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Isolation and partial characterization of cholesterol pronucleating hydrophobic glycoproteins associated to native biliary vesicles

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Cholesterol is transported both in unilamellar phosphatidylcholine vesicles and in bile salts-mixed micelles in native bile. The vesicular carrier of biliary lipids apparently has a well defined protein profile with a potent cholesterol crystallization-promoting activity. This study was conducted to identify and further characterize these vesicular proteins and to test the effect of isolated vesicular proteins on the cholesterol crystal formation in supersaturated model bile. The results confirmed that proteins are a constant component of highly purified biliary vesicles both in hepatic and gallbladder bile. Immunoglobulins (IgA, IgG and IgM) and albumin are associated to the purified hepatic biliary vesicles. Furthermore, four different hydrophobic glycoproteins with a molecular mass of 130, 114, 86, and 62–67 kDa were isolated. These glycoproteins showed no reactivity with anti-human whole serum or anti-immunoglobulin antibodies, suggesting that these proteins are biliary-specific. Isolated 130, 114 and 62–67 kDa vesicular glycoproteins significantly decreased the cholesterol nucleation time in artificial model bile. We concluded that some, but not all, vesicular-bound hydrophobic glycoproteins have cholesterol pronucleating activity and they may be involved in the pathogenesis of cholesterol gallstone disease.

Biliary vesicle; Cholesterol crystallization; Glycoprotein

1. INTRODUCTION

Phospholipid vesicles are considered to be the primary carrier of cholesterol in bile [1–4]. In hepatic bile these vesicles are very stable, however, in concentrated gallbladder bile they exhibit a strong tendency to aggregation [5,6], thus leading to cholesterol crystal formation. Biliary vesicles are commonly found associated to cholesterol crystals in gallbladder bile specimens of patients with cholesterol gallstones [7]. Biliary cholesterol crystallization is a critical step in the pathogenesis of cholesterol gallstone disease [8].

In contrast to model bile systems [6], in native lithogenic gallbladder bile no correlation has been found between the lipid composition of the vesicular carrier and the cholesterol nucleation time [7,9]. Factors influencing the metastability of this carrier in lithogenic bile are not well defined. A number of biliary proteins that promote, or inhibit cholesterol crystal formation have been isolated [10–14]. In recent studies, we have isolated highly purified cholesterol biliary vesicles from native bile of cholesterol gallstone patients [15]. The purified vesicular fraction obtained with this method contained a unique and well-defined protein profile of six polypeptides from 52 to 200 kDa. Lipid solutions containing these vesicular proteins showed a potent cholesterol pronucleating activity in supersaturated model bile [15].

In the present study we isolated and partially characterized the vesicular-bound proteins and tested their effect on the cholesterol nucleation time of model bile.

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2. MATERIALS AND METHODS

2.1. *Isolation and purification of biliary vesicles*

Hepatic bile samples were obtained from 13 patients with indwelling T-tubes, 5 to 7 days after cholecystectomy and choledochotomy for cholesterol gallstone disease. Bile was collected early in the morning and it was immediately processed as previously described [15]. In 4 patients we also studied gallbladder bile obtained at the moment of the surgical procedure. Biliary vesicles were isolated from native bile specimens by rapid density gradient ultracentrifugation and purified by sequential gel filtration chromatography [15].

2.2. *Gel electrophoresis and immunoblotting*

Proteins from purified biliary vesicles were precipitated with 8% (w/v) trichloroacetic acid and delipidated with ethanol/diethylether (1:3, v/v). Then, SDS-PAGE was performed under reducing and non-reducing conditions according to Laemmli [16]. Proteins were visualized by Coomassie brilliant blue staining. For immunoblotting, after SDS-PAGE proteins were electroblotted onto nitrocellulose [17], incubated with rabbit polyclonal anti-human whole serum, anti-albumin, anti-IgAα, -IgGγ or -IgMμ chain antibodies (Sigma Chemicals Co., St. Louis, MO, USA) and stained with protein A-alkaline phosphatase conjugate.

2.3. *Concanavalin A affinity chromatography and Triton X-114 phase separation of vesicular proteins*

The presence of *N*- or *O*-linked glycoside residues to vesicular proteins were determined by concanavalin A-affinity chromatography [10]. To evaluate their relative hydrophobic/hydrophilic behaviour the vesicular proteins were analyzed by phase separation in Triton X-114 [18].

2.4. *Electroelution and renaturation of vesicular proteins from SDS-polyacrylamide gels*

To isolate a sufficient quantity of the individual vesicular proteins for the cholesterol nucleation time assays, we electroeluted each protein from preparative non-reducing SDS-PAGE. To allow recovery of vesicular proteins in apparent native conditions for cholesterol nucleation experiments we applied the renaturation method of Hager et al. [19]. Re-electrophoresis and Coomassie blue staining of each isolated protein fraction demonstrated a single polypeptide band with the same original molecular weight of every isolated protein.

2.5. *Cholesterol nucleation time assays*

The effect of the individual purified vesicular proteins electroeluted and renaturated from SDS-PAGE on the cholesterol crystal nucleation time of supersaturated model bile was determined by the method of Groen et al. [10]. Nucleation time represents the interval (in days) between zero time and the first detection of cholesterol crystals under polarizing microscopy. Electroeluted and renaturated vesicular proteins were resuspended in model bile buffer and were added to artificial bile to achieve a final protein concentration of 20 μg/ml. Equal volumes of control solutions obtained from electroelution of gel pieces without proteins were added to model bile as control mixtures. The nucleation time of these control mixtures did not differ from that of the original model bile.

2.6. *Chemical analysis*

Cholesterol, phospholipids, bile salts and protein concentrations in hepatic and gallbladder bile and in vesicular fractions were determined by standard techniques as previously described [20,21]. The cholesterol saturation index was determined using Carey's critical tables [22]. Relative composition of vesicular proteins were assessed with a densitometer unit (Shimadzu, Tokyo, Japan) after SDS-PAGE and Coomassie blue staining.

3. RESULTS

Lipid composition of hepatic and gallbladder bile from study patients are given in Table I. The major constituents by weight of the isolated and purified biliary vesicles are phospholipids (63.5 ± 4.6%, mean ± 1 S.D., $n = 13$) and cholesterol (35.5 ± 5.4%, $n = 13$); less than 1% of total lipids corresponds to bile salts. A low (1 ± 0.2%, $n = 13$) but constant amount of protein is associated with the vesicular material.

Non-reducing 10% SDS-PAGE of purified native biliary vesicles isolated from hepatic bile shows the presence of a constant protein profile composed of six polypeptides with molecular masses of 200, 130, 114, 86, 62–67 and 52 kDa (Fig. 1, lane b). Under reducing conditions the 200 kDa protein is resolved into a number of low molecular weight polypeptides in the range between 25–80 kDa (Fig. 1, lane 3b). Also, the 52 kDa protein changed its migration to 66 kDa apparent mol. wt. in reducing SDS-PAGE. The 130, 114, 86 and 62–67 kDa mol.wt. proteins did not show variations of electrophoretic migration under reducing conditions (Fig. 1, lane 1b vs. 3b).

Table I

Composition and cholesterol nucleation time of hepatic and gallbladder bile from patients with gallstone disease

	Hepatic bile	Gallbladder bile
Biliary lipid concentration (mM)		
Bile acids	22 ± 13	83 ± 12
Phospholipids	6.4 ± 3.1	17.3 ± 7.8
Cholesterol	5.7 ± 2.8	7.4 ± 2.2
Protein concentration (g/l)	1.8 ± 0.7	5.4 ± 1.9
Cholesterol saturation (%)	111 ± 55	137 ± 28
Vesicular cholesterol (%)	68 ± 8	26 ± 6
Cholesterol nucleation time (days)	15 ± 4	3 ± 1

Values represent the mean ± 1 S.D. of bile samples from 13 different patients.

In some patients, we were also able to obtain gallbladder bile that contained a low but significant vesicular cholesterol fraction (range 18–33%, $n = 4$). Vesicles were isolated and purified as described above for hepatic bile samples. As shown in Fig. 1, lane 2 a similar protein profile as in hepatic biliary vesicles was found under non-reducing SDS-PAGE.

Immunoblotting of vesicular proteins separated on SDS-PAGE under non-reducing conditions were first carried out with rabbit anti-human whole serum antibody. Only two major bands corresponding to the 200 and 52 kDa proteins became visible (result not shown). Under reducing conditions (Fig. 2), anti-albumin antibody recognized only the 66 kDa band (lane 3), confirming that this protein corresponded to biliary albu-

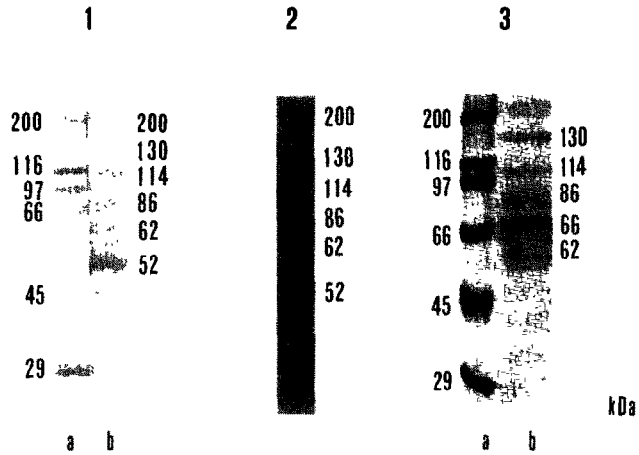

Fig. 1. SDS-PAGE of vesicular proteins stained with Coomassie blue. 100 μg of total protein were applied to each lane. (1) Lane a, protein molecular weight markers; lane b, hepatic biliary vesicles under non-reducing conditions. (2) Vesicles harvested from gallbladder bile under reducing conditions. (3) Lane a, molecular weight markers; lane b, hepatic biliary vesicles under reducing conditions.

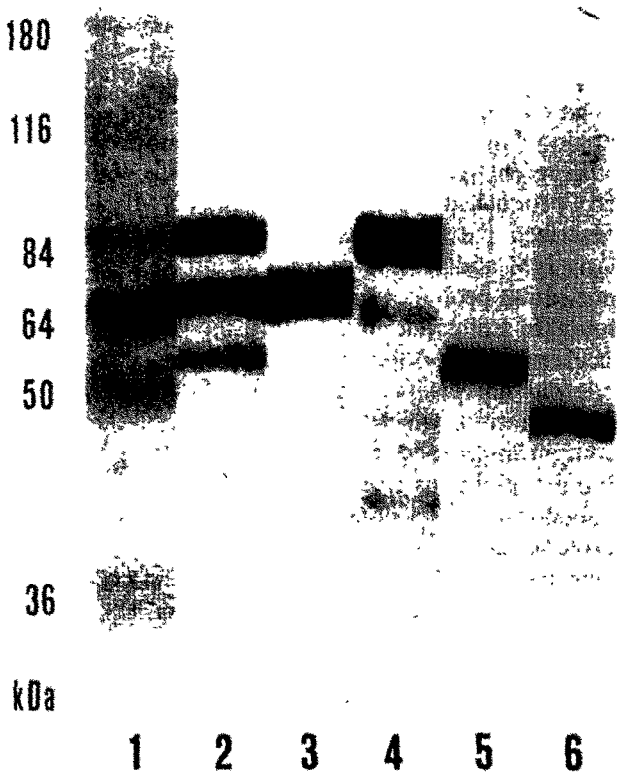

Fig. 2. Western blot of vesicular proteins separated by reducing SDS-PAGE. Lane 1, prestained molecular weight markers; lane 2, anti-human whole serum; lane 3, anti-albumin; lane 4, anti-IgMμ chain; lane 5, anti-IgAα chain; lane 6, anti-IgGγ chain.

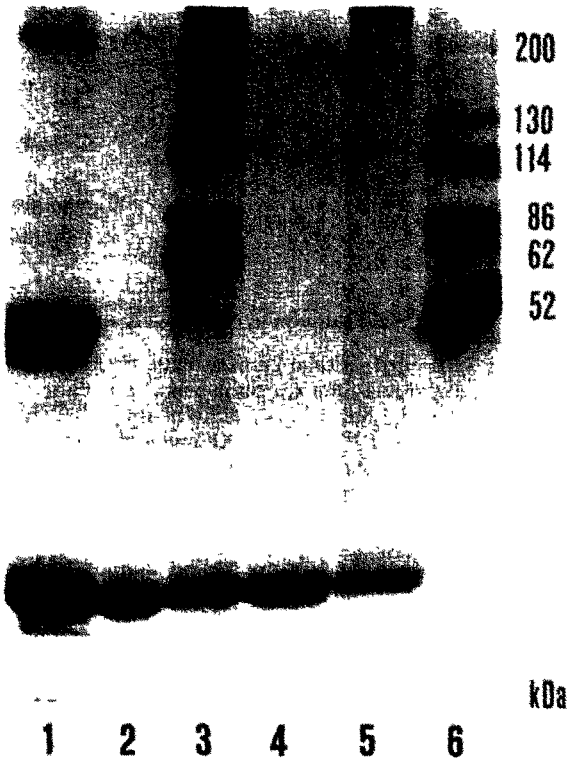

Fig. 3. SDS-PAGE of vesicular proteins after concanavalin-A affinity chromatography. Lane 1, unbound fraction; lane 2, washout of the column with buffer; lane 3, eluate of the bound fraction using buffer containing α-D-methylmannopyranoside; lane 4 and 5, wash-out of the column with glycoside buffer; lane 6, original vesicular protein sample. The dark band shown at the bottom of the lanes corresponds to Con-A released from the column.

min. Anti-immunoglobulin antibodies (anti-IgAα, -IgGγ and -IgMμ chain) were bound to the 200 kDa mol.wt. band (result not shown) and after reducing SDS-PAGE the low molecular weight bands generated from the 200 kDa protein, immunoreacted with the anti-human Ig heavy chain antibodies (Fig. 3, lane 4,5,6) indicating that immunoglobulins are associated with the purified biliary vesicles. The 130, 114, 86, and 62–67 kDa proteins showed no immunoreaction with any of the anti-human serum proteins tested, both in non-reducing and reducing PAGE, suggesting that they could be specific biliary proteins.

The relative distribution of vesicular proteins from hepatic bile was assessed by scanning of Coomassie brilliant blue stained SDS-PAGE with a densitometer unit. The 200 and 86 kDa proteins were found in minor amounts in most samples and were absent in some of them. Albumin (the 52 kDa band in non-reducing SDS-PAGE) was the predominant protein accounting for 35.6 ± 5.7% (mean ± S.D., n = 7) of the total vesicular protein content. Interestingly, the 130, 114, and 62–67 kDa proteins represent on average 10.7 ± 5.9, 14.2 ± 7.5 and 26.2 ± 17.1% and together they were the most predominant in four samples.

Fractionation of vesicular proteins was carried out on Con A-Sepharose chromatography. Fig. 3, lane 1 shows that, as expected, the 52 kDa protein (albumin) was completely recovered in the unbound fractions. Moreover, part of the 200 kDa and also some of the other proteins were recovered in this fraction too. The 130, 114, 86 and 62–67 kDa were found in the Con A eluate

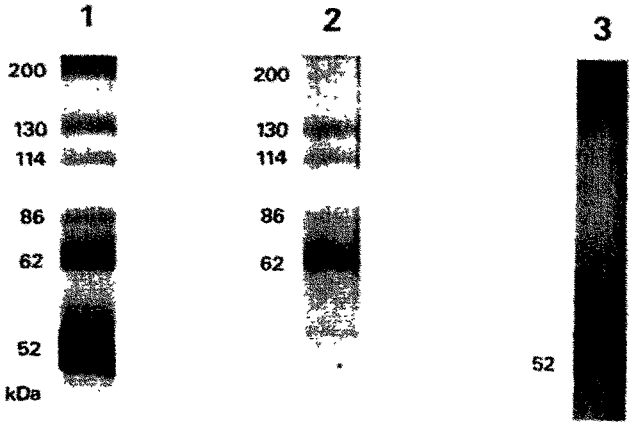

Fig. 4. SDS-PAGE of vesicular proteins after phase separation in Triton X-114. Lane 1, total vesicular proteins; lane 2, detergent phase; lane 3, aqueous phase.

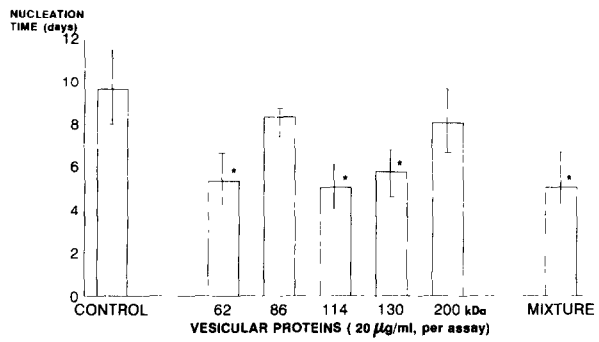

Fig. 5. Effect of isolated vesicular proteins on the cholesterol crystal nucleation time of supersaturated artificial model bile. Each column represents the mean ± 1 S.D. of 5 to 6 different assays. In a series of experiments a mixture of 3.5 μg/ml of each protein was added to the assay. The asterisks indicate a significant difference at the $P < 0.05$ from the control (Student's t-test).

together with the 200 kDa protein (Fig. 3, lane 3). These results suggest that most of the vesicular proteins are glycoproteins.

Vesicular-bound proteins were analyzed by phase separation in Triton X-114 solution. As shown in Fig. 4, lane 2, the 130, 114, 86, 62–67 and a small fraction of the 200 kDa glycoproteins had a strong hydrophobic behaviour with depletion from the aqueous phase and complete recovery in the detergent phase. In contrast, the 52 kDa and the major part of the 200 kDa mol.wt. protein were recovered in the aqueous phase. These findings indicate that most of the vesicular-bound proteins possess hydrophobic domains with the ability to bind detergents, suggesting that these glycoproteins could be bound by hydrophobic interaction to the core of the lipid bilayer of the biliary vesicles.

To assess the cholesterol nucleation influencing activity of the vesicular proteins, supersaturated model bile was mixed with aliquots containing isolated and renaturated vesicular proteins. The nucleation time of these mixtures was compared with control mixtures prepared as described. As shown in Fig. 5, the 130, 114, and 62–67 kDa glycoproteins significantly decreased the nucleation time of model bile by 50% at a concentration of 20 μg/ml. At this concentration, the 200, 86 and 52 kDa proteins lacked nucleation promoting activity (result with the 52 kDa protein, corresponding to albumin by Western blotting, is not shown). Finally, mixtures of all vesicular proteins at the same total concentration (approximately 3.5 μg/ml of each one) reduced the cholesterol nucleation time in the same proportion.

4. DISCUSSION

The major finding of this study is that native purified biliary vesicles contain a constant protein profile, both from hepatic and gallbladder bile which by mass represents approximately 1% of vesicle constituents. We found a series of three hydrophobic glycoproteins (molecular weights of 130, 114 and 62–67 kDa) with potent cholesterol crystallization-promoting activity. Isolated and purified 86 kDa-hydrophobic glycoprotein without apparent pronucleating activity and a series of immunoglobulins.???

In recent years, several biliary proteins with cholesterol pronucleating or antinucleating activity have been described [10–15]. Biliary immunoglobulins, particularly IgM and IgA, have been identified as cholesterol nucleation promoter proteins at concentrations usually found in gallbladder bile [13]. In the present study, vesicular immunoglobulins isolated from native hepatic bile did not exhibit pronucleating effect at 20 μg/ml concentration, which corresponds to one-third of the concentration used in the study by Harvey et al. [13]. Immunoglobulins isolated from the vesicular fraction only constituted a minor component of total vesicular proteins, as compared to the hydrophobic glycoproteins with cholesterol nucleation promoting effect (the 130, 114, 62–67 kDa proteins) and albumin. The 130 kDa glycoprotein found in this study may be the 130 kDa pronucleating glycoprotein described by Groen et al. in both hepatic and gallbladder bile of patients with gallstones [10,11].

With respect to the mechanism of cholesterol crystallization promoting activity, we could speculate that the hydrophobicity of the pronucleating glycoproteins determines their binding to the core of the lipid bilayer of the biliary vesicles, favouring vesicular aggregation and fusion, the two critical pronucleating events in the complex pathophysiological cascade of the cholesterol crystal formation in gallstone disease. It is interesting to note that gallbladder mucin also accelerates cholesterol crystal nucleation in model bile [23], probably by a molecular mechanism dependent on the hydrophobic domains of the mucin protein core [24]. Present results are consistent with the current hypothesis of the pathogenesis of cholesterol gallstone disease and they strongly suggest that cholesterol crystal formation and growth may be finally determined by a series of different biliary factors, including the presence of proteins, originated in hepatic as well as in gallbladder bile.

Presently, the nature, origin and functional significance of the four vesicular hydrophobic glycoproteins described in this study remain unknown. Because biliary vesicles probably represent the primary mechanism of biliary lipid secretion, they might be related to the process of sorting, intracellular transport and canalicular secretion of the precursor carrier(s) of biliary lipids. The true functional significance of the vesicular glycoproteins could become apparent after their identification, quantitation and correlation to the secretory process of biliary lipids.

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