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High Vesicular Cholesterol and Protein in Bile Are Associated with Formation of Cholesterol but Not Pigment Gallstones

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To examine the differentiating parameters between cholesterol and pigment gallstones, we compared the nucleation times, concentrations of biliary lipid and protein, and the distribution of vesicular cholesterol in gallbladder bile of 16 patients with cholesterol, eight patients with black pigment gallstones, and nine gallstone-free control patients. Cholesterol monohydrate crystals were present in the fresh bile of only the cholesterol gallstone group. The nucleation time was significantly faster in the cholesterol stone group (3.3 ± 3.2 days) than in the other two groups (pigment stone: 15.8 ± 6.6, control: 16.9 ± 5.7). The cholesterol saturation indices and the distribution of vesicular cholesterol were significantly higher in the cholesterol gallstone group than those in the other two groups. The total biliary protein concentration was significantly ($P < 0.01$) higher in the cholesterol gallstone group [2.57 ± 1.91 (SD) mg/ml] than that in the black pigment stone group (1.09 ± 0.59). All parameters in patients with black pigment gallstone were essentially similar to the controls. We conclude that the presence of cholesterol crystals, rapid nucleation time, high vesicular cholesterol distribution, elevated cholesterol saturation index, and high protein concentration are associated with cholesterol gallstones but not with black pigment gallstones.

KEY WORDS: pigment gallstone; vesicle; biliary protein.

Supersaturation of bile with cholesterol is a prerequisite for cholesterol gallstone formation. However, bile is also frequently saturated in normal controls. Better distinction between patients with cholesterol stones and normal controls can be made by measuring the nucleation time, the appearance time of cholesterol monohydrate crystals in isotropic bile (1).

The finding of two cholesterol carriers, micelles and vesicles, in bile (2–4) have further advanced our understanding of the pathogenesis of cholesterol gallstone disease. It has been demonstrated that cholesterol monohydrate crystals nucleate from vesicles (5–7) and that the nucleation time correlates significantly with the amount of cholesterol carried in vesicles (8). The nucleation time of gallbladder bile from pigment stone patients has been reported to be longer than that of bile from cholesterol stone patients (9–11). Despite the importance of the vesicle, there has been little study of the relationship between the distribution of vesicular cholesterol and the nucleation time and how this relationship may differ between the bile of patients with cholesterol gallstones and those with pigment gallstones.

It is also reported that the biliary total protein concentration is related to the nucleation time (8,

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12) and that there is a higher protein concentration in gallbladder bile samples associated with cholesterol crystals or cholesterol gallstones (9, 12). There has been little study, however, of the role of biliary protein in black pigment gallstone formation.

In the present study, we compared the differences in vesicular cholesterol concentration, biliary protein concentration, the nucleation time, and the presence of cholesterol monohydrate crystals in fresh bile among the three groups of patients with cholesterol gallstones, black pigment gallstones, and without hepatobiliary disease.

MATERIALS AND METHODS

Patients. Twenty-four patients with gallbladder stone disease and nine control patients without hepatobiliary disease participated in this study. All patients had normal liver function. The nine control patients consisted of seven patients with gastric cancer, one with colonic cancer, and one with adenoma of the duodenum. Their mean age was 60 ± 10 years old. The absence of gallstones was confirmed by preoperative ultrasonography and by intraoperative palpation. Twenty-four gallbladder stone cases were classified into one of three categories (cholesterol, brown pigment, and black pigment stones) based on visual inspection and chemical analysis (13) as described below. Sixteen patients had cholesterol gallstones and eight had black pigment stones. Their mean ages were 53 ± 12 and 57 ± 14 years, respectively. In each patient, the gallbladders were well functioning as determined by preoperative drip infusion cholangiography and total biliary lipid concentration greater than 5 g/dl. Gallstone patients with nonvisualized gallbladders were excluded from this study. All patients gave informed consent preoperatively. The gallbladder bile was completely needle aspirated to avoid stratification (14) and care was taken to avoid the contamination with blood.

Bile Samples. Gallbladder bile samples thus obtained were immediately transported to the laboratory in a sterile test tube kept at 37° C in the dark. The fresh bile samples were examined for the presence of cholesterol monohydrate crystals and ultracentrifuged at 37° C for 2 hr at 10^5g (Hitachi 55P-72, Tokyo, Japan) to obtain the isotropic cholesterol crystal-free bile samples. The isotropic samples were subsequently used for the studies described below. A part of each isotropic bile sample was stored at −20° C for later chemical analysis.

Nucleation Time. The nucleation time was determined as described by Holan et al (1) with certain modifications (15). The isotropic bile samples were taken into the sterile glass tubes at 37° C, in the dark, under nitrogen. An aliquot was immediately examined to confirm the absence of any cholesterol monohydrate crystals and subsequently examined daily for the appearance of cholesterol monohydrate crystals under a polarized microscope (Nikon XTP-II, Tokyo, Japan) maintained at 37° C. All samples were observed for a period of 21 days. When cholesterol crystals did not appear during the observation period, the nucleation time was recorded as 21 days. The sterility of all bile samples studied was verified by bacteriological culture.

Gel Filtration Chromatography. Separation of vesicles from micelles by gel filtration chromatography was performed basically as described elsewhere (16). Fresh isotropic bile samples (0.4 ml) were incubated with 0.2 μCi of [^{3}H]cholesterol ([1α,2α(n)-^{3}H]cholesterol, 46 Ci/mmol, Amersham, Buckinghamshire, England) and 0.2 μCi of [^{14}C]phospholipid (L-3-phosphatidylcholine, 1-palmitoyl-2-[1-^{14}C]-oleoyl, 54 mCi/mmol Amersham) at 37° C for 1 hr with gentle shaking. The labeled bile sample was applied to a Sephacryl S-300 superfine (Pharmacia LKB, Uppsala, Sweden) column (10 × 350 mm) at 37° C with a flow rate of 15 ml/hr. Blue Dextran (Sigma, St. Louis, Missouri, mol wt 2 × 10^6) was used as a marker for the void volume. All columns were eluted with a buffer (50 mM Tris HCl, pH 8.0, 1.5 mM EDTA disodium salt, 150 mM NaCl, 0.02% sodium azide, 10 mM sodium cholate). Thirty-five fractions of 1 ml each were collected using a fraction collector and a part of each fractionated sample was counted in the liquid scintillation counter (LSC 3500, Aloka, Tokyo, Japan) after adding Ready-Solve scintillation cocktail (Beckmann, Fullerton, California). Quench correction was made by means of the external standardization technique. The percent and concentration of the cholesterol carried in the vesicles was calculated as previously described (8).

Biliary Lipids and Protein. Individual bile acids and cholesterol were simultaneously quantified by capillary gas–liquid chromatography as we have previously described (17). Phospholipids were determined by the method of Bartlett (18). The cholesterol saturation index (CSI) was calculated based on the critical tables provided by Carey (19).

Total biliary protein was measured by the fluorescamine method (20) as applied to bile samples (21). Briefly, isotropic bile was diluted 100-fold with deionized water. Protein was precipitated from 1 ml of the diluted sample with 1 ml of 20% trichloroacetic acid. The solution was incubated for 30 min at 4° C, and centrifuged at 1700g for 30 min at 4° C. The resulting pellet was washed with 3 ml of diethyl ether–ethanol 3:1 (v/v) for delipidation and then recentrifuged. The final pellet was dissolved in 0.5 ml of borate buffer (pH 9.0) and incubated overnight at 4° C. After the addition of 1 ml borate buffer to the solution, 0.5 ml of fluorescamine (Sigma, St. Louis, Missouri) solution (0.02% in acetone) was added and the fluorescence was measured at an emission wavelength of 480 nm and an excitation wavelength of 390 nm with a fluorescence spectrophotometer (Hitachi 650-10S, Tokyo, Japan).

Gallstone Analysis. The dried gallstone was homogeneously crushed to a powder, weighed, and dissolved in a dimethylsulfoxide–acetone–1 N HCl (90:9:1 v/v/v) solution, ultrasonified, and then centrifuged. The supernatant obtained after two more repeated extractions was subjected to determination of cholesterol, fatty acids, and bilirubin as reported from our laboratory (13). The calcium content was also measured by atomic absorption spectrometry (Shimadzu AA 630-01). Twenty-four gallbladder stone cases thus analyzed could be divided into 16 cholesterol stone and eight black pigment stone cases.

TABLE 1. BILE LIPID COMPOSITION

	Gallstones		
*Composition**	*Cholesterol* (N = *16*)	*Black pigment* (N = *8*)	*Controls* (N = *9*)
Total bile acids (mM)	133.6 ± 44.4	175.5 ± 69.0	199.0 ± 65.1b
Cholesterol (mM)	15.5 ± 5.8	11.8 ± 6.5	15.0 ± 6.0
Phospholipids (mM)	46.8 ± 21.7	40.6 ± 9.9c	58.6 ± 19.0
TLC (g/dl)	10.8 ± 3.8	12.2 ± 4.2	14.9 ± 4.6b
CSI	1.11 ± 0.26	0.78 ± 0.21a‡	0.74 ± 0.14a
Total protein (mg/dl)	2.57 ± 1.91	1.09 ± 0.59a	1.43 ± 1.24
Cholesterol crystal†	12/16 (75%)	0/8 (0%)	0/9 (0%)

*TLC: total lipid concentration; CSI: cholesterol saturation index.
†The presence of cholesterol monohydrate crystal in fresh bile.
‡a,b Significantly different from cholesterol gallstone group (a, $P < 0.01$; b, $P < 0.05$); and c, from the control group ($P < 0.05$).

Statistics. Values are represented as the mean ± SD. The statistical significance of any difference between groups was evaluated by one-way analysis of variance followed by Student's *t* test. Linear regression analysis was done by the method of least squares and the significance was assessed by estimating the correlation coefficient. Probability values less than 0.05 were considered to be significant.

RESULTS

Biliary Lipid and Gallstone Compositions. Biliary lipid compositions are shown in Table 1. The concentration of each biliary lipid and the total lipid concentration in cholesterol gallstone patients were similar to those in black pigment gallstone patients. The concentrations of total bile acid and total lipid in control patients were significantly higher than those in the cholesterol gallstone patients. The CSI were significantly higher in the cholesterol gallstone patients (1.11 ± 0.26) than in the black pigment gallstone patients (0.78 ± 0.21, $P < 0.01$) or in the controls (0.71 ± 0.14, $P < 0.01$). Cholesterol monohydrate crystal was present in 75% of the cholesterol gallstone patients but absent in the other two groups. The only difference between the pigment stone and the control group was that the pigment stone group had a significantly lower concentration of phospholipid.

The chemical compositions of cholesterol and black pigment stones are provided in Table 2. The cholesterol content was approximately 85% dry weight in cholesterol stones while it was only 2% in black pigment stones. More bilirubin, fatty acids, and calcium were present in black pigment stones as compared with cholesterol gallstones.

Nucleation Time. The nucleation time was significantly faster in the bile of cholesterol stone patients (3.3 ± 3.2 days) than in the black pigment stone patients (15.8 ± 6.6) or the controls (16.9 ± 5.7) ($P < 0.01$; Figure 1). The nucleation time did not differ between the black pigment stone and the control groups.

Vesicular Cholesterol Distribution. The percent of biliary cholesterol carried by vesicles are shown in Table 3. The distribution of vesicular cholesterol was significantly higher in the cholesterol gallstone patients than in the black pigment stone patients or

TABLE 2. CHEMICAL COMPOSITION OF GALLBLADDER STONES*

	Cholesterol stone (N = *16*)	*Black pigment stone* (N = *8*)
Cholesterol	850 ± 112.2a	23 ± 20.7
Bilirubin	15 ± 23.2a	85 ± 39.1
Fatty acids	21 ± 18.9	39 ± 35.5
Calcium	10 ± 15.5	45 ± 52.1

*Values are expressed in mg/g dry weight (mean ± SD); a; $P < 0.01$.

Fig 1. Nucleation time in cholesterol and black pigment gallstone patients and in the control patients.

TABLE 3. DISTRIBUTION OF CHOLESTEROL AND PHOSPHOLIPIDS IN VESICLES*

	Gallstones		
	Cholesterol (N = 16)	Black pigment (N = 8)	Controls (N = 9)
Cholesterol (%)	22.9 ± 13.3	5.6 ± 5.0a	5.0 ± 4.1a
Phospholipids (%)	9.0 ± 6.7	1.6 ± 1.8a	1.2 ± 1.1a

*Significantly different from the cholesterol gallstone group: a, $P < 0.01$. No significant difference was found between the pigment gallstone and control group.

controls. No significant difference was found between the black pigment stone group and the control group.

Biliary Protein. The total protein concentration in gallbladder bile was significantly higher in the cholesterol gallstone patients than in patients with black pigment stones (Table 1). The difference between controls and cholesterol gallstone groups was not significant even though the value was 1.8 times higher in the cholesterol gallstone group.

Correlations Between Parameters. The nucleation time was inversely correlated with the percent of biliary cholesterol in vesicles (Figure 2A, $r = -0.744$, $P < 0.01$), with the CSI (Figure 2B, $r = -0.769$, $P < 0.01$), and with the total biliary protein concentration (Figure 2C, $r = -0.411$, $P < 0.05$). It should be stressed that five of 16 patients with cholesterol gallstones had higher concentrations of biliary protein than others (Figure 2C). These patients had cholesterol crystals in their fresh bile and showed the rapid nucleation time within one day. No significant correlation was found between the total lipid concentration and the nucleation time. The total lipid concentration did not correlate with the biliary protein concentration. The percent of biliary cholesterol in vesicles correlated with the CSI (Figure 3A, $r = 0.827$, $P < 0.01$) and with the total biliary protein concentration (Figure 3B, $r = 0.384$, $P < 0.05$).

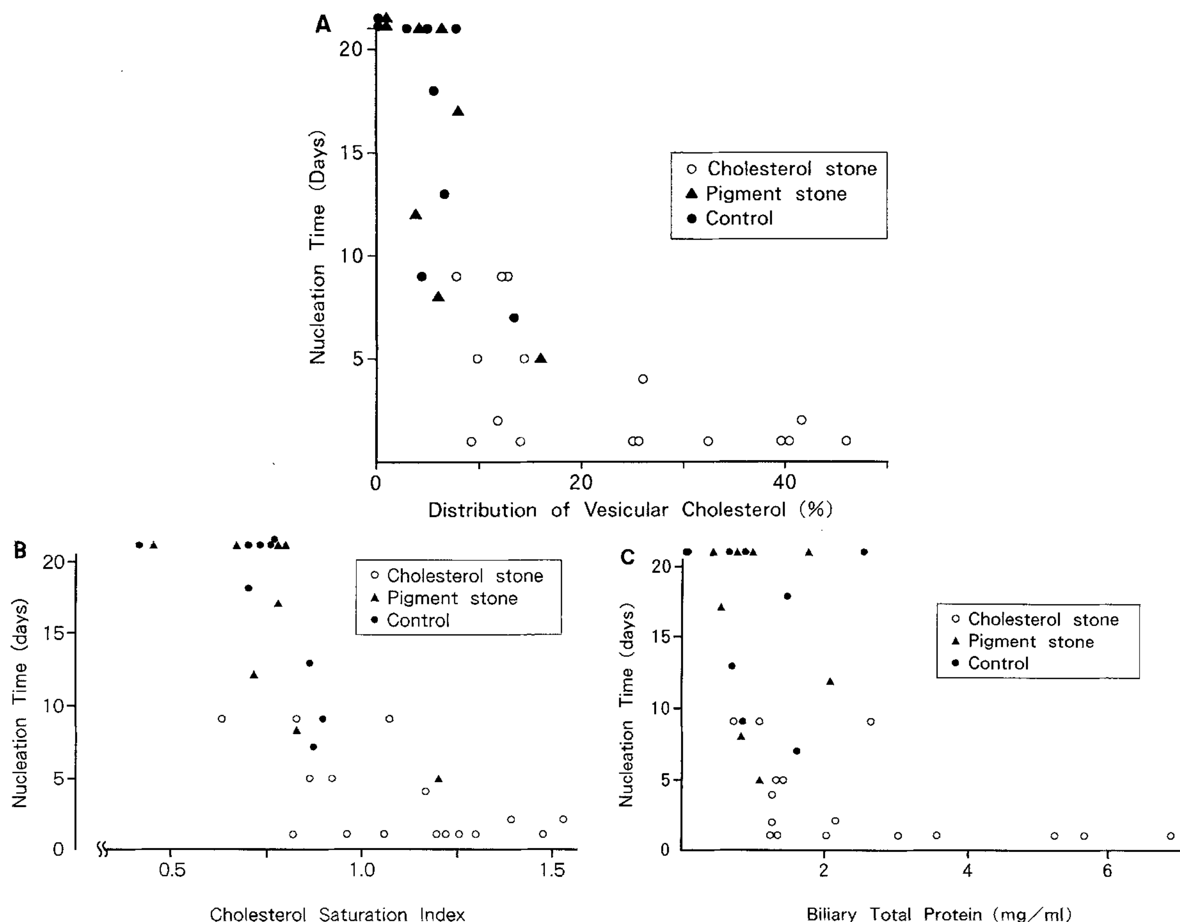

Fig 2. Correlation between the nucleation time and (A) the percent of biliary cholesterol in vesicles; (B) the cholesterol saturation index; and (C) biliary protein concentration.

Fig 3. Correlation between the percent of biliary cholesterol in vesicles and (A) the cholesterol saturation index or (B) the biliary protein concentration.

DISCUSSION

Based on the significant difference in the cholesterol content of gallstones, one could predict that the precipitation of cholesterol is necessary to form a cholesterol gallstone but not a pigment gallstone. This was confirmed by the present study in concordance with others (9–11, 22). However, the nucleation time could not distinguish the black pigment stone patients from the gallstone-free controls.

An important finding in the present study was that a significantly higher distribution of vesicular cholesterol was observed in the cholesterol gallstone patients than in the black pigment stone and control patients. The distribution of vesicular cholesterol was similar between the black pigment stone and the control group, which both had slower nucleation times. Although there is now little doubt that vesicles play an important role in cholesterol gallstone disease, a question may arise as to the method by which vesicles are separated from micelles. One critical factor may be the bile salt concentration in the elution buffer. The ideal bile salt concentration in the elution buffer may be the intermicellar bile salt concentration with or without the bile salt concentration in a simple micelle. However, the determination of the correct intermicellar bile salt concentration in each bile sample is not practical and is complicated at present. In native bile, the intermicellar bile salt concentration depends upon various factors such as biliary lipid composition, individual bile salt species, and biliary protein. Even though gel permeation chromatography is conventional and may be artifactual, the method has been widely used and is reported to be appropriate. Since neither the correct bile salt species nor the correct intermicellar bile salt concentration has been used in all the literature, exact quantification of vesicular lipids awaits a nonperturbing separation method without shifting cholesterol between vesicles and micelles. Significant correlation ($r = -0.74$) between the distribution of biliary cholesterol in vesicles and the nucleation time agrees with the results of Harvey et al (8).

To further examine the parameters possibly differentiating the three groups, biliary lipid and protein concentrations were quantified and compared. Although the total biliary lipid concentration was not significantly different between the two stone type groups, the cholesterol saturation index and the total protein concentration were significantly higher in the cholesterol gallstone group than in the black pigment stone group. Supersaturated bile with cholesterol may enhance the secretion of protein, and extensive cholesterol nucleation will occur in the presence of high protein concentration. The association of high protein concentration with rapid nucleation time has been demonstrated recently by Strasberg et al (9) and Gallinger et al (12). An inverse relationship between biliary protein and nucleation time was observed in the present study. In contrast to cholesterol supersaturated bile, there was a lower concentration of biliary protein in bile of the black pigment stone group as well as the control group. The lack of correlation between the total protein concentration and the total lipid concentration suggests that the biliary protein concentration is independent of water absorption (concentrating function) by the gallbladder. The results suggest that protein in the gallbladder plays an important role in cholesterol gallstone formation but does not contribute to the formation of black pig-

ment stones. Thus, the concentration of biliary protein can differentiate the cholesterol gallstone group from others but can not differentiate between pigment stone and control patients.

The interesting observation that the distribution of vesicular cholesterol correlates not only with the CSI but also with the total protein concentration in gallbladder bile indicates that the high CSI and protein concentrations may increase the distribution of vesicular cholesterol and reduce bile metastability. Groen et al (23) have demonstrated that a concanavalin A binding protein shifts the cholesterol molecule from the micellar to the vesicular phase.

With respect to all of the parameters examined, the values in the black pigment stone group were similar to controls, in contrast to the cholesterol gallstone group.

In conclusion, the presence of cholesterol crystals in fresh bile, the nucleation time, the cholesterol saturation index, biliary protein, and the distribution of vesicular cholesterol are associated with cholesterol gallstones. All parameters in black pigment gallstone patients are similar to the gallstone-free control patients.

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