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Cholesterol crystallization-promoting activity of aminopeptidase-N isolated from the vesicular carrier of biliary lipids

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Different hydrophobic glycoproteins are associated to native biliary vesicles, which are the major carrier of biliary cholesterol. Some of these proteins promote cholesterol crystallization, a key step in cholesterol gallstone formation. This study was specifically conducted to identify the 130 kDa biliary vesicle-associated glycoprotein and to determine its in vitro effect on the cholesterol crystal formation time. The 130 kDa vesicular glycoprotein was identified as aminopeptidase-N by amino acid sequencing and specific enzymatic assay. Polyclonal antibodies raised against aminopeptidase-N allowed us to determine its concentration in human hepatic bile, which varied from 17.3 to 57.6 μg/ml. Aminopeptidase-N showed a concentration-dependent cholesterol crystallization activity when it was added to supersaturated model bile at a concentration range usually found in native bile. Because of this promoting effect on in vitro cholesterol crystal formation, we suggest that biliary aminopeptidase-N may play a critical role in the pathogenesis of cholesterol gallstone disease.

Biliary vesicle; Aminopeptidase-N; Cholesterol crystallization; Cholesterol gallstone formation

1. INTRODUCTION

Cholesterol crystallization from supersaturated human bile is a key step in the pathogenesis of cholesterol gallstone disease [1,2]. Recently, different biliary proteins have been described with cholesterol nucleation influencing activity. Mucin [3], immunoglobulins [4] and other small molecular weight glycoproteins, such as a 130 kDa glycoprotein [5], presumably aminopeptidase-N [6], fibronectin [7], phospholipase C [8] and α-1-acid glycoprotein [9], have been reported as pronucleating biliary proteins. However, the real pathophysiological significance of these cholesterol pronucleating factors still remains to be defined.

Nowadays, there is general agreement that cholesterol crystal formation occurs from biliary vesicles [10–12], the major carrier of biliary cholesterol [13–16]. Cholesterol crystals probably nucleate as a consequence of aggregation and fusion of these cholesterol-rich vesicles [17,18]. Thus, nucleation promoting factors must presumably be interacting with the vesicular carriers to induce biliary cholesterol crystallization.

To elucidate the major protein factors involved in accelerated cholesterol nucleation time of bile from patients with gallstones, we have been interested in studying the association of putative pronucleating proteins with native biliary vesicles. First, we isolated and purified native biliary vesicles from cholesterol gallstone patients [19]. These vesicles were mainly composed of cholesterol and phospholipids, but also contained a characteristic and well-defined protein profile [19,20], including some biliary-specific hydrophobic glycoproteins (130, 114, 86 and 62–67 kDa molecular weights) [20]. In addition, we demonstrated that lipid solutions containing the vesicle-associated proteins have in vitro cholesterol pronucleating activity [19], which was dependent of protein concentration, but not related to lipid composition. Furthermore, we have shown that the 130, 114, 62–67 kDa vesicular glycoproteins significantly decrease the cholesterol nucleation time of artificial model bile [20].

The aims of this study were to isolate and identify the vesicle-associated 130 kDa glycoprotein, to quantitate its concentration in human hepatic bile and to test its in vitro cholesterol pronucleating activity at concentrations usually found in native bile.

2. MATERIALS AND METHODS

2.1. *Bile sampling*

Hepatic bile was obtained from cholesterol gallstone patients through indwelling T-tubes after cholescystectomy and choledocotomy. Bile samples were collected in sterile tubes containing 0.05% chloramphenicol, 3 mM sodium azide, 0.2 mM thymerosal, 1 mM PMSF and 1 mM leupeptin as preservatives. Bile was immediately processed for biliary vesicle isolation.

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2.2. *Isolation and purification of biliary vesicles*

Firstly, bile samples werer centrifuged at 10,000 × *g* for 10 min and filtered through 1-μm filters. Then, 16% metrizamide (Sigma Chemicals Co. St. Louis, MO, USA) was directly dissolved in bile and bile–metrizamide mixture was ultracentrifuged at 160,000 × *g* for 200 min at 10°C to isolate biliary vesicles [21]. The vesicular fraction in the top of the centrifuge tube was further purified by two sequential gel filtration chromatographic steps, as previously described [19,20].

2.3. *Gel electrophoresis*

Proteins were precipitated with 8% (w/v) trichloroacetic acid from whole bile, micellar fraction and purified vesicles as required and, then, delipidated using ethanol/diethylether (1:3, v/v) prior to electrophoresis. Proteins were resuspended in reducing sample buffer and 9% SDS-PAGE was performed by standard techniques [22]. Gels were stained with Coomassie brilliant blue or silver staining.

2.4. *Isolation and renaturation of the 130 kDa vesicular glycoprotein*

The 130 kDa glycoprotein associated to purified biliary vesicles was isolated by electroelution from preparative polyacrylamide gels. The Coomassie blue-stained 130 kDa protein band was excised from gels. The gel Coomassie blue-stained 130 kDa protein band was excised from gels. The gel slices were transferred to a dialysis bag containing 192 mM glycine and 25 mM Tris-HCl (pH 7.4) and, then, electroelution was performed overnight at 50 mA in a horizontal chamber with 96 mM glycine and 12.5 mM Tris-HCl (pH 7.4). The electroeluted protein was concentrated by ultrafiltration and, finally, lyophilized. The purification of the 130 kDa vesicular glycoprotein was evaluated by re-electrophoresis as described above.

The 130 kDa protein was renaturated after preparative SDS-PAGE, as previously described [23,24], to obtain the protein under native conditions for cholesterol crystallization assays. The region of the polyacrylamide gels containing the 130 kDa polypeptide was excised and eluted by diffusion in elution buffer (0.1% SDS, 0.1 mM EDTA, 5 mM DTT, 150 mM NaCl and 50 mM Tris-HCl, pH 7.4). After overnight elution, the SDS was removed from the protein by acetone precipitation and the precipitated protein was resuspended in 6 M guanidine hydrochloride. Subsequently, removal of guanidine was performed by P-10 Bio-Rad gel filtration as previously reported [24].

2.5. *Amino acid sequencing of the 130 kDa vesicular protein*

The 130 kDa glycoprotein purified from biliary vesicles was re-electrophoresed. Then, it was electroblotted to a polyvinylidine difluoride (PVDF) membrane in appropriate transfer buffer (10% methanol, 10 mM CAPS, pH 10.0). The blotted protein was evidenced by Coomassie blue and the band was cut out for amino acid sequencing. The protein was directly sequenced from the PDVF membrane using an Applied Biosystems gas-phase sequencer equipped with an on-line PTH analyzer. The amino acid sequence of the 130 kDa protein was compared with the Protein Identification Resource National Biomedial Research Foundation (USA) data base.

2.6. *Antibody preparation*

The purified 130 kDa protein was emulsified with complete Freund's adjuvant and 50 μg was injected in the popliteal lymph node of white females New Zealand rabbits. Intradermal booster doses (100 μg) were administered weekly for three times. Blood samples from the marginal ear vein were taken to detect the production of specific antibodies by immunoblotting [25].

2.7. *Quantitative immunoblotting*

The concentration of the 130 kDa glycoprotein was measured by quantitative immunoblotting in hepatic bile. Five microliters of different hepatic bile samples were processed in duplicate for reducing SDS-PAGE [22] and immunoblotting [25]. The rabbit anti-130 kDa protein polyclonal antibody was used as the primary antibody. Adsorbed immunoglobulins were detected by using Protein A-coupled alkaline phosphatase and the color reaction was developed with 3-bromo-4-chloro-5-indolyl-phosphate and nitroblue tetrazolium as substrates. Quantitation was performed by densitometry (Shimadzu, Tokyo, Japan) of immunoblotted bile samples in comparison with previously known amounts of purified 130 kDa vesicular glycoprotein as a standard. The densitometric measurements of these standards gave a linear correlation curve within the protein concentrations employed in the procedure and all native bile samples were within this range.

2.8. *Cholesterol observation time assays*

Crystal observation time (the time interval between a bile sample was cleared of crystals and the first detection of typical cholesterol crystals) of native hepatic bile was performed by polarizing microscopy. The effect of purified and renaturated 130 kDa vesicular glycoprotein on cholesterol crystal formation of supersaturated model bile was determined by the method described by Groen et al. [5]. After renaturation, the 130 kDa protein was filtered through 0.22-μm filters and aliquots were added to artificial bile to obtain an increasing protein concentration in the assays. Cholesterol nucleation assays were performed in triplicate for each protein concentration. The time of crystal appearance was observed as described above. In all studies, control assays were performed by adding the same volume aliquots of the renaturating solution, without protein, to model bile. These control experiments did not show significant modifications in the cholesterol crystal appearance time of the original model bile.

2.9. *Chemical analysis*

Cholesterol, phospholipids, bile salts and total protein concentration in hepatic bile was determined as previously described [21,26]. The cholesterol saturation index was calculated using Carey's critical tables [27]. The renaturation of the vesicle-associated aminopeptidase-N was tested by assaying its enzymatic activity [28].

2.10 *Statistical analysis*

The correlation coefficients and the statistical significance of the correlations were performed on a Macintosh computer, using SYSTAT analytic software. P values < 0.05 were considered significant.

3. RESULTS

Hepatic bile was obtained from patients with cholesterol gallstones. The lipid and protein composition of these bile samples are presented in Table I. Each sample was separately processed to obtain purified 130 kDa vesicular glycoprotein.

Firstly, isolated and purified biliary vesicles were prepared by ultracentrifugation and, subsequently, gel filtration chromatography, as previously described [19–

Table I

Lipid and protein composition, cholesterol saturation and nucleation time of human hepatic bile from cholesterol gallstone patients

	Hepatic bile
Biliary lipid concentrations (mM)	
Bile salts	21 ± 5.7
Phospholipids	4.9 ± 0.7
Cholesterol	2.3 ± 0.8
Cholesterol saturation (%)	199 ± 59
Cholesterol nucleation time (days)	12 ± 5
Total protein concentration (mg/ml)	5 ± 1.8
Aminopeptidase-N concentration (mg/ml)	0.04 ± 0.01

Bile was obtained from six patients with T-tube bile fistula, one week after surgery. Values represent the mean ± 1 S.D.

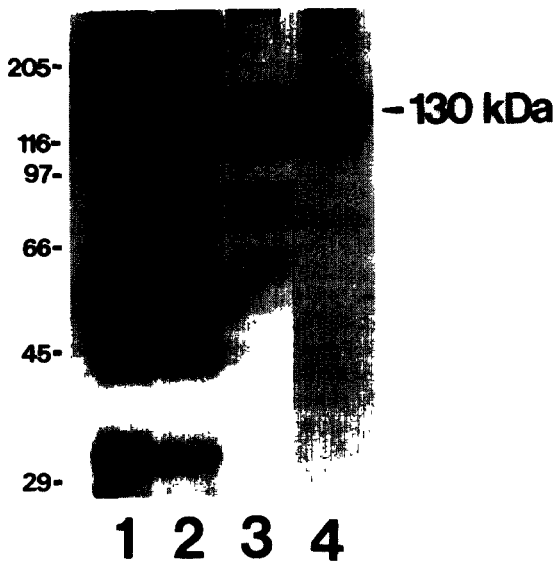

Fig. 1. SDS-polyacrylamide gel electrophoresis of native bile, micellar and vesicular fractions and purified 130 kDa vesicular glycoprotein. Lane 1, whole native bile; lane 2, biliary micellar fraction; lane 3, purified biliary vesicles; lane 4, purified 130 kDa vesicular glycoprotein. 50 μg of total protein were applied to each lane. Protein molecular weight markers are indicated at the left margin.

21]. The major composition of purified biliary vesicles were cholesterol and phospholipids (molar ratio of 1.1) (result not shown). A minor (0.8 ± 0.2, w/w), but constant, protein component was always detected in the purified vesicular fraction, which was further employed for isolation of the vesicle-associated 130 kDa glycoprotein.

The protein composition of the purified biliary vesicles was analyzed by SDS-PAGE, as shown in Fig. 1. As previously described [19,20], biliary vesicles (lane 3) had a characteristic protein profile composed of 130, 114, 86, 62–67 and 52 kDa polypeptides, which is clearly distinctive from the protein pattern of whole bile and the micellar fraction (lane 1 and lane 2, respectively).

The 130 kDa vesicular polypeptide was isolated by electroelution of the protein band excised from polyacrylamide gels. The purification of the electroeluted 130 kDa glycoprotein was evaluated by re-electrophoresis and silver staining (lane 4). After the isolation procedure, the 130 kDa protein was qualitatively the only band detected, without presence of the others vesicular proteins.

The identification of the 130 kDa vesicular glycoprotein isolated from purified biliary vesicles was performed by direct amino acid sequencing and protein data bank searching. As shown in Fig. 2, there was a complete identity between the 130 kDa vesicular glycoprotein sequence and the previously reported amino acid sequence of the human intestinal aminopeptidase-N [29]. The determined sequence corresponds to the cytoplasmic region and the transmembrane hydrophobic domain of this plasma membrane-associated ectoenzyme [30].

To measure the biliary concentration of aminopeptidase-N by quantitative immunoblotting, rabbit polyclonal antibodies were prepared against the 130 kDa glycoprotein isolated from biliary vesicles. The immune serum exclusively recognized its specific antigen in Western blot assays of purified biliary vesicles, without a positive reaction with the other vesicle-associated proteins (result not shown). In five human hepatic biles, the mean concentration of aminopeptidase-N was 40 μg/ml (range from 17.3–57.6 μg/ml). This amount represents about 0.8% of the total biliary protein concentration (Table I). These measurements of biliary aminopeptidase-N were further considered to design the appropriate experiments to analyze the effect of this protein on in vitro cholesterol nucleation time assays.

Prior to the cholesterol nucleation time experiments, purified biliary aminopeptidase-N was obtained in native conditions by purification and renaturation procedures of the 130 kDa glycoprotein isolated from SDS-PAGE of biliary vesicles. Renaturation of the purified aminopeptidase-N was demonstrated by testing the presence of its specific enzymatic activity. Aminopeptidase-N specific activity was 2.47 mU/μg under renatured conditions after purification from biliary vesicles.

Finally, the putative cholesterol crystallization-promotin activity of the vesicle-associated aminopeptidase-N was assessed by measuring its effect on the cholesterol crystal observation time of artificial model bile. Renatured aminopeptidase-N was added to supersaturated model bile at increasing protein concentrations (from 1 to 20 μg/ml) in the assays. As represented in Fig. 3, biliary aminopeptidase-N showed a concentration-dependent promoting effect on the cholesterol crystal observation time. The nucleation time was significantly correlated ($r = -0.74$, $P < 0.001$) in a reciprocal manner with the concentration of aminopeptidase-N used in these experiments. Because the low yield of the purification procedure to obtain sufficient amount of biliary aminopeptidase for nucleation time assays, we used

Biliary vesicle
130 kDa glycoprotein

AKGFYIS**KSLGILGILL**

MAKGFYIS**KSLGILGILLGVAAVCTIIALSVVYS**
10 20 30

Human intestinal
aminopeptidase-N

Fig. 2. Amino acid sequence of the amino-terminus of the biliary vesicle-associated 130 kDa glycoprotein. Amino acid sequencing was performed directly from the 130 kDa vesicular glycoprotein electroblotted to a PVDF membrane. For comparison, the amino acid sequence of the human intestinal aminopeptidase-N is also shown [29].

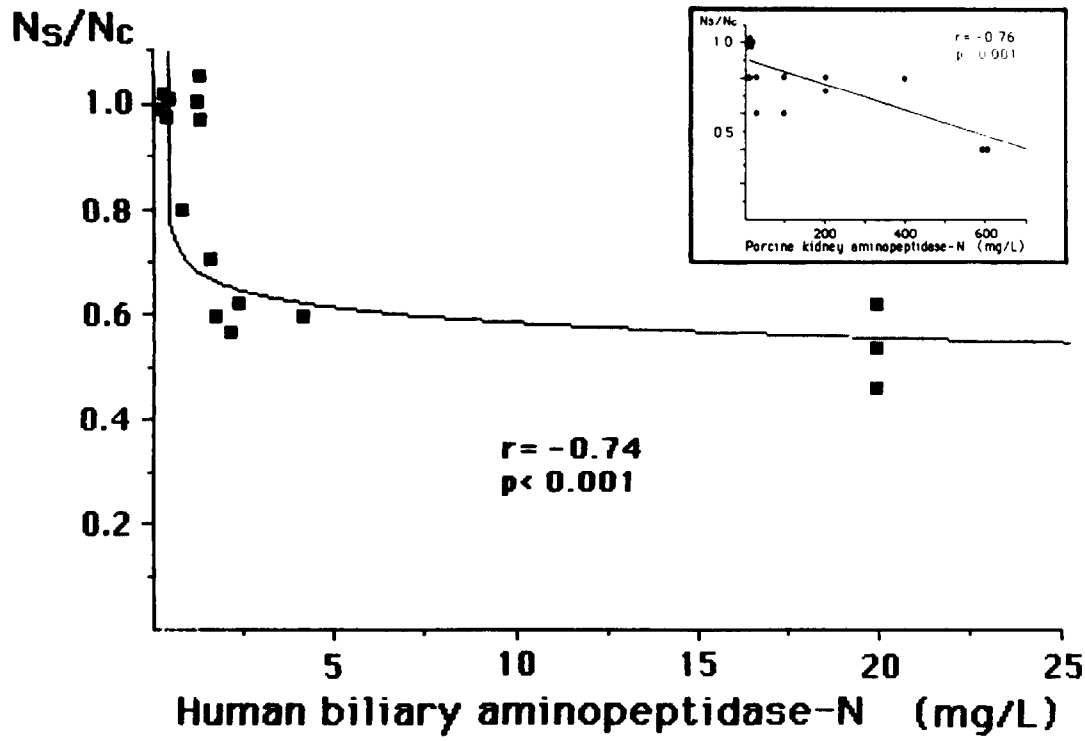

Fig. 3. Effect of aminopeptidase-N on the cholesterol crystal observation time of supersaturated model bile. Renatured biliary aminopeptidase-N and porcine kidney aminopeptidase-N (inset) were added at increasing protein concentrations. The cholesterol crystallization influencing activity is represented on the ordinate by the ratio between the nucleation time (in days) of assays with aminopeptidase-N (Ns) and the nucleation time of the respective control assays (Nc). The nucleation time of the control model biles varied from 9 to 12 days (10 ± 1 days, mean ± 1 S.D.). Then a Ns/Nc ratio less than 0.8 was considered as a significant cholesterol nucleation promoting activity of aminopeptidase-N.

commercial available porcine kidney aminopeptidase-N (Sigma Chemicals Co., St. Louis, MO, USA) to analyze its effect on the cholesterol crystallization time at a concentration range commonly found in hepatic and, presumably, in gallbladder bile. It was assumed that the gallbladder bile concentration of aminopeptidase-N is ten times higher than the concentration measured in hepatic bile. As shown in the inset of Fig. 3, porcine kidney aminopeptidase-N also showed a significant concentration-dependent cholesterol crystallization promoting effect at a range concentration presumably present in human gallbladder bile ($r = -0.76$, $P < 0.001$).

4. DISCUSSION

The present study demonstrates that one of the hydrophobic glycoproteins associated to native biliary vesicles is aminopeptidase-N, which possessed an in vitro concentration-dependent cholesterol crystallization activity.

The 130 kDa vesicular glycoprotein was identified as aminopeptidase-N by amino acid sequencing and specific enzymatic assay. The amino acid sequence analysis revealed that this protein is present in bile as a whole molecule, including its hydrophobic transmembrane domain [29]. This finding is consistent with the evidence that most of the biliary aminopeptidase-N is hydrophobic [28] and with our previous report that the 130 kDa vesicular glycoprotein is partitioned into the detergent phase during Triton X-114 phase separation assays [20]. These experimental data allow us to postulate that aminopeptidase-N may be associated to biliary vesicles as a transmembrane protein, with its hydrophobic region (from Leu-11 to Val-32) inserted into the lipid bilayer core of the cholesterol-phospholipid unilamellar vesicles.

Aminopeptidase-N has been localized in the canalicular plasma membrane domain of the hepatocyte [31,32]. Although it has been suggested that canalicular ectoenzymes could be secreted into bile by a direct detergent effect of bile salts [33], the cellular mechanisms involved in the secretion of biliary aminopeptidase-N remain to be elucidated.

The major finding of this study was that aminopeptidase-N promoted in vitro cholesterol crystallization in a dose-dependent manner when it was added to artificial supersaturated model bile, using a concentration range present in human hepatic bile and, presumably, in native gallbladder bile. In a preliminary report, Offner et al. have also shown that biliary aminopeptidase-N possessed a cholesterol pronucleating effect [6]. We think

that aminopeptidase-N could represent the well-characterized, but still unidentified, potent cholesterol nucleation-promoting protein previously described by Groen et al. [5]. Further immunological and protein analysis of the biliary 130 kDa concanavalin A binding protein are required to examine this possibility.

The molecular mechanisms involved in the cholesterol crystallization promoting activity of aminopeptidase-N are presently unknown. In artificial cholesterol-rich phosphatidylcholine vesicles, it has been shown that cholesterol phase-separated domains reduce repulsive hydration and steric forces between vesicles and generate discontinuity and point defects in the lipid membrane inducing vesicle aggregation and fusion, respectively [34,35]. It can be hypothesized that the hydrophobic domain, inserted in the lipid bilayer of biliary vesicles, may play a critical role in the cholesterol pronucleating effect of biliary aminopeptidase-N. This hydrophobic amino acid stretch could be interacting with either cholesterol or phospholipids of the vesicular membrane providing an appropriate local environment for cholesterol phase separation within the plane of the lipid bilayer, leading to crystal formation and growth.

The demonstration of in vitro pronucleating activity of biliary aminopeptidase at concentrations usually found in native bile and the quantitation of aminopeptidase-N in abnormal bile from cholesterol gallstone patients represent two initial key steps in the elucidation of the putative role of aminopeptidase-N in the pathogenesis of cholesterol gallstones. In a preliminary study, we have also shown that there is a significant reciprocal correlation between the concentration of biliary aminopeptidase-N and the cholesterol crystal observation time in native gallbladder bile of cholesterol gallstone patients [36]. This suggestive result represents another necessary, but not sufficient, criteria for supporting a definitive role aminopeptidase-N in the pathogenesis of cholesterol gallstones. The next steps are to look for quantitative or qualitative differences of biliary aminopeptidase-N between patients with and without gallstones and to define the relative relevance of this cholesterol pronucleating glycoprotein as compared with other biliary pronucleating factors. Thus, further investigations are required to determine the significance of the biliary aminopeptidase-N in the pathophysiology of cholesterol gallstone disease.

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