Interaction of cholesterol-crystallization-promoting proteins with vesicles

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In this study, the interaction of mucin and concanavalin A-binding proteins isolated from human bile with cholesterol/phospholipid vesicles was investigated. Using resonance energy transfer assays originally developed by Struck, Hoekstra and Pagano [(1981) *Biochemistry* 20, 4093–4099], no significant protein-induced fusion or aggregation of vesicles was demonstrated. Instead of fusion, these proteins induced destabilization of cholesterol/phospholipid vesicles, as monitored by release of

entrapped carboxyfluorescein. A good correlation ($\rho = 0.81$) was obtained between the extent of leakage and the nucleation-promoting activity of the concanavalin A-binding proteins. We conclude that aggregation or fusion of cholesterol/phospholipid vesicles is not an obligatory step in cholesterol crystallization. Biliary protein-induced crystallization seems to be preceded by vesicle disruption.

INTRODUCTION

Cholesterol crystallization in bile is the result of a complex pathway involving many steps. At the concentrations of bile acid, phosphatidylcholine and cholesterol found in most human biles, cholesterol will be carried by different carriers [1–3]. According to the phase diagrams [3,4], these carriers are mixed micelles, unilamellar cholesterol/phospholipid vesicles and lamellar structures consisting of stacked bilayers of cholesterol and phosphatidylcholine, although the existence of the latter carrier has recently been challenged [5]. A number of studies suggest that vesicles are the main precursor for cholesterol crystal formation [6–8]. The stability of these vesicles can probably be modulated by proteins. Lee and Smith [9] have shown that gallbladder mucin promotes crystallization of cholesterol from isolated cholesterol/phosphatidylcholine vesicles. We have shown that the protein fraction that binds to concanavalin A (Con A) exerts the same effect [10]. The mechanism by which these proteins act is still unknown. Halpern et al. [11,12] have suggested that vesicle aggregation and fusion precede cholesterol crystal formation in human bile. As both mucin and Con A-binding glycoprotein (CABG) act on isolated vesicles, one could predict that these pronucleators induce vesicle aggregation and fusion. In the present study this hypothesis was tested using established assays for vesicle aggregation and fusion.

MATERIALS AND METHODS

Materials

N-(7-Nitro-1,2,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine Rhodamine B sulphonyl)phosphatidylethanolamine (Rh-PE) were obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Carboxyfluorescein was purchased from Eastman Kodak (Rochester, NY, U.S.A.). Con A-Sepharose and Sephadex G-25 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Egg yolk phosphatidylcholine, cholesterol and methyl α -D-mannopyranoside were from Sigma (St. Louis, MO, U.S.A.). Taurocholate was purchased from Fluka (Buchs, Switzerland). Albumin was obtained from Boehringer (Mannheim, Germany).

Protein isolation

Human gallbladder mucin was isolated as described by Smith and Lamont [13]. CABG was isolated from T-tube bile derived from cholesterol gallstone patients as described by Groen et al. [10]. Protein preparations were dialysed against 50 mM Hepes buffer (pH 7.4) containing 100 mM NaCl and 0.02 % NaN_3 .

Resonance energy transfer assays

Aggregation

Vesicle aggregation and fusion were determined essentially as described by Düzgünes et al. [14]. NBD-PE or Rh-PE was incorporated at a concentration of 1 mol % in separate vesicle preparations by mixing the lipids in chloroform with equimolar concentrations of phosphatidylcholine and cholesterol. After the lipids had been dried in a rotary evaporator, the lipid film was lyophilized to remove all residual organic solvent. The dried lipid film was hydrated in 50 mM Hepes/100 mM NaCl/0.1 % NaN_3 (pH 7.4) and sonicated for 30 min at 70 W in a Branson B12 sonicator on ice under a stream of ultrapure nitrogen. The two vesicle preparations were mixed (1:1, v/v) with either CABG or mucin (1–2 mg/ml) and incubated under nitrogen at 37 °C. The final phosphatidylcholine and cholesterol concentrations in the incubation were both 2 mM. At certain time points a portion of the mixture was diluted in 50 mM Hepes/100 mM NaCl/0.1 % NaN_3 (pH 7.4). Resonance energy transfer was monitored by exciting NBD at 450 nm and measuring emission at 530 nm using a Perkin–Elmer 3000 fluorimeter spectrometer (Gouda, The Netherlands). Data were expressed as percentage decrease in NBD fluorescence and normalized to the NBD fluorescence in the control incubation without added protein at $t = 0$.

Fusion

NBD-PE and Rh-PE were incorporated at a concentration of 2 mol % each in cholesterol/phosphatidylcholine vesicles as described above. Lipid mixing was monitored by incubation of the CABG or mucin (1–2 mg/ml) with labelled vesicles and a 20-fold excess of unlabelled vesicles. The final concentration of phosphatidylcholine and cholesterol in the incubation was 2 mM each. Lipid mixing was expressed as percentage of the NBD

signal compared with maximum fluorescence (100%). To obtain the value for maximum fluorescence, a sample of the labelled vesicles was mixed with a 20-fold excess of unlabelled vesicles and sonicated. Resonance energy transfer was monitored as described above.

Carboxyfluorescein-leakage assay

Carboxyfluorescein-loaded small unilamellar vesicles were prepared as follows. Solutions of phosphatidylcholine in chloroform/methanol (10:1, v/v) alone or together with cholesterol in equimolar concentrations were prepared. Organic solvent was evaporated by using a rotary evaporator, followed by lyophilization. The dried lipid film was hydrated in 200 mM carboxyfluorescein dissolved in 50 mM Hepes/100 mM NaCl/0.1% NaN₃ (pH 7.4). Carboxyfluorescein was purified as described by Ralston et al. [15]. The lipid mixture was sonicated on ice and under nitrogen for 15 min in a Branson B12 sonicator with an output of 70 W. Subsequently the preparation was centrifuged for 1 h at 10000 *g* and the pellet was discarded. Vesicles were separated from free carboxyfluorescein by fractionation on a Sephadex G-25 column. The vesicles were eluted in the void volume of the column. Vesicle preparations with a background fluorescence of less than 5% of the maximum were used in the experiments. The vesicles were incubated at 37 °C with either CABG or mucin (1–2 mg/ml) at final phosphatidylcholine and cholesterol concentrations of 2 mM each. Vesicle leakage was quantified by dilution of a portion of the mixture in 50 mM Hepes/100 mM NaCl/0.1% NaN₃ (pH 7.4) and estimating fluorescence (excitation 492 nm, emission 516 nm). Maximum fluorescence was obtained after the addition of Triton X-100 at a final concentration of 0.1%. The experiments were carried out in duplicate. Variation between the duplicates was generally less than 3%.

Determination of nucleation-promoting activity

Model bile was prepared as described by Kibe et al. [16] with slight modifications. Taurocholate in methanol/water (7:3 v/v) was mixed with phosphatidylcholine and cholesterol in chloroform. The mixture was flushed with nitrogen at 50 °C to remove organic solvent and subsequently lyophilized. The dry lipid film was solubilized in 50 mM Hepes/100 mM NaCl/0.02% NaN₃ buffer (pH 7.4) and incubated for 24 h at 56 °C. The final concentrations of biliary lipids were: taurocholate 130 mM, cholesterol 13 mM, phosphatidylcholine 30 mM. Nucleation-modulating activity was measured as described previously [17]. Briefly, model bile was mixed 1:1 (v/v) with CABG or mucin and the nucleation time of the mixture was compared with the nucleation time of model bile mixed 1:1 (v/v) with 50 mM Hepes/100 mM NaCl/0.02% NaN₃ buffer (pH 7.4). Each day a 10 µl portion of the sterile mixtures was screened microscopically at a magnification of 100–400×. The time interval to the first appearance of the typical birefringent cholesterol crystal was called nucleation time [18]. The nucleation time of the control varied from 6 to 8 days when different batches of model bile were used. Cholesterol and phosphatidylcholine concentrations were determined using standard enzymic procedures [19,20].

Statistical analysis

The correlation between nucleation-promoting activity and induction of carboxyfluorescein leakage by CABG was analysed by the non-parametric Spearman's rank correlation test.

RESULTS

To investigate the potency of CABG and mucin to induce vesicle aggregation, cholesterol/phospholipid vesicles were incubated with the protein fractions in the physiological concentration range 1–2 mg/ml, and the change in NBD fluorescence at 530 nm was monitored. In control incubations either no protein or 1 mg/ml albumin was added to monitor potential aspecific absorption. No difference between the incubation with albumin and the incubation without added protein was observed (results not shown). Figure 1 shows the results of a typical experiment in which CABG from three different patients and purified gallbladder mucin were tested. NBD-derived fluorescence decreased in all incubations. The magnitude of resonance energy transfer was similar in all incubations, including the controls. No significant biliary protein-induced aggregation was observed. Similar results were obtained with CABG from eight other patients and three batches of gallbladder mucin. Compared with earlier studies using these assays, we used relatively long incubation times. Although diffusion of NBD-PE and rhodamine-

Figure 1 Effect of mucin and CABG on resonance energy transfer between NBD-PE- and Rh-PE-labelled vesicles

Mucin and CABG (1–2 mg/ml) were mixed in a 1:1 ratio with NBD-PE (1 mol%)- and Rh-PE (10 mol%)-labelled vesicles. At each time point indicated a portion of the mixture was diluted and fluorescence at 530 nm was determined. Data are given as means of duplicate incubations. Variation in the readings of duplicate samples was less than 5%. Data are expressed as decrease in NBD fluorescence compared with the start of the assay. ●, CABG 1 (1.2 mg/ml); ○, CABG 2 (1.4 mg/ml); ▲, CABG 3 (1.8 mg/ml); ■, mucin (1 mg/ml); ◆, control albumin (1 mg/ml).

Figure 2 Effect of mucin and CABG on lipid mixing

Mucin and CABG (1–2 mg/ml) were incubated with 2 mol% NBD-PE/Rh-PE-labelled vesicles in the presence of a 20-fold excess of unlabelled vesicles. At each time point indicated a sample of the mixture was diluted and fluorescence at 530 nm determined. Maximum fluorescence (100%) was obtained with vesicles containing both NBD-PE and Rh-PE (1 mol% each). ●, CABG 1 (1.2 mg/ml); ○, CABG 2 (1.4 mg/ml); ▲, CABG 3 (1.8 mg/ml); ■, mucin (1 mg/ml); ◆, control albumin (1 mg/ml). Values are means of duplicate determinations.

Figure 3 Effect of mucin and CABG on integrity of phospholipid/cholesterol vesicles

Mucin, CABG (1–2 mg/ml) and albumin (1 mg/ml) were incubated with carboxyfluorescein-loaded cholesterol/phospholipid vesicles. At each time point indicated a sample of the mixture was diluted and fluorescence at 516 nm was measured. Leakage was expressed as percentage of maximum fluorescence (100%) obtained after addition of 0.1% Triton X-100. ●, CABG 1 (1.2 mg/ml); ○, CABG 2 (1.4 mg/ml); ▲, CABG 3 (1.8 mg/ml); ■, mucin (1 mg/ml); ◆, control albumin (1 mg/ml); +, buffer.

PE through the aqueous phase has been shown to be low [14], it could induce artifacts. Therefore we also investigated aggregation by studying the effect of the proteins on vesicle-induced light scattering. The extent of light scattering increased, indicating that aggregation or fusion had indeed occurred. However, similarly to the results shown in Figure 1, no difference between CABG, mucin and controls was observed (results not shown).

A disadvantage of both the resonance energy transfer assay and the light-scattering experiments was that no distinction could be made between vesicle aggregation and fusion. Therefore the experiments were repeated using vesicles containing both NBD-PE and Rhodamine-PE and a large excess of unlabelled vesicles. In this set-up a decrease in resonance energy transfer indicates mixing of lipid, suggesting that a fusion event has occurred [14]. Neither mucin nor CABG induced lipid mixing above control values, indicating that no extra vesicle fusion had occurred (Figure 2). As both promoting factors have been shown to induce nucleation from isolated vesicle fractions, some reactivity of these factors with the vesicles was expected. Therefore effects of the protein on vesicle integrity were investigated. Cholesterol/phospholipid vesicles were loaded with carboxy-

Figure 4 Dose-response effect of mucin on carboxyfluorescein leakage from cholesterol/phospholipid vesicles

Mucin was incubated with cholesterol/phospholipid vesicles at the concentrations indicated. The extent of carboxyfluorescein leakage was determined at $t = 20, 40, 70$ and 110 h and corrected for leakage in the control incubation. Data are given for leakage at 40 h but similar curves were obtained for the other time points.

Figure 5 Effect of mucin and CABG on vesicle integrity

Mucin and CABG (1–2 mg/ml) were incubated with carboxyfluorescein-loaded phospholipid vesicles as indicated in the legend to Figure 3. The final concentration of lecithin was 2 mM. ●, CABG 1 (1.2 mg/ml); ○, CABG 2 (1.4 mg/ml); ▲, CABG 3 (1.8 mg/ml); ■, mucin (1 mg/ml); ◆, control albumin (1 mg/ml).

fluorescein at a self-quenching concentration of 200 mM. These loaded vesicles were incubated with the protein fractions and the release of fluorescence as a measure of vesicle integrity was monitored. Figure 3 depicts the results for three different CABG fractions, albumin and mucin. Mucin and all the CABG fractions induced vesicle leakage, but albumin seemed to stabilize the vesicles somewhat. Mucin had the strongest vesicle-destabilizing effect. Almost all the protein fractions showed hyperbolic kinetics, but 100% vesicle leakage was never reached. Similar results were obtained with 15 other CABG fractions and three batches of mucin.

Figure 4 shows a dose-response curve for biliary mucin. A non-linear relation was observed. At low mucin concentrations the extent of carboxyfluorescein leakage depended almost linearly on the amount of protein. In the higher concentration range the amount of vesicles probably determined the rate of carboxyfluorescein leakage as well.

To test whether proteins were also capable of inducing vesicle leakage in the absence of cholesterol, the experiments were repeated with vesicles composed of phosphatidylcholine only. As can be seen in Figure 5, such vesicles are more labile, as monitored

Figure 6 Correlation between vesicle leakage and nucleation-promoting activity of the CABGs

CABG was isolated from bile of 15 different patients. Nucleation-promoting activity was determined by mixing the protein fractions with model bile as described in the Materials and methods section. Nucleation time was defined as the time of appearance of the first microscopically visible cholesterol crystal. The nucleation time of the control incubation (no protein) was 6.5 days ($n = 4$). The effect of the different CABGs on carboxyfluorescein leakage was determined as described in the Materials and methods section. Data are given for leakage at 40 h; values were corrected for leakage in the control incubations (5%).

by the relatively high carboxyfluorescein release in the control incubation. The proteins are also capable of destabilizing these vesicles further, indicating that the interaction with cholesterol is not the primary event in vesicle permeabilization. To investigate whether the effect of CABG on vesicle leakage correlated with nucleation-promoting activity, the effect of the proteins on the nucleation time of a model bile was determined in parallel. Figure 6 shows that for the 15 different CABG fractions tested, correlation between nucleation-promoting activity and vesicle leakage was significant ($\rho = 0.81$, $P < 0.05$).

DISCUSSION

It is now generally accepted that proteins play an important role in cholesterol crystallization in human bile. Little is known about the molecular mechanism of protein-induced cholesterol nucleation. Halpern et al. [11] concluded from video-enhanced microscopy studies with human bile that vesicle fusion or aggregation precedes cholesterol nucleation. Almost no vesicle aggregation occurred in bile from control patients, suggesting that nucleating factors were responsible for the phenomenon. Although the video-enhanced microscopic pictures of Halpern et al. were very suggestive of vesicle fusion, they did not prove it nor a very close membrane-to-membrane aggregation. In the present study we have attempted to visualize these processes more quantitatively by using established vesicle aggregation/fusion and leakage assays. Although CABG and mucin induce cholesterol nucleation of isolated vesicles, our results suggest that this does not occur via vesicle aggregation or fusion, as no significant change in resonance energy transfer was apparent in the presence of either CABG or mucin. However, some interaction of the proteins with vesicles was evident, as, in another set of experiments, entrapped carboxyfluorescein was released from the vesicles on incubation with the proteins. Formation of cholesterol crystals in human bile is a slow process and the same sluggish kinetics were observed for CABG-induced vesicle leakage. The CABG isolated in this study is a mixture of many proteins [21–23]. In recent years several putative promoting proteins have been isolated from this fraction. One of these is a phospholipase C-like activity [24,25]. Phospholipase C from *Clostridium perfringens* has been shown to induce vesicle fusion [26]. As total CABG does not induce vesicle fusion, the phospholipase C-like activity present in CABG is different or not high enough. Another candidate for the observed effect on vesicle leakage is aminopeptidase N. This nucleation-promoting transmembrane enzyme has recently been shown to be co-isolated with biliary vesicles, indicating tight association [23,27]. The enzyme, which is present in CABG [22,23], could enter the bilayer when incubated with isolated cholesterol/phospholipid vesicles, thereby inducing rearrangement of lipid and possibly vesicle leakage. This scenario can also be applied to other amphipathic proteins with promoting activity such as α_1 -glycoprotein [28] but also to the non-Con A-binding mucin. It has been demonstrated that mucin contains hydrophobic binding sites which bind phospholipid and cholesterol [9]. In addition to these hydrophobic sites, the carbohydrate moiety of mucin plays a role in the interaction with the vesicles. This was suggested by a recent study demonstrating that mucins with different lectin-binding affinity showed significant

variation in induction of vesicle leakage [29]. Possibly, the charge on the carbohydrate chains regulates the interaction of mucin with the vesicles.

We realize that the model system employed in this study does not resemble the actual situation in bile. Therefore our results do not exclude the possibility that vesicle aggregation or fusion may occur in the presence of the high bile-salt concentration prevailing in human bile. However, our results do indicate that vesicle fusion or aggregation is not a mandatory step in cholesterol crystal formation.

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