecretion of CPs, especially those in vesicular phase, may be an important change in the early stage of cholesterol gallstone formation.

Key words: Bile; Concanavalin A binding proteins; Cholesterol gallstone; Chromatography

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INTRODUCTION

Protein in bile has been considered for more than 3 decades as a contributing factor to the pathogenesis of gallstone formation[1]. But the essential progress in our understanding of this process was made just only 10 years ago, when Groen and colleagues[2] used lectin affinity chromatography to isolate and purify a potent pronucleating glycoprotein which has since been recognized as the bile-form aminopeptidase N. Since then, numerous nucleation promoting and inhibiting biliary proteins have been characterized[3-5]. But, as of yet, nearly nothing has been done in the research field of the most important or key nucleating proteins.

Concanavalin A-binding proteins (CPs), a group of biliary proteins containing almost all the well known pronucleating proteins, provided an opportunity to study the nucleation-effecting proteins systematically. Vesicular proteins are functionally location-specific pronuncleating proteins, their concanavalin A-binding regions may exert important actions in gallstone formation. In this article, the authors determined the concentrations and nucleation activities of total biliary proteins, biliary CPs and vesicles related to CPs, so as to elucidate the relationship between changes in quantity and quality of biliary CPs and gallstones formation and study systematically the importance of CPs in pathogenesis of gallstone formation.

MATERIALS AND METHODS

Patients

Sixty-six patients were enrolled in this study, including 43 with cholelithiasis (36 with cholesterol gallstones, 7 with pigment gallstones), 9 with gallbladder cholesterosis, 2 each with gallbladder adenomyomatosis and adenoma, and 10 with non-biliary diseases (5 with

Table 1 Concentrations of proteins in human gallbladder bile (g/L, $\bar{x} \pm s$)

Patient group	*n*	Total protein	CP concentration
Cholesterol gallstones	36	4.1 ± 1.5	0.39 ± 0.11
Pigment stones	7	3.2 ± 1.8	0.26 ± 0.12
Gallbladder cholesterosis	9	4.4 ± 1.6	0.40 ± 0.09
Gallbladder adenomyomatosis	2	3.8 ± 1.2	0.29 ± 0.19
Gallbladder adenoma	2	3.9 ± 1.1	0.26 ± 0.14
Non-biliary diseases	10	4.5 ± 1.6	0.27 ± 0.09

[a]$P < 0.05$ *vs* other groups: [b]$P < 0.01$ *vs* pigment gallstones and non-biliary diseases. CP: Concanavalin A-binding protein

Table 2 Nucleation time of human gallbladder bile ($\bar{x} \pm s$)

Patient group	*n*	Nucleation time (d)
Cholesterol gallstones	36	7.8 ± 4.2
Solitary	20	5.3 ± 2.7
Multiple	16	10.1 ± 5.6
Pigment gallstones	7	14.7 ± 6.0[1]
Gallbladder cholesterosis	9	6.1 ± 3.7
Stone-free diseases	14	16.3 ± 3.2[2]

Notes: Those ([1]3 cases, [2]8 cases) without nucleation after 21 d were recorded as 21 d.

colon cancer, 2 with gastric cancer, 1 each with peptic ulcer perforation and pancreatic pseudocyst). All the patients met the following criteria: No acute inflammation of the gallbladder, and no obstruction at the cystic duct or common bile duct; no abnormality in liver biochemistry, and negative bile culture; total lipid concentration in bile of > 50 g/L; serum protein concentration within normal range. Bile was sampled by aspiration according to the standard method[6] during operation and then stored at -20 ℃. Observation of cholesterol monohydrate crystal in bile was carried out immediately after sample obtainment.

Stone classification was based on gross inspection and cholesterol content. Cholesterol gallstone was identified by cholesterol dry weight percentage > 70%; pigment gallstone was identified if the cholesterol dry weight percentage was < 30%.

Isolation of biliary vesicles and quantitation of vesicular protein

Biliary vesicles were isolated from 10 mL bile of each patient according to the method described by Amigo *et al*[7], and further purified according to the method described by Stone *et al*[8]. Vesicular protein concentrations were determined using a pooled concentrated eluent that had undergone ultrafiltration.

Quantitative and nucleating activity analysis of CPs

CPs isolated from 1 mL bile were the bound fractions for concanavalin A affinity chromatography, and determined quantitatively using the Coomassie bright blue method. Nucleation activities were determined as follows: Lyophilized CPs prepared from 1 mL gallbladder bile as stated above were put into 1 mL artificial bile, and then incubated at 37 ℃. Nucleation time (NT) was determined according to Holan's method[9], and the NT ratio was calculated as the relative value to the blank control; final cholesterol crystal concentration (FCCC) was that after 3 wk at 37 ℃, and its relative percentage to the blank control was the FCCC percentage.

Preliminary study of the distribution of CPs in vesicles

To determine the distribution of CPs in the vesicular phase of native bile, concanavalin A was first linked to horseradish peroxidase (HRP) (con A-HRP, 1:1 molar ratio) according to Guo's report[10]. One drop of the con A-HRP solution was added into one drop of bile on a 200-mesh zinc grid that was covered with carbon film, and stored overnight in moist saturation at 37 ℃. The excess liquid was soaked out the next morning and dyed with 0.01% diaminobenzidine (DAB) solution (with dropwise addition of H_2O to bring the volume up to 20 mL) for 15 min. The prepared zinc grids were then gently washed 3 times with buffer solution, dried and examined by a JEM-1200EX electronic microscope at a magnification of 30000 and using an accelerating voltage of 80 KV.

The binding characteristics of CPs to vesicles and micelles in model bile were determined using gallbladder bile samples from 6 patients with cholesterol gallstones and 6 with pigment gallstones. The samples were processed to harvest CPs as described above; after 20 min of ultracentrifugation at 10000 rpm, the CPs were then added into model bile to a final concentration of 0.2 g/L. After incubation at 37 ℃ for 2 wk, the contents of vesicular and micellar protein in these solutions were analyzed.

Table 3 Cholesterol crystals and biliary proteins ($\bar{x} \pm s$)

Patient group	*n*	Total protein (g/L)	CPs (g/L)
Gallstones	36		
With crystal	29	4.9 ± 2.7	0.43 ± 0.14
Without crystal	7	3.4 ± 2.1	0.29 ± 0.16
Gallstone-free	30		
With crystal	14	4.1 ± 1.9	0.40 ± 0.17
Without crystal	16	3.2 ± 2.1	0.31 ± 0.17

Table 4 Influence of Concanavalin A-binding proteins on nucleating activities of model bile ($\bar{x} \pm s$)

Patient group	*n*	Nucleation time ratio	Final cholesterol crystal concentration percentage
Cholesterol gallstones	5	0.57 ± 0.21	143.4 ± 12.6
Pigment gallstones	5	0.71 ± 0.19	121.3 ± 27.4
Gallbladder cholesterosis	5	0.44 ± 0.23	152.7 ± 18.4
Non-biliary diseases	5	0.73 ± 0.11	130.4 ± 15.7

Preparation of model bile

According to the method described by Kibe *et al*[11], this model bile has a CSI of 1.2, total lipid concentration of 100 g/L and cholesterol/lecithin molar ratio of 4. The concentration of biliary protein was determined following the method described by Gallinger *et al*[12]. Dunnett's *t*-test was used to compare the mean values between test groups and control group, and the *U* test was used to compare the mean values between two groups. A probability of < 0.05 was considered significant.

RESULTS

Concentration of total biliary protein and nucleating activities in human gallbladder bile

Table 1 presents our observations of no significant differences in total biliary protein concentration for the patients with cholesterol gallstones and/or gallbladder cholesterosis as compared to other groups. But, a significantly higher concentration of biliary CPs was found in patients with cholesterol gallstones and/or gallbladder cholesterosis ($P < 0.05$). A very significant difference in biliary CPs was also found between patients with gallbladder cholesterosis and pigment gallstone as well as those with non-biliary diseases ($P < 0.01$). Gallbladder bile from patients with cholesterol gallstones and/or gallbladder cholesterosis nucleated more rapidly than those with pigment gallstone and/or other stone-free diseases ($P < 0.001$, Table 2).

Relationship between cholesterol crystals and biliary protein

As shown in Table 3, both the gallstone and stone-free patients with crystals have a higher concentration of total biliary protein than those without crystals, but significant differences were only found between gallstone patients with crystals and stone-free patients without crystals ($P < 0.05$). Biliary CP concentrations, however, were significantly higher in all patient groups with crystals than in the crystal-free patients ($P < 0.05$).

Nucleation activities of biliary CPs

The nucleation time induced by CPs from patients with cholesterol gallstones and gallbladder cholesterosis was significantly shorter than that from patients with non-biliary diseases and pigment gallstones ($P < 0.05$). An even more significant difference was found between patients with gallbladder cholesterosis and pigment gallstone as well as those with non-biliary diseases ($P < 0.01$). CPs from patients with cholesterol gallstones or gallbladder cholesterosis caused a significantly higher FCCC ratio than those with pigment

gallstones and non-biliary diseases ($P < 0.01$, Table 4).

Distribution of CPs in vesicle phase

Using a concanavalin A-affinity staining method, we observed many dark brown spherical vesicles of varied size in native bile. After incubation of artificial bile with CPs for 2 wk, the binding percentage of CPs to vesicles was 2.4% ± 0.9% in patients with cholesterol gallstones, which was significantly higher than that in patients with pigment gallstones (0.9% ± 0.5%, $P < 0.01$).

DISCUSSION

Both pro- and antinucleating proteins have been found in human bile, and dozens of pronucleating proteins and several antinucleating proteins have been discovered in recent years; however, which among them plays the most important or key role in cholesterol nucleation remains unknown. CPs are glycoproteins that contain almost all the nucleating proteins reported. Systematic studies of CPs may provide some approaches for identifying these key factors, but up to now few reports have dealt with this issue.

Theoretically, increase of nucleating proteins in bile would cause an elevation of total protein level[13], but inconsistent results have been reported because too many factors may influence the results. Our current data showed that patients with stone-free diseases had a higher level of biliary total protein than those with cholesterol gallstone (but with no statistical significance; Table 1). Nucleation time, however, was arbitrarily much faster in patients with cholesterol gallstones than those with pigment gallstones (Table 2), indicating that the differences between them arose from their differences in quantity and quality of nucleating proteins. Our results, as shown in Table 1, demonstrated a higher concentration of biliary CPs in patients with cholesterol gallstones and gallbladder cholesterosis than those with pigment gallstones and stone-free diseases, and a stronger nucleating activity for CPs from the former than the latter, indicating that the differences in CPs determined nucleation of cholesterol in bile.

The presence of cholesterol crystal in bile often indicates a high nucleating and crystal growth activity. In the current study, the concentration of biliary CPs in patients with cholesterol crystals was found to be higher than in those without the crystals; moreover, the concentration of biliary CPs in stone-free patients with crystals was higher than that in in gallstone patients without crystals (Table 3), which suggests that elevation of nucleating proteins was not secondary to gallstone formation.

Owing to their special location, vesicular proteins have been considered as a very important group of pronucleating proteins[14], and it was predicted that CPs, which include almost all the pronucleating proteins, also exist in the vesicular phase. We confirmed this prediction by an affinity staining method devised to display the presence of CPs in the vesicular phase. This investigation of CPs bound to artificial vesicles also demonstrated that only a small part of the CPs could bind to vesicles and the binding percentage was much higher for CPs from patients with cholesterol gallstones than that from patients with pigment gallstones. These results imply that vesicle-binding CPs may exert some special effects on cholesterol nucleation in human bile.

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