**RESULTS:** The protein component, the type of linkage between glycan was an extensively glycosylated (37.3%) monomer polypeptide and carbohydrate components. Using two different enzymatic approaches (HRP-DSA, HRP-ConA, HRP-WGA) and was deglycosylated 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.
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 °C until processed.

Protein purification procedure Pooled bile specimens were separated on a molecular sieving chromatography column (BioGel A-5m, 5x100 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 microace 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 °C 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[9] described.

SDS-PAGE SDS-PAGE(5-12 %) was developed in a buffer system described by Laemmli[10]. Aliquots (100 µl) of protein and bile samples were resolubilized with a sample buffer (60 mmol/L Tris-HCl, 2 % SDS, 10 % glycero, 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-HP conjugate The lectin-HP 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 conjugate of DSA-HRP, WGA-HRP and Con A-HRP was respectively. The membrane was blocked with 1 % BSA overnight at 37 °C. The completion of the electrophoretic run, gels were fixed in a 50 % methanol, 10 % acidic acid solution for 6 h and stained with Coomassie blue.

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 °C. 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 °C in 6 N HCl/0.2 % phenol containing noreulcein 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, pH8.6, 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 °C. The molecular weight of deglycosylated polypeptide backbone was then detected using SDS-PAGE.

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 °C. This was followed by further incubation with 2 U/ml of endo-α-N-acetyl-galactosaminidase for 12 h at 37 °C. 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 °C. 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 °C. 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,N-Asp-Asn-Ser-Gln-His-Arg-Tyr-Val-Phe-Ile, which was different from α3-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 | mmol/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                          |
| NH₂        | 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   |
|----------------------|------|------|------|
| Purified 33.5 kDa protein | 0.57 | 1.52 | 1.63 |
| + N-deglycosylation   | 1.08 | 1.01 | 0.98 |
| + O-deglycosylation   | 0.58 | 1.61 | 1.54 |
| + Proteolysis         | 1.12 | 0.87 | 0.99 |

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, CS=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.[9], 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 α1-acid protein[12], immunoglobulinF, aminopeptidase N[4,6], and a pronase resistant carcinoembryonic antigen-related cell adhesion molecule 1 most recently described by Jirsa et al.[8], and some unidentified proteins such as 200 kDa pro-nucleating glycoprotein[13]. But still most of the activity has not been identified[8]. In this study we purified and characterized a novel promoting-nucleating 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 vesicle 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-aminoterminal sequencing of H-N-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 pronase 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 α1-acid protein. Abei et al[29] reported that α1-acid protein was 37 % glycosylated with mannos, 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 degraded 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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