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• BASIC RESEARCH •

Purification and characterization of 33.5 kDa vesicular protein in human bile

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Abstract

AIM: The present study was undertaken to purify and partially characterize the 33.5-kilodalton (33.5 kDa) vesicular protein in human bile and to explore the possible molecular mechanisms of the initial crystal nucleation process.

METHODS: The 33.5 kDa vesicular protein was isolated by ultracentrifugation and further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions. The purified 33.5 kDa vesicular protein was subjected to N-terminal amino acid sequencing and amino acid analysis. Cholesterol crystallization activity was detected by cholesterol crystal growth assay. The sugar chain of the 33.5 kDa vesicular protein was analyzed by dot-immunobinding assay of lectin coupled to a peroxidase (HRP-DSA, HRP-ConA, HRP-WGA) and was deglycosylated using two different enzymatic approaches (*N*-deglycosylation and *O*-deglycosylation) to determine the molecular weight of the protein component, the type of linkage between polypeptide and carbohydrate components.

RESULTS: The 33.5 kDa vesicular protein with complicated glycan was an extensively glycosylated (37.3 %) monomer and these sugar chains strongly bound to DSA, but did not bind to ConA. Amino acid sequencing indicated that the protein was unique. The 33.5 kDa vesicular protein exhibited potent cholesterol crystallization promoting activity *in vitro* with derived crystal growth curve indices It, Ig, Ic presented as 0.57, 1.52, and 1.63 respectively. Both enzymatic proteolysis and *N*-deglycosylation of the protein removed all activity.

CONCLUSION: These data suggest the 33.5 kDa vesicular protein may be responsible for the pathogenesis of cholesterol gallstone disease, and the sugar chains play an important role in pro-nucleating process.

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INTRODUCTION

Cholesterol nucleation process represents a critical step in the cholesterol gallstone formation. Cholesterol pro-nucleating and anti-nucleating proteins can accelerate or retard the rate of cholesterol crystallization in supersaturated bile, and thus may play important roles in cholesterol crystallization[1-3]. From 1988, both inhibitors and promoters of cholesterol crystallization have been isolated from human bile and characterized[4-7]. The major cholesterol crystallization promoting activity was localized at the concanavalin A-binding fraction of biliary glycoproteins (CABG). These proteins include mucin[8], immunoglobulins[9-11], α_1-acid protein[12], aminopeptidase N[4], low-density protein-lipid complex[5,13], and some unidentified proteins such as 70 kDa[14] and 200 kDa[15] pro-nucleating glycoproteins. Abei *et al*[16] provided comparative data regarding the relative potency of these different glycoprotein promoters and found that α_1-acid protein accounted for the greatest portion (33 %) of the net biliary Con A-bound promoting activity derived from currently defined and well-identified glycoproteins. But still more than 60 % of total Con A-bound promoting activity remains unaccounted for. It was speculated that there was still some other more important proteins involved in cholesterol nucleation process.

Lecithin vesicles are the primary cholesterol carriers in bile supersaturated with cholesterol and have been shown to play an important role in the nucleation of cholesterol. This nucleation takes place after aggregation and fusion of cholesterol-rich biliary vesicles, a process modulated by biliary proteins. Miquel *et al*[17] found a potent cholesterol pro-nucleating activity in purified biliary vesicles. Further study demonstrated that this activity was related with specific vesicular proteins including immunoglobulins IgA, IgG and IgM[18].

In this study, a novel 33.5 kDa vesicular protein obtained from human gallbladder bile of cholesterol gallstone patients was isolated, purified and partially characterized. We attempted to determine whether pro-nucleating activity occurred in the 33.5 kDa vesicular protein and to detect whether the protein was lectin-specific. Our results showed that the 33.5 kDa vesicular protein exhibited potent pro-nucleating activity *in vitro*, which depends on intact structure of peptide and sugar chain, and especially bound DSA lectin.

MATERIALS AND METHODS

Materials

Sodium salts of taurocholic(STC) and taurodeoxycholic (STDC) greater than 99 % purity, cholesterol(CH), egg lecithin and Tween 20 were obtained from Fluka Company. Metrizamide, nitrocellulose sheets, and all the chemicals for SDS-PAGE were obtained from Sigma Chemical Co. Datura stramonium agglutinin (DSA), wheat germ agglutinin (WGA), concanavalin A(Con A), peroxidase(HRP), and Sephadex G150 were also from Sigma Chemical Co. Periodic acid($NaIO_4$) was purchased from Wako Pure Chemical. *N*-glycosidase F, endo-α-N-acetyl-galactosaminidase, neuraminidase, and *Pronase K* were purchased from Boehringer Mannheim Corp., Germany, and 0.22 μm micropore filters were obtained from Millipore Corp., Bedford, MA. USA.

Methods

Patients and bile collection All patients gave written informed consent to participate in the study, which was approved by the ethical committee. Gallbladder bile was obtained from three patients by directly puncturing the gallbladder with a sterile 19G needle at cholecystectomy for cholelithiasis. The bile (20 ml) was immediately transported to the laboratory and stored at -80 ℃ until processed.

Protein purification procedure Pooled bile specimens were separated on a molecular sieving chromatography column (BioGel A-5m, 5×100 cm), eluted with 10 mmol/L Tris-HCl buffer to remove soluble mucin glycoprotein. The main fraction was centrifuged at 10 000 rev/min for 10 minutes at room temperature. The upper fraction was filtered through 0.22 μm micropore filters, and metrizamide (13 % w/v) was directly dissolved in the elution and centrifuged at 45 000 rev/min for 3.5 h at 10 ℃ in a Vti-50 vertical rotor (Beckman Instruments Inc., USA). The top opalescent vesicular fraction was collected by tube puncturing and loaded on SDS-PAGE under nonreducing conditions. The 33.5 kDa vesicular protein lane was resected according to the protein marker position and dialyzed in Tris-HCl buffer and concentrated as Ma *et al*[19] described.

SDS-PAGE SDS-PAGE(5-12 %) was developed in a buffer system described by Laemmli[20]. Aliquots (100 μl) of protein and bile samples were resolubilized with a sample buffer (60 mmol/L Tris-HCl, 2 % SDS, 10 % glycerol, pH 6.8). On completion of the electrophoretic run, gels were fixed in a 50 % methanol, 10 % acidic acid solution for 6 h and stained with Coomassie blue.

Preparation of lectin-HRP conjugate The lectin-HRP conjugate of DSA-HRP, WGA-HRP and Con A-HRP was made according to Guo *et al*[21]. Briefly, 5 mg HRP was dissolved in 0.5 ml distilled water, then added with 0.5 ml 60 mmol/L $NaIO_4$ and kept at 4 ℃ for 30 minutes. Five mg lectin such as DSA, WGA and Con A was mixed with HRP and 0.1 mol/L α-methyl mannose for Con A, and N-acetylglucosamine for DSA and WGA was added to protect the glycan binding site of the lectin. The reaction mixture was dialyzed in 50 mmol/L carbonate buffer (pH 9.5) and centrifuged at 4 000 rev/min for 10 minutes. The supernatant was removed and the pellet was dissolved and dialyzed in sodium phosphate buffer (20 mmol/L, pH 7.4).

Lectin affinity staining Five, 10, 15 μg/ml of purified 33.5 kDa vesicular proteins were blotted to nitrocellulose membrane respectively. The membrane was blocked with 1 % BSA overnight at 37 ℃. Subsequent incubation of the membrane with 1:500 peroxidase-labeled Datura stramonium agglutinin (DSA), wheat germ agglutinin (WGA), concanavalin A(Con A) in the same solution was followed by washing three times in the TTBS buffer (0.05 % Tween 20, 0.1 mol/L Tris-HCl, pH7.5) and chemiluminescent detection.

Amino acid analysis The purified 33.5 kDa vesicular protein was hydrolyzed for 16 hours at 115 ℃ in 6 N HCl/0.2 % phenol containing norleucine as an internal standard. After incubation, samples were dried and redissolved in 100 μl of NaS sample dilution buffer (Beckman Instruments Inc., USA) and run on a Beckman model 7300 Amino Acid Analyzer.

Amino acid sequencing The amino-terminal sequences of the 33.5 kDa vesicular protein were subjected to N-terminal amino acid sequencing with an automated sequencer (model 477A: Protein Sequencer, Applied Biosystems). Determined sequences were compared with those well-identified glycoproteins in the Pub-Med NCBI human gene bank database.

Enzymatic deglycosylation The 33.5 kDa vesicular protein was treated with *N*-glycanase enzyme according to supplier's specifications based on the work of Elder and Plummer *et al*[22,23]. Five hundred μg 33.5 kDa vesicular protein boiled for 5 minutes was diluted with 0.1 mmol/L sodium phosphate buffer, pH 8.6, 10 mmol/L 1, 10-phenanthroline, and then mixed with 10 U *N*-glycanase, and the reaction mixture was incubated for 24 h at 37 ℃. The molecular weight of deglycosylated polypeptide backbone was then detected using SDS-PADE.

In the *O*-deglycosylation study, the vesicular protein was diluted with 10 mmol/L calcium acetate, 20 mmol/L sodium cacodylate buffer (pH 7.0) and was incubated with 10 U/ml of neuraminidase for 12 h at 37 ℃. This was followed by further incubation with 2 U/ml of endo-α-N-acetyl-galactosaminidase for 12 h at 37 ℃. Finally, the mixture was examined using SDS-PAGE.

Proteolysis studies One hundred μg of 33.5 kDa vesicular protein was dissolved in 50 μl ammonium bicarbonate (25 mmol/L, pH 11), and then incubated with 1.5 U *Pronase K* for 24 h at 37 ℃. After incubation, the sample was concentrated and loaded on SDS-PAGE.

Cholesterol crystal growth assay Supersaturated model bile was prepared with a cholesterol saturation index of 1.4, a total lipid concentration of 125 g/L, and a bile acid/phospholipid ratio of 4.4. This model bile was made as Busch *et al*[24,25] described. In brief, this lipid mixture was evaporated to dryness, lyophilized, and then resolubilized with 20 mmol/L Tris-HCl/150 mmol/L NaCl (TBS), pH 7.4 at 55 ℃. After filtration (0.22 μm), 25 μl of this model bile mixed with 50 μg protein or its enzymatic samples was diluted with 475 μl TBS/10 mmol/L STDC solution. After 20 minutes, absorbance at a single wavelength within the visible range (700 nm) was sequentially measured. The cholesterol crystal growth curves of the supersaturated model bile without (control) and with (experimental) protein samples were thus generated for each sample. The three growth curve parameters were derived: growth index Ig=maximal slope of experimental curve/maximal slope of control, crystal index Ic=final crystal concentration of experimental/final crystal concentration of control, time index It=onset time of experimental/onset time of control.

Statistical analysis

The cholesterol crystal growth curves were compared by using analysis of variance (ANOVA) at each time to determine whether difference existed between the study groups. When the ANOVA was statistically significant ($P<0.05$), the Dunnett's multiple comparison procedure was made to compare each of the study groups to the control group.

RESULTS

Purification and identification of novel 33.5 kDa glycoprotein

The bile was divided into three fractions after ultracentrifugation (Figure 1). The top opalescent vesicular fraction was collected by tube puncture and the targeted vesicular protein was further separated by SDS-PAGE. The protein profile from three different gallstone patients with Coomassie blue staining is shown in Figure 2. The protein marker is shown at lane 1 and a single band of 33.5 kDa protein at lanes 2-4 on SDS-PAGE was stained under nonreducing condition. Amino acid analysis of the purified glycoprotein showed that the protein was composed of 153 amino acid residues of which almost one third were the following amino acids: glutamine/glutamic acid and asparagines/aspartic acid (Table 1). N-amino-terminal sequencing of the protein showed H_2N-Asp-Asn-Ser-Gln-His-Arg-Tyr-Val-Phe-Ile, which was different from α_1-acid protein, Ig, aminopeptidase N and phospholipase C. Lectin staining showed higher affinity for *Datura stramonium agglutinin* (DSA) than for wheat germ agglutinin (WGA) and concanavalin A(Con A)(Figure 3). *N*- deglycosylation studies showed disappearance of the original 33.5 kDa protein and the presence of a new 21kDa band on SDS-PAGE (Figure 4), indicating

the protein was heavily glycosylated (37.3 %) and the connection mode between polypeptide and carbohydrate components was *N*-linkage. Proteolysis studies showed the protein was sensitive to *Pronase K* digestion.

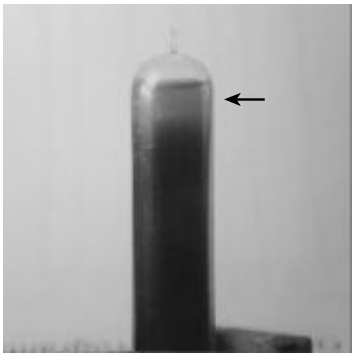

Figure 1 Pretreated bile centrifuged at 45 000 rev/min and divided into three fractions. Horizontal arrows indicate the vesicular phase bile.

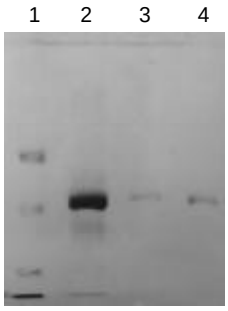

Figure 2 Purified 33.5 kDa vesicular proteins from three different bile samples run on SDS-PAGE. Lane 1: protein marker, Lanes 2-4: the 33.5 kDa vesicular protein.

Table 1 Amino acid composition of 33.5 kDa vesicular protein

Amino acid	nmol/total protein	No. of residues/mol protein
Asp/Asn	6.761	19
Thr	4.488	13
Ser	1.589	5
Glu/Gln	10.434	30
Gly	2.242	6
Ala	2.864	8
Val	2.501	7
Ile	3.226	9
Leu	4.782	14
Tyr	1.937	6
Phe	2.966	8
Lys	4.777	14
His	0.840	2
Arg	2.645	8
Pro	1.411	4
NH_2	11.297	32
Total	64.76	153

Cholesterol crystal growth assay

Figure 5 depicts the promoting effect of 33.5 kDa vesicular protein on cholesterol crystal growth curve at the concentration of 100 μg/ml. The protein strongly promoted cholesterol crystallization, accelerated the onset and increased the total quantity of crystal plates with derived crystal growth curve indices It, Ig, Ic presented as 0.57, 1.52, 1.63 respectively. But no promoting activity was detected in the same supersaturated model bile after incubation with *N*-glycanase enzyme or complete protein degradation (Table 2).

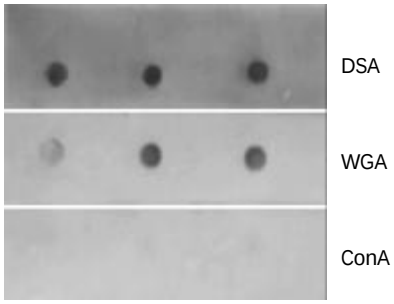

Figure 3 Lectin affinity staining with DSA, WGA, Con A labeled with peroxidase. The 33.5 kDa vesicular protein was strongly connected with DSA, and weakly bound to WGA, but did not react with Con A.

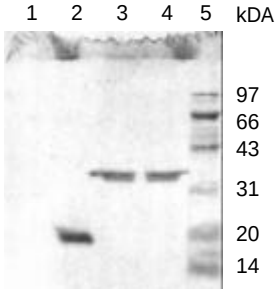

Figure 4 SDS-PAGE (reduced condition) of the 33.5 kDa vesicular protein after *N*-deglycosylation, *O*-deglycosylation and proteolysis. Complete disappearance was observed after incubation with *Pronase K* at lane 1. A single 21 kDa band was stained after treated with *N*-glycanase at lane 2, but no change of the protein after enzymatic *O*-deglycosylation at lane 3. The band of lane 4 and lane 5 represented the 33.5 kDa vesicular protein and protein marker respectively.

Table 2 Effect of 33.5 kDa vesicular protein on activity indices of cholesterol crystallization (100 μg/ml)

	It	Ig	Ic	*P* value
Purified 33.5 kDa protein	0.57	1.52	1.63	<0.05[a]
+ *N*-deglycosylation	1.08	1.01	0.98	<0.05[b]
+ *O*-deglycosylation	0.58	1.61	1.54	<0.05[a]
+ Proteolysis	1.12	0.87	0.99	<0.05[b]

a: compared with control, b: compared with 33.5 kDa vesicular protein group.

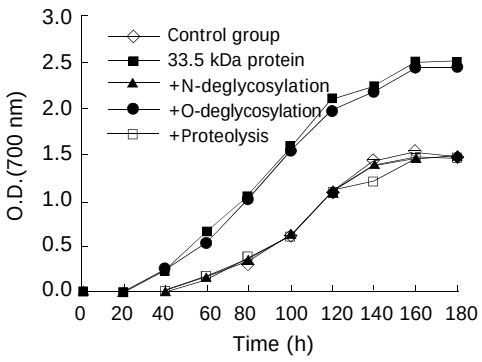

Figure 5 Promoting effect of 33.5 kDa vesicular protein and its enzymatic products on cholesterol crystal growth curves in model bile (TL=125 g/L, BA/PL=4.4, CSI=1.4). All curves are given as the mean ±SD, n=4. P<0.05 *vs* control at each time.

DISCUSSION

Since the first report of the presence of pro-nucleating activity in cholesterol patient' s bile by Burnstein *et al*[26], many groups have tried to purify and identify the active protein-related components[16,17,25,27,28]. Of particular interest are the presence and role of concanavalin A-binding fraction of biliary glycoproteins (CABG), which have a potent cholesterol crystallization-promoting activity. Proteins thought to explain this activity included α_1-acid protein[12], immunoglobulins[9-11], aminopeptidase N[4,6], and a pronase resistant carcinoembryonic antigen-related cell adhesion molecule 1 most recently described by Jirsa *et al*[29], and some unidentified proteins such as 200 kDa pro-nucleating glycoprotein[15]. But still most of the activity has not been identified[30]. In this study we purified and characterized a novel promoting-nucleation glycoprotein with molecular weight of 33.5 kDa in vesicular bile of cholesterol gallstone patients. In 1992, Miquel *et al*[17] isolated and purified human vesicles with potent cholesterol-nucleation-promoting activity, and found that this protein-related activity belonged to immunoglobulins. Although they were from the same vesicular bile, the difference between the immunoglobulin family of glycoprotein and the 33.5 kDa vesicular protein was obvious. We took considerable care to rule out the possibility that the present glycoprotein shared similar features with the immunoglobulins. First, the potent cholesterol-nucleation-promoting vesicular protein had a strong activity of accelerating the onset and increasing the total quantity of crystals appearance and was unique to have a high affinity for *Datura stramonium agglutinin* (DSA), and did not bind to *concanavalin A* (Con A). This was different from the previously described promoting-nucleation glycoprotein. Amino acid sequencing study further demonstrated that the 33.5 kDa vesicular protein with N-amino-terminal sequencing of H_2N-Asp-Asn-Ser-Gln-His-Arg-Tyr-Val-Phe-Ile, was a novel glycoprotein from vesicular bile.

In additional experiments, the 33.5 kDa vesicular protein could not only accelerate onset of nucleation, but also induce rapid cholesterol crystallization growth. We speculate the factor identified in this study may play an important role in the initial stage of the gallstone formation. To study the underlying mechanism and pathophysiological significance of the peptide and carbohydrate moiety, the 33.5 kDa vesicular protein was treated with glycanase enzyme and p*ronase* respectively. Incubation with *N*-glycanase resulted in disappearance of the original 33.5-kilodalton band and presence of a strong 21-kilodalton band on SDS-PAGE, and no cholesterol crystallization promoting activity of 33.5 kDa vesicular protein was detected in supersaturated model bile. It suggested that the sugar chain might be responsible for the promoting-nucleation activity. This striking characteristic of the vesicular protein was very similar to α_1-acid protein. Abei *et al*[12] reported that α_1-acid protein was 37 % glycosylated with mannose, sialic acid content, and some other multiple antennae and the carbohydrate moiety were essential to the promoting activity of glycoprotein. In addition, vesicular glycoprotein was completely degradated and no promoting activity existed after proteolytic digestion.

In conclusion, our results indicate that, the 33.5 kDa vesicular protein with complicated glycan and high affinity for DSA, is a novel and unique pro-nucleating glycoprotein, which exhibits potent cholesterol crystallization promoting activity *in vitro*. However, further studies are needed to evaluate the predictive value, concentration, relative potency and origin of the 33.5 kDa vesicular protein before we can ascertain its specific role in the pathogenesis of cholesterol gallstone disease.

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