

Biliary Proteins in Patients with and without Gallstones

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Background: Nucleation from supersaturated bile of calcium salts of cholesterol and bilirubinate is essential in the formation of gallstone. Nucleation requires gallbladder mucin and its main component, glycoprotein, may contribute to gallstone formation by providing a nidus or matrix for precipitation of lipid components. However, biliary protein patterns of patients with gallstones have not been completely explored.

Methods: We have tried to extract, isolate and characterize the proteins in patients with gallstones and without gallstones. 21 bile samples were obtained from patients with different types of gallstones and with no stones at cholecystectomy. Biliary protein concentrations were measured by Lowry and Bensadoun methods, and individual glycoproteins from each of the patients were compared by silver staining and densimetric quantification of Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

Results: 1) Among 16 gallstones, 5 were cholesterol stones, 5 were calcium bilirubinate stones, and 6 were black pigment stones. 2) The mean protein concentration was highest in bile with cholesterol stones (47.6 mg/ml), 24.2 mg% in bile without gallstones, and 15.9 mg/ml in brown pigment stones. 3) Cholesterol gallstones were found to have 14.2 KD glycoproteins, whereas pigment stones were found to have 66 KD glycoproteins.

Conclusions: Gallbladder proteins from both cholesterol and pigment stones play an important role in the nucleation and growth of calcium salt crystals.

Key Words: Gallstone, Biliary protein, Nucleation

INTRODUCTION

Biliary proteins have been suggested to play an important role in bile nucleation and gallstone formation. Supersaturation of gallbladder bile is the first step in gallstone formation¹⁾ and nucleation from supersaturated bile of calcium salts of cholesterol or bilirubinate have been implicated in

gallstone formation²⁾. Normal bile contains factors that inhibit nucleation, whereas lithogenic biles contain factors that promote nucleation³⁾. Although the precise role of nucleation has not been well characterized, recent study suggest that these factors are probably proteins⁴⁻⁶⁾. It should be also suggested that nucleation requires an organic matrix, composed of small, soluble acidic regulatory proteins bound to a structural framework of high molecular weight gallbladder mucins.

Several investigators have reported results of measurements of total protein concentrations in biles of gallstone patients, but they differ in their opinions with different results⁷⁻¹⁰⁾. The idea that

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there might be a specific differences in the protein compositions in patients with cholesterol and pigment gallstones may provide information as to the nature of nucleating or antinucleating factors in bile. Little data are available, however, to compare the biliary protein patterns of patients with cholesterol or pigment stones and those without gallstones. We have tried to extract, isolate, and characterize the proteins in patients with gallstones and without gallstones.

METHODS

1. Collection of Bile

Bile samples were obtained from patients with different types of gallstones (5 cholesterol, 5 brown pigment, and 6 black pigment) and 5 with no gallstones. Gallbladder bile was collected by needle puncture at cholecystectomy and stored frozen at -50°C under light protection.

2. Composition of Stones

Approximately 0.5 mg of dessicated powdered gallstone and 100 mg of KBr were jointly ground finely using mortar and pestle. The mixture was redessicated for at least 1wk and then compressed in an evacuated stainless steel die at 22000 lb/in for 12 min. The resultant wafer was subjected immediately to infrared spectroscopy (Perkin-Elmer Corp.) Pigment content (calcium bilirubinate) was determined from absorbance at 1624 cm^{-1} , phosphate at 1030 cm^{-1} and carbonate at 865 cm^{-1} as described by Trotman et al⁽¹¹⁾.

3. Protein Assay

Proteins in bile were precipitated with trichloroacetic acid (TCA) and delipidated with ether/ethanol,

sodium deoxycholate ($10\text{ }\mu\text{l}$) were added to the sample ($200\text{ }\mu\text{l}$), samples were then centrifugated at 1400 rpm 10 min, and the supernatant was aspirated and mixed with acetone. The mixture was centrifugated again at 1400 rpm for 10 min. and dried with nitrogen gas. Total protein concentrations were then measured using albumin as a standard by Lowry and Bensadoun technique^(12,13). Individual glycoproteins from each patient were compared by silver staining and densitometric quantification of SDS (Sodium Dodecyl Sulfate) Polyacrylamide Gel Electrophoresis^(14,15). Molecular weight and purity were determined by SDS-PAGE in 50 mM Tris-HCl, pH 8.3, containing 0.1% sodium dodecyl sulfate with a 5~10% linear acrylamide gradient, developed at 25~35mA for 4~5hrs, and protein bands were identified by silver staining of the gel. Standard proteins used to determine molecular weight were as follows; phospholipase (97K), bovine serum albumin (66K), ovalbumin (45K), glyceraldehyde-3-phosphate dehydrogenase (36K), carbonic anhydrase (29K), trypsin inhibitor (29K), α -lactalbumin (14.2K), and myoglobin digests (8K, 6K).

RESULTS

1. Characteristics of the Patients

Three groups of patients were studied and the characteristics of the patients are summarized in Table 1. The three study groups were similar with respect to age, sex, laboratory studies, risk factors, etc. The incidence of bacteria was 0 in bile with black pigment stones, 20% with cholesterol stone and 100% with brown pigment stones. Most frequently isolated bacterial species was *E. coli* (50%), and others were *pseudomonas aeruginosa*,

Table 1. Patients' Characteristics

	Cholesterol	Pigment stone		Control
		Brown	Black	
Age	48.2	54.6	53.3	47.3
Sex(M:F)	3:2	3:2	3:3	4:0
Bile culture	20%(1/5)	100%(5/5)	0	0
GPT(IU)	73.6 ± 83.48	90.8 ± 41.61	38.5 ± 20.71	53.5 ± 13.30
GOT(IU)	38.6 ± 33.3	72.2 ± 24.9	22.6 ± 12.31	48.2 ± 12.97
Cholesterol	189.8 ± 30.39	157.2 ± 62.12	135.3 ± 38.01	166.5 ± 46.10
WBC	6340 ± 1366.7	8600 ± 1847.9	7966 ± 3972.7	5650 ± 1302.0
Hgb	12.4 ± 0.93	12.1 ± 0.85	12.0 ± 1.30	12.3 ± 1.43

Blackpigment Brown pigment Ca. Bilirubinate stone Protein Conc (mg/ml)

Control Cholesterol Black Brown Stone Type

Citrobacter freundii and *Clostridium baratii*.

2. Classification of Gallstones

As determined from infrared spectra, the three types of gallstones could be classified according to their compositions. Among 16 gallstones, 5(31%) were cholesterol stones, 5 (31%) were calcium bilirubinate stones, and 6 (38%) were black pigment stones (Fig. 1). Pigment accounted more than 50% of pigment gallstone. Calcium for phosphate and carbonate were detectable in about 80% of black pigment stones in varying concentrations, and the levels were significantly lower in brown pigment stones than in black pigment stones. Calcium bilirubinate was present in all brown pigment stones and represents a greater proportion than in black pigment stones.

3. Protein Analysis

1) Composition of Gallstone Protein

The protein yield of repeated extraction was not statistically different in three types of gallstones. The mean protein concentration was highest in bile with cholesterol gallstones (47.6 ± 25.9 mg/ml; 19~75), 24.2 ± 17.3 mg/ml in bile with black pigment stones (10~51), 16.5 ± 4.4 mg/ml in bile without

stones (11~20), and the concentration was lowest in bile with brown pigment stones (15.9 ± 18.9 mg/ml; 7.3~51) (Fig. 2).

2) SDS-PAGE of the Gallstone Proteins

Fig. 3 shows the protein patterns on a SDS-polyacrylamide gel. Proteins from the stones consisted largely of major bands below 66 KD. Left first lane shows molecular weight protein standards and the other lanes show several different protein fractions of 3 different gallstones. Of the glycoproteins identified from SDS-PAGE, differences among groups were noted in the bands at 66000, 45000, and 14200 daltons. (Fig. 4) Cholesterol gallstones were found to have 14.2KD glycoprotein and brown and black pigment stones were found to have 66KD glycoproteins. Patients without gallstones had a 29KD glycoprotein, whereas patients with gallstones did not have any 29KD glycoprotein. All patients with gallstones had 45KD glycoproteins, whereas patients without gallstones did not have 45KD glycoprotein.

DISCUSSION

Gallstones have become a major public health problem in all parts of the world¹⁶⁾, and the incidence has been increasing in the Orient, even in our country^{17,18)}. There are two major classes of gallstones: cholesterol gallstones and pigment gallstones on the basis of their major composition, characteristics and pathogenesis¹⁹⁾. Cholesterol stones consist of at least 70% cholesterol, often with alternating layers of brown to black pigments, in which case they are called mixed stones²⁰⁾. Pigment stones are subclassified into two groups: black pigment stones and brown pigment stones²¹⁾. Black pigment stones contain much insoluble black pigment that is highly crosslinked

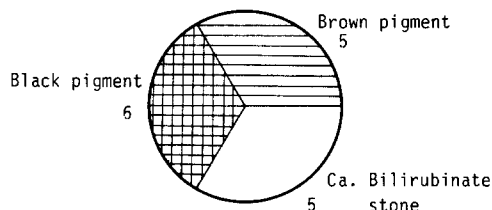


Fig. 1. Type & composition of gallstones by IR spectrophotometer.

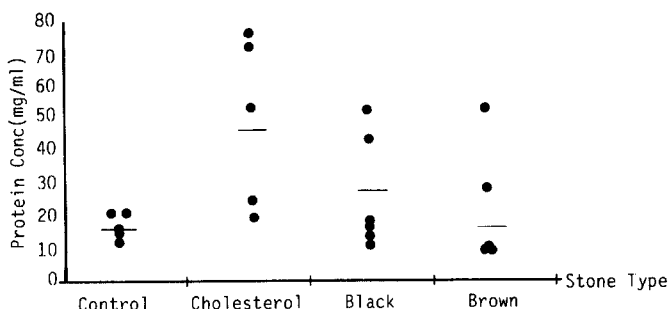


Fig. 2. Biliary protein concentrations in patients with or without gallstones.

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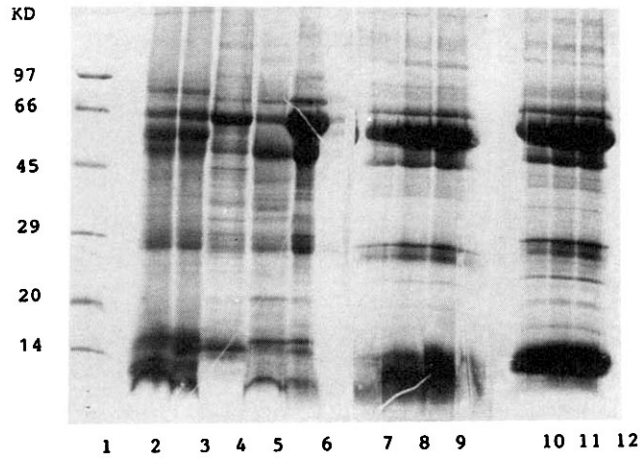


Fig. 3. SDS-PAGE of proteins from different types of gallstones. Lane 1, low molecular weight protein standards; land 2~6, protein fractions of cholesterol stones; lane 7~9, protein fractions of brown pigment stones; lane 10~12, protein fractions of black pigment stones.

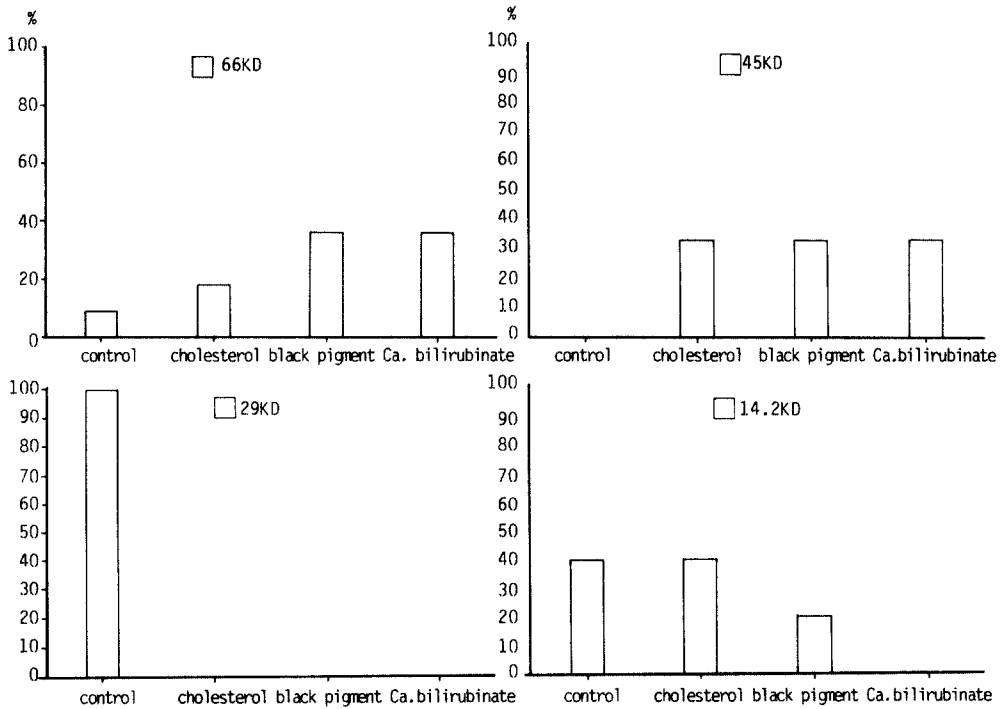


Fig. 4. Relative abundance of specific glycoproteins in human bile.

network polymer or tetrapyrroles²²⁾, which is mixed with calcium salts of bilirubinate, carbonate and/or phosphate^{11,21)}. Brown pigment stones consist

mainly of the insoluble calcium bilirubinate, calcium soaps of fatty acids, and a significant proportion of cholesterol^{23,24)}.

The classification of gallstones has been based on morphological and chemical analysis but has been limited by the variety and insolubility of many of the components²⁴⁾. The introduction of solid state analytical techniques such as X-ray diffraction and infrared spectroscopy has permitted identification of insoluble components of gallstones^{25,26,27)}. The infrared spectroscopy has an advantage that it could identify the components in pigment stones without requiring gallstones crystallinity and solubilization^{22,23,28)}.

Gallstone pathogenesis can be broken down into three distinct stages: 1) supersaturation, 2) nucleation, and 3) stone growth. Early investigators focussed primarily on cholesterol saturation, and most studies revealed that normal bile may be supersaturated and pathological bile is always supersaturated. However, supersaturation is not a sufficient condition for nucleation and gallstone formation^{7,29,30)}. Cholesterol monohydrate crystals precipitate much more rapidly from lithogenic biles than from normal biles, due to potent factors that promote nucleation of cholesterol³¹⁾. Normal bile also contains potent proteins that inhibit nucleation of cholesterol and calcium salts from supersaturated solutions including abnormal biles^{3,5,32)}. These inhibitory proteins are diminished in the gallbladder bile of patients with gallstones^{33,34)}. A great deal of interest has recently focused on the identification of the potential pronucleating factors.

In the present experiment, there was no statistical difference in protein composition among three types of gallstones. Harvey et al also reported that the glycoprotein concentrations in three types of gallstones were not significantly different³⁵⁾. The mean protein concentrations in our study were a little bit higher than those from Harvey's study and those determined by Lowry and Bradford³⁶⁾. However, the mean protein concentration was higher (47.6 ± 25.9 mg/ml) in cholesterol gallstones than in pigment stones or no stones, and it is still possible that the onset of nucleation may be associated with a temporary increase in protein concentration which then returns to normal levels once nucleation has occurred.

As in most biomineralization processes in mammals, there should be alterations of the biomineralization system in cholelithiasis: 1) alterations of mucin, 2) alterations in nucleating proteins, and 3) alterations in antinucleating proteins³⁵⁾.

Marked increase in the concentration of cholesterol-nucleating protein has been found in

the bile from patients with cholesterol gallstones. This protein, however, is not shown to be increased in concentration in the bile of pigment gallstones. We agree with Strasberg's groups that there should be an alteration in protein in lithogenic bile, and these proteins may be nucleated cholesterol or calcium bilirubinate from gallbladder bile⁶⁾.

The present result is similar to our previous results which were done with extraction of gallstones³⁸⁾. We still need to revise the extraction method to get more protein.

We try to determine the molecular weight and purity by SDS-PAGE electrophoresis, and the protein bands could be identified by silver staining of the gel. SDS-PAGE showed major bands at approximate molecular weight 66, 45, 29, and 14.2 KDa. The lowest molecular weight bands stained heavily, indicating many protein residues³⁹⁾. Cholesterol gallstones were found to have 14.2 KDa glycoproteins, whereas pigment stones had 66 KDa glycoproteins. All patients with gallstones had 45 KDa glycoproteins, whereas patients without gallstones didn't. These results suggest that some of these proteins may serve as nucleating agents. The present result differs from the 7 KDa bile-proteins isolated from T-tube bile by Etter-Kjelsaas and Kuenzle⁴⁰⁾. The present study, however, would appear to confirm the result of Shimizu et al. with both experiments suggesting that both cholesterol and pigment gallstones contain acidic proteins with many phosphorylated residues⁴¹⁾. They thus resemble the low molecular weight proteins from normally calcifying tissues (eg. phosphorylations in dental enamels and osteocalcin from bone), that regulate the biomineralization processes by binding calcium salts³⁹⁾. They are generally small glycoproteins, which are acidic due to a high content of aspartate, glutamate, phosphorylated/ sulfated serine/ threonine, and often gamma-carboxy-glutamate residues^{42,43)}. Pancreatic and urinary calculi were also found to contain similar proteins^{44,45)}.

We thought that the acidic phosphorylated proteins from gallstones may play a similarly important role in the initiation of nucleation process. However, it remains to be determined whether the protein functions as a promoter or inhibitor of nucleation. Moreover, it is known that the same protein can act as nucleating or antinucleating factors at different concentrations, or under different conditions⁴⁶⁾.

We propose that lithogenic bile may induce an

increase in gallbladder protein secretion and gallbladder proteins from both cholesterol and pigment stones could play an important role in the nucleation and growth of the calcium salt crystals. Further studies are necessary to purify and characterize the biliary proteins.

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