Quantitative assessment of comparative potencies of cholesterol-crystal-promoting factors: relation to mechanistic characterization

Tomoji NISHIOKA, Susumu TAZUMA¹, Gunji YAMASHITA and Goro KAJIYAMA

First Department of Internal Medicine, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima 734, Japan

The crystallization of cholesterol is affected by various factors in bile. The present study evaluated the relative importance of cholesterol-nucleation-promoting factors and partially characterized the mechanisms of their action. Model bile with an identical relative composition of cholesterol, egg-yolk phosphatidylcholine and taurocholate, except for replacing phosphatidylcholine (5–20%) with dilinoleoyl-phosphatidylcholine or taurocholate (10–30%) with taurodeoxycholate. Cholesterol crystallization was quantitatively assessed spectrophotometrically and morphologically estimated by the laser-scattering diffraction analyser and video-enhanced microscopy in the absence and presence of concanavalin A-binding glycoprotein isolated from human bile. In a series of experiments, lipid distribution among particulate species was determined after isolation by FPLC. In

all experiments, cholesterol crystallization was dose-dependently enhanced with a rank order of: concanavalin A-binding glycoprotein > dilinoleoyl-phosphatidylcholine > taurodeoxycholate. No morphological alteration was evident for vesicles and crystals, but the cholesterol/phospholipid ratio in vesicles was increased significantly by replacement with dilinoleoyl-phosphatidylcholine and excess cholesterol. A high proportion of relatively hydrophilic phosphatidylcholine species such as dilinoleoyl-phosphatidylcholine and excess cholesterol in bile cause a redistribution of cholesterol to increase a vesicular cholesterol/phospholipid ratio, eventually promoting cholesterol crystallization, whereas concanavalin A-binding glycoprotein acts via differing mechanisms.

INTRODUCTION

The crystallization of cholesterol from gallbladder bile that is supersaturated with cholesterol is a key step in the formation of a gallstone. Such crystallization is believed to follow the aggregation and fusion of unilamellar vesicles rich in cholesterol [1]. Cholesterol is solubilized together with phospholipid vesicles in bile, and together with phospholipids and bile salts in mixed micelles [1–3]. Since the formation of mixed micelles requires more phospholipids than cholesterol, a larger amount of phospholipids is removed relative to cholesterol in the conversion of vesicles into mixed micelles. As a result, the residual vesicles become progressively richer in cholesterol and depleted of phospholipids, and their cholesterol-to-phospholipid (C/P) ratio increases steadily. The cholesterol-rich vesicles tend to aggregate, leading to the precipitation of cholesterol crystals [3,4]. Cholesterol/phospholipid vesicles are present in gallbladder bile of humans, and play a key role in the nucleation-of-cholesterol-crystals process [3,5]. Based on video-enhanced microscopy studies of human bile, Halpern et al. [3] reported that vesicle aggregation and fusion precede cholesterol nucleation. The crystallization of cholesterol is affected by the presence of various factors in bile, such as biliary proteins [6–11], phospholipid species [4,12–14] and bile-salt species [15,16]. The biliary proteins that modulate cholesterol crystallization include concanavalin A (Con A)-binding glycoproteins (CABG), which are promoters, and the apolipoproteins A-1 and A-2, and helix pomatia-bound glycoprotein, which are inhibitors [6–11,17]. Thus the formation of cholesterol gallstones depends on an imbalance among these factors.

Recent findings suggest that biliary phospholipids are im-

portant in the solubilization of cholesterol in bile. We previously showed that bile metastability, as reflected by nucleation time and the time required for the growth of cholesterol crystals, is affected by the net balance of the hydrophobic and hydrophilic properties of phosphatidylcholine [13]. We found that synthetic phosphatidylcholine containing polyunsaturated fatty acids, a relatively hydrophilic phospholipid, binds less tightly to cholesterol than does synthetic phosphatidylcholine containing saturated fatty acids [4,18]. We also found that the hydrophobic/hydrophilic balance of phosphatidylcholine affects the morphology of the cholesterol crystals and the size distribution of the vesicles and crystals [13]. In the present study, we used dilinoleoyl-phosphatidylcholine (DLPC) to represent a relatively hydrophilic phospholipid containing polyunsaturated fatty acids.

A higher proportion of deoxycholic acid (DCA) in bile has been found in patients with cholesterol gallstones compared with those without [15]. DCA directly and indirectly affects the nucleation of cholesterol. DCA causes the hypersecretion of a mucous glycoprotein that promotes the nucleation of cholesterol [15].

Despite such findings, the precise mechanisms by which biliary proteins and lipids promote the crystallization of cholesterol are unclear. The present study used supersaturated model bile (MB) solutions to clarify the mechanisms by which such substances affect cholesterol nucleation. We also compared the activity of these various promoting factors to determine the relative potency in achieving the crystallization of cholesterol.

This work was presented in part at the 97th Annual Meeting of the American Gastroenterological Association, May 13, 1997, in Washington, DC, and was published as an abstract [18a].

Abbreviations used: BA/PL ratio, bile acid/phospholipid ratio; Con A, concanavalin A; CABG, Con A-binding glycoprotein; CCG assay, cholesterol-crystal-growth assay; C/P ratio, cholesterol/phospholipid ratio; CSI, cholesterol saturation index; DCA, deoxycholic acid; DLPC, dilinoleoyl-phosphatidylcholine; EYPC, egg-yolk phosphatidylcholine; I_{CGR} , crystal-growth-rate index; I_{CM} , crystal mass index; IMC, intermixed micellar and vesicular bile salt concentration; I_{nt} , nucleation time index; MB, model bile; STC, sodium taurocholate; STDC, sodium taurodeoxycholate; TLC, total lipid concentration.

¹ To whom correspondence should be addressed (e-mail stazuma@mcai.med.hiroshima-u.ac.jp).

MATERIALS AND METHODS

Materials

Egg-yolk phosphatidylcholine (EYPC), dilinoleoyl-phosphatidylcholine (DLPC), sodium taurocholate (STC) and sodium taurodeoxycholate (STDC) were obtained from Sigma (St. Louis, MO, U.S.A.). Cholesterol was purchased from Wako Pure Chemical Industries (Osaka, Japan). Bio Gel A-5m for gel-permeation chromatography was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Con A lectin–Sephacrose, Sepharose 6B-CL and Superose 6[®] were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). *N*-Acetyl- α -D-galactosamine was purchased from Sigma, and *N*-acetyl- α -D-mannopyranoside was purchased from Wako Pure Chemical Industries. SDS, acrylamide, bisacrylamide, ammonium persulphate and *N,N,N',N'*-tetramethylethylenediamine were purchased from Bio-Rad Laboratories. Glassware was washed with 99% methanol and rinsed with distilled water before drying.

Preparation of MB solutions

MB solutions were prepared according to a previously described method [19]. Aliquots of stock solutions [STC in methanol, phosphatidylcholine in chloroform/methanol (9:1, v/v) and cholesterol in chloroform/methanol (2:1, v/v)] were first added to a volumetric flask to yield the desired amount of lipids. The organic solvent was next evaporated in a stream of nitrogen until the mixture condensed to a viscous paste. The mixture was lyophilized to achieve complete solvent removal and then stored at -80°C . The required volumes of Tris/NaCl (25 mM/150 mM) buffer containing 0.02 NaN_3 (pH 7.45) were added to the lyophilized lipids. These solutions were flushed with nitrogen, sealed and incubated at 55°C for 12 h while shaking at 150 rev./min (Double Shaker NR-3; Taitec, Saitama, Japan), until the solution became microscopically isotropic.

To compare the effects of the various effector substances on cholesterol crystallization, we prepared MB solutions of identical lipid composition under the following conditions. The control MB system had a cholesterol saturation index (CSI) of 1.7, a total lipid concentration (TLC) of 9.13 g/dl, 29 mM EYPC, 116 mM STC, 19 mM cholesterol and a final bile acid-to-phospholipid molar ratio (BA/PL) of 4.0. This control MB system was altered by: (1) adding cholesterol (CSI 1.8), (2) replacing 10% of the EYPC with DLPC, (3) replacing 10% of the STC with STDC, or (4) adding CABG (100 $\mu\text{g}/\text{ml}$). The CSI was determined from the critical table of Carey [20].

To investigate the dose dependence of each promoting factor, we prepared MB solutions under the following conditions: (1) control MB with the same lipid composition as described above, (2) changing the amount of cholesterol from CSI 1.8 to 1.6, (3) replacing 5, 10 and 20% of the EYPC with DLPC, and (4) replacing 10, 20 and 30% of STC with STDC. All MB systems had equal TLCs, except for those with altered cholesterol.

Protein purification procedure

Human gallbladder bile was obtained by needle aspiration at surgery in patients with cholesterol gallstones. CABG was isolated essentially as described by Groen et al. [10]. Briefly, bile was centrifuged for 60 min at 100 000 *g* (Model L5-50 with a 50.3 Ti rotor; Beckman Instruments, Fullerton, CA, U.S.A.) to remove microcrystals and insoluble mucins. The supernatant was applied to a Con A–Sephacrose column (1.6 cm $\times$ 30 cm), which was then equilibrated with 10-column bed-volumes of the previous starting buffer. Each column was allowed to equilibrate

overnight at 4°C to absorb completely the antigen from the applied sample. Each column was then washed extensively, first with 3-column volumes of starting buffer to remove non-specific binding materials, next with 12-column volumes of starting buffer containing 30 mM STC to remove lipids, and finally, with 5-column volumes of starting buffer to remove STC. The glycoproteins bound to the column were then eluted with 5-column bed-volumes of the eluting buffer, which contained 0.5 M α -D-methyl mannopyranoside in the starting buffer. The Con A-bound fraction was concentrated, and the buffer was replaced with 30 mM ammonium persulphate, using an ultra-filtration system containing a UK ultrafilter (Advantec; Toyo Roshi Co., Tokyo, Japan). The residual content of lipids in the buffer-exchanged Con A-bound fractions were below the limit of detectability. As a final step, the solution was lyophilized in a microvial.

SDS/PAGE

An SDS/polyacrylamide gel (8% non-gradient) was exposed to the buffer system described by Laemmli [21] in an electrophoresis cell (Mini-Protein 2; Bio-Rad Laboratories). The 8% non-gradient gel was used under reducing conditions that entailed only the addition of 5% (v/v) β -mercaptoethanol to the following sample buffer: 62.5 mM Tris, 2% (w/v) SDS, 20% (v/v) glycerol and 0.001% (w/v) Bromophenol Blue at a pH of 6.8 and non-reducing conditions. Aliquots containing 10 μg of protein were boiled for 3 min in the presence of the sampling buffer. After completion of the electrophoresis, the gel was fixed and stained with silver.

Conventional nucleation time study

Nucleation time was determined essentially as described by Holan et al. [22]. The isotropic MB solutions were filtered through a sterile 0.20 μm filter (Advantec; Toyo Roshi) and aliquots (720 μl each) of these MBs were mixed into 1 ml glass microvials with either CABG samples (80 μl) or the other solution (80 μl of Tris/NaCl buffer). The vials were flushed with nitrogen, sealed and incubated at 37°C while gently shaking. Time zero of nucleation was defined as 15 min after thermal equilibration. Samples were observed every 24 h under video-enhanced differential interference microscopy to detect the crystals and any morphological changes of the cholesterol crystals during their production.

Cholesterol-crystal-growth (CCG) assay

To evaluate the comparative potencies of the promoting factors on cholesterol nucleation in the supersaturated MB systems, the appearance of cholesterol crystals and their growth were quantitatively assayed using a modification of the method described by Busch et al. [23]. The isotropic MB solutions were filtered through a sterile 0.20 μm filter (Advantec; Toyo Roshi) and transferred into 1 ml glass microvials. The vials were flushed with nitrogen, sealed and incubated at 37°C while gently shaking. Time zero was defined as 15 min after thermal equilibration. A 30 μl aliquot of each MB solution was sampled in an ELISA plate. A 100 μl volume of Tris/NaCl buffer containing 120 mM STDC was added to each MB solution to dissolve vesicles, and absorbance was measured 30 min later at a wavelength of 620 nm using a spectrophotometer (ImmunoMini NJ-2300; System Instruments, Tokyo, Japan). This absorbance signifies the turbidity of the cholesterol crystals. Measurements were performed every 24 h. The crystal-growth-rate index (I_{cgr}), which

represents the effect on the maximum growth rate, was calculated as the ratio of the maximum slope of the experimental curve to that slope of the control curve. Final crystal mass was decided at the plateau point of each nucleation curve.

Measuring the dimensions of vesicles and crystals

The dimensions of the vesicles and crystals were determined using a laser-diffraction particle-size analyser (SALD-2000; Shimadzu, Kyoto, Japan) [4]. This apparatus analyses the diffraction and scattering patterns of laser light when measuring the sizes of particles of powders between 0.03 and 280 μm . The diffraction pattern of forward-scattered laser light was analysed using the Fraunhofer theory for particles from 280 μm down to about 0.08 μm in diameter. Particles smaller than 0.08 μm in diameter were analysed using the Mie and Side scattering theories. Using these algorithms, the microcomputer in this apparatus calculates the distribution of particle size. MB solutions (30 μl each) were placed in cuvettes and diluted with 7 ml of Tris/NaCl buffer containing the intermixed micellar and vesicular bile salt concentration (IMC) of STC. Particles with diameters of 0.6–1.0 μm were identified as vesicles and their aggregates. Particles with diameters of 10–100 μm were identified as cholesterol crystals, whereas those about 10 μm in diameter were considered micro-crystals (spiral and needle-like), and those over 100 μm in diameter were considered mature plate-like crystals. Measurements were performed at different time intervals until a state of equilibrium was reached in each sample.

Video-enhanced contrast microscopy

One drop of each MB solution was placed between two cover glasses at frequent intervals during its incubation. These samples were observed using video-enhanced contrast microscopy (Nikon, Tokyo, Japan). The images were monitored with a video camera control system (C-2400; Hamamatsu Photonics, Hamamatsu, Japan) and a video monitor (PMV-1442Q; Sony, Tokyo, Japan).

Separation of lipid particles

The separation of the lipid particles in each solution of MB was performed with an FPLC system. The isotropic MB solution was filtered through a sterile 0.20 μm filter and equilibrated at 37 °C for 1 h. The IMCs of the MB solutions were determined by the method of Donovan and Jackson [24]. In brief, 500 μl of MB solution was placed in the Centricon 10[®] (Amicon, Beverly, MA, U.S.A.) and centrifuged for 15 min at 1500 *g*. The concentration of bile salts in the filtrates was measured, then corrected by a factor that considers the Donnan equilibrium equation to obtain thus an accumulated IMC value. After 4 h of incubation at 37 °C, 500 μl of each MB solution was applied to a Superose 6[®] column, which had been equilibrated with Tris/NaCl buffer that contained the IMC of STC, and was eluted with the same buffer at a flow rate of 0.3 ml/min. A total of 30 fractions of 1 ml each was then collected and assayed for cholesterol and lecithin to identify the various particulate fractions.

Analytical procedures

Phosphatidylcholine concentrations were measured by the methods of Bartlett [25] and Fiske and Subbarrow [26], and cholesterol concentrations were determined enzymically with a commercially available assay kit that evaluates cholesterol esterase and cholesterol oxidase (Nihon Shoji, Osaka, Japan). Bile acid concentrations were measured enzymically, using 3- α

hydroxysteroid dehydrogenase (Daiichi Kagaku, Tokyo, Japan). Protein concentrations were assayed by the Coomassie Blue dye-binding assay, using a commercially available assay kit (Bio-Rad Laboratories) that requires trichloroacetic acid precipitation.

Statistical analysis

The statistical analyses of the CCG curves were performed using analysis of variance at each time point to determine whether differences existed among the groups. The statistical analyses of the lipid distributions in the MB systems were performed using a Student's *t* test.

RESULTS

Comparative effect of promoting factors in the CCG assay

The effect of each promoting factor on cholesterol crystallization (nucleation and growth) kinetics was assessed by the CCG assay in the supersaturated MB systems, and the growth curves for each MB system are shown in Figure 1. The I_{CGR} and final crystal

Figure 1 Relative promoting activity of each promoting factor on cholesterol crystallization as assessed by the CCG assay

The MBs used in the CCG assay had the following compositions: (1) ■, Control MB; CSI 1.7, TLC 9.13 g/dl; (2) ●, the addition of cholesterol (CSI 1.8); (3) ◆, replacing 10% of STC with 10% STDC; (4) ▲, replacing 10% of EYPC with 10% DLPC; and (5) □, the addition of CABG (100 $\mu\text{g/ml}$). Each data point represents a mean value $\pm$ S.D. ($n = 4$). The rank order for the promoting activity of each MB is CABG $\geq$ DLPC $>$ CSI 1.8 $>$ STDC = control. * $P < 0.05$, ** $P < 0.01$, compared with the control.

Table 1 Promoting activity of each promoting factor on cholesterol crystallization as estimated by CCG assay

I_{nt} is measured by the onset time of crystal detection with video-enhanced microscopy; I_{CGR} is the maximal growth rate (maximum slope of the experimental relative to control curves); I_{CM} is the final crystal mass relative to the control. * $P < 0.05$, ** $P < 0.01$, compared with the control ($n = 4$).

MB composition	Activity indices		
	I_{nt}	I_{CGR}	I_{CM}
Control MB	1.00	1.00	1.00
CSI 1.8 MB	0.76*	1.33*	1.41*
10% STDC MB	1.00	0.96	1.04
10% DLPC MB	0.52**	2.94**	2.26**
100 $\mu\text{g/ml}$ CABG MB	0.52**	3.02**	2.07**

Figure 2 Time course of changes in the size distribution of vesicles and crystals in MB solutions as calculated by a laser-diffraction particle-size analyser

z-axes show the number of hours of observation, performed at various time intervals. Vesicles and/or cholesterol crystals in each MB system were detected.

mass index (I_{CM}) values for the experimental MB systems compared with those of the control MB system are shown in Table 1. The CABG and DLPC MB systems had increased I_{CGR} and I_{CM} values, whereas the CSI 1.8 MB system had a shortened nucleation time index (I_{nt}). The CABG MB system clearly showed the most potent promoting activity. The DLPC MB system also enhanced cholesterol crystallization to nearly the same magnitude as that seen with CABG. The STDC MB system did not significantly promote cholesterol crystallization. The ranking

order of the I_{CGR} values for the MB systems was CABG > 10% DLPC > CSI 1.8 > control = 10% STDC, and the ranking order of the I_{CM} values was 10% DLPC MB > CABG > CSI 1.8 > 10% STDC > control.

Dimensions of vesicles and cholesterol crystals

The time course of the changes in the distribution of the particle sizes for each MB solution is shown in Figure 2. In all of the MB

Figure 3 Particles present in CABG MB solutions

Representative patterns of crystallization in CABG MB after the time periods indicated, observed by video-enhanced microscopy.

solutions, vesicular formation was detected soon after incubation began, and these vesicles gradually grew until the early stages of crystal precipitation, after which they decreased slightly in diameter. In the DLPC and CABG MB systems, the crystals became rapidly nucleated between 52 and 76 h after incubation began. Numerous microcrystals were observed during this time interval, and these gradually matured into monohydrate plate-like crystals. In contrast, plate-like crystals began forming during the early stages of nucleation in the control MB and STDC MB solutions.

Morphological changes in vesicles and cholesterol crystals

Vesicular formation and morphological changes of the cholesterol crystals were observed by video-enhanced microscopy. Vesicle formation and aggregation were rapid in all MB solutions. Needle-like crystals were continuously observed during the study, and transformed into spiral shapes. Then plate-like crystals appeared. As a final step, the classical monohydrate crystal became the predominant form in the MB solution; neither the morphology of the vesicles nor the formation of the crystals in any MB solution was altered. Representative patterns of crystallization in CABG MB are shown in Figure 3.

Effect of promoting factors on distribution of cholesterol among lipid particles

The effects of each promoting factor on the preferential pattern of distribution of cholesterol among lipid particles in the super-saturated MB solutions were evaluated. The values of the IMC of each MB solution did not significantly differ from one another (results not shown). In contrast, the elution profiles of the MB solutions determined by Superose 6[®] column chromatography showed two peaks, representing the vesicular and micellar fractions. The differences of these profiles among the MB systems are shown in Figure 4. As further described in Table 2, these profiles showed that the amount of cholesterol and the C/P ratios were significantly increased in the vesicles of the 10% DLPC MB system. The C/P ratio in the vesicles of the CSI 1.8 MB system was also significantly greater than that of the

Figure 4 Representative elution profiles of each MB system using Superose-6[®] column chromatography

Each column was equilibrated with Tris/NaCl buffer containing STC at its IMC concentration, and eluted with the same buffer. Void and total volumes (V_o and V_t respectively) are indicated by arrows. The initial eluting lipid is the vesicular fraction, and the later eluting sharp peak is the micellar fraction. ○, cholesterol; ●, phospholipid. The composition of each MB solution was the same as for those shown in Figure 1 ($n = 4$).

control MB system. Neither the amount of cholesterol in the vesicles nor the C/P ratio in the vesicles of the CABG and STDC MB systems differed from those of the control MB solutions.

Dose dependence of each promoting factor

The dose dependence of each promoting factor on the amount of crystal mass seen over the time course of each experiment was estimated by the CCG assay (Figures 5–7). Replacing 5, 10 and 20% of EYPC with equal percentages of DLPC in the MB

Table 2 Effects of promoting factors on distribution of cholesterol within lipid particles in MB solution

Values are given as means $\pm$ S.D. ($n = 4$). * $P < 0.05$ compared with the control, as assessed by Student's t test.

	Vesicle		Micelle	
	Cholesterol (%)	C/P ratio	Cholesterol (%)	C/P ratio
Control	33.79 $\pm$ 4.15	2.27 $\pm$ 0.19	66.18 $\pm$ 4.09	0.44 $\pm$ 0.03
CSI 1.8	35.60 $\pm$ 3.40	2.69 $\pm$ 0.22*	64.40 $\pm$ 3.39	0.44 $\pm$ 0.16
STDC	31.49 $\pm$ 4.56	2.36 $\pm$ 0.07	68.55 $\pm$ 0.45	0.44 $\pm$ 0.03
DLPC	38.85 $\pm$ 7.86	2.52 $\pm$ 0.08*	61.14 $\pm$ 7.86	0.40 $\pm$ 0.04
CABG	30.23 $\pm$ 4.56	2.37 $\pm$ 0.21	69.70 $\pm$ 2.47	0.45 $\pm$ 0.01

Figure 5 Dose dependence of the promoting activity of the phosphatidylcholine species on cholesterol crystallization

The MB system used in the assay had the following composition: ●, control MB (CSI 1.7, TLC 9.13 g/dl); ○, replacement of 5% EYPC with DLPC; ■, replacement of 10% EYPC with 10% DLPC; □, replacement of 20% EYPC with 20% DLPC. Each data point represents a mean value $\pm$ S.D. ($n = 4$). * $P < 0.05$, ** $P < 0.01$, compared with the control.

system enhanced the growth rate of crystals dose dependently (Figure 5). Although replacing 10 and 20% STC with STDC in the MB system did not significantly affect crystal growth, replacing 30% STC with STDC in the MB system promoted that growth (Figure 6). A distinct dose-dependent effect of cholesterol content on crystal growth was observed (Figure 7), with CSI 1.8 being nearly equal to 30% STDC in its effect on the MB in the CCG assay.

DISCUSSION

The pathogenesis of gallstone disease consists of complex steps involving the presence of cholesterol supersaturation of the gallbladder bile, lipid-vesicle formation and aggregation, which culminates in the phase separation of cholesterol and the nucleation of cholesterol crystals [1–6]. Although a variety of factors affect this process, few investigations have compared the potencies of these factors. This background led us to use supersaturated MB solutions, to clarify the mechanisms of action of cholesterol-nucleating-effector substances and to rank them in order of activity.

A major finding of our study was that the CABG isolated from the cholesterol gallstone patients promoted nucleating activity,

Figure 6 Dose dependence of the promoting activity of bile acid species on cholesterol crystallization

The MB system used in the CCG assay had the following composition: ●, Control MB (CSI 1.7, TLC 9.13 g/dl); ○, replacement of 10% STC with STDC; ■, replacement of 20% STC with STDC; □, replacement of 30% STC with 30% STDC. Each data point represents a mean value $\pm$ S.D. ($n = 4$). * $P < 0.05$, ** $P < 0.01$, compared with the control.

Figure 7 Dose dependence of the promoting activity of cholesterol content on cholesterol crystallization

The MB system used in the CCG assay had the following composition: ○, control MB (CSI 1.7, TLC 9.13 g/dl); ●, decreased cholesterol (CSI 1.6); ■, increased cholesterol (CSI 1.8). Each data point represents a mean value $\pm$ S.D. ($n = 4$). * $P < 0.05$ compared with the control.

as shown by others (Figure 1), but with no major effects on the distribution of biliary lipid particulate species (Table 2). This finding contrasts with the data of Groen [11], which demonstrate that Con A eluted from human bile induces a shift of cholesterol and phospholipids from bile salt micelles to the vesicles. This discrepancy could be related to differences in experimental design, e.g. in incubation time and in the procedure for separating the lipid particles. Groen [11] used an incubation time of 48 h and density-gradient ultracentrifugation to separate the vesicular and micellar fractions. In contrast, we determined the lipid distribution of our MB systems in the presence of CABG by Superose 6® column chromatography with an eluent containing an IMC of STC, estimated by the method of Donovan and Jackson [24]. The incubation time of our MB system with CABG

was much shorter (for 4 h) than that of Groen [11]. Under our conditions, the physico-chemical state of the MB solution may have been more physiological, suggesting that the failure of CABG to affect lipid distribution represents the physiological state. de Bruijn et al. [6] reported that the nucleation-promoting action of CABG is mediated, in part, by the vesicle disruption, using a unique vesicle-monitoring system with carboxyfluorescein. Thus the cholesterol-crystallization-promoting action of CABG, which occurs under physiological circumstances, could not be based on the redistribution of lipids among biliary lipid particulate species, but seemingly on the alteration of cholesterol metastability within individual vesicular lipid bilayers. Such an alteration in the physico-chemical state of the vesicular lipids could lead to the enhancement of cholesterol-crystal formation.

The fact that a 10% replacement of the control MB system with DLPC promoted CCG and increased the vesicular C/P ratio was another major finding. An increased concentration of phospholipid in the bile is known to have a greater effect on the nucleation of cholesterol than does an increased bile acid concentration [12]. Di-saturated synthetic phosphatidylcholine species also prolong the nucleation time as compared with EYPC or mono-unsaturated phosphatidylcholine [4,13,18]. As previously described, the degree of hydrophobicity is determined by fatty acyl chain saturation as indexed by the retention time of reversed-phase HPLC [13]. Thus a phosphatidylcholine species dominantly containing saturated fatty acyl chains is defined as a relatively hydrophobic phospholipid, whereas a relatively hydrophilic phosphatidylcholine species contains unsaturated fatty acyl chains predominantly. In principle, relatively hydrophilic phosphatidylcholine that contains mainly unsaturated fatty acid binds less tightly to cholesterol than does synthetic phosphatidylcholine, which contains mainly saturated fatty acids. Thus the hydrophilic property of phosphatidylcholine affects bile metastability, as reflected by the degree of cholesterol-crystal nucleation. Several studies have shown that cholesterol interacts differently with DLPC compared with other PC molecular species such as dipalmitoylphosphatidylcholine or palmitoyloleoylphosphatidylcholine. The difference in interaction has been attributed to the geometry of di-unsaturated aliphatic chains, which prevents stable interaction with cholesterol [13,18,27]. The present study used DLPC as a representative hydrophilic phosphatidylcholine to replace 10% of EYPC in the control MB system, and found a promoting effect on cholesterol crystallization (Figure 1, Table 1) and an increase in the vesicular C/P ratio (Table 2). Its promoting effect at 10% nearly equalled that of 100 µg/ml CABG. Further replacement studies with DLPC for EYPC revealed a dose-dependent effect (Figure 5). These findings suggest that hydrophilic phosphatidylcholine preferentially migrates into the bile salt micelles independently from that migration of cholesterol. The increased C/P ratio in the vesicle makes the vesicles physically unstable. When the phosphatidylcholine species change, the high ratio of cholesterol to phosphatidylcholine in the vesicles dictates the rapidity of the nucleation of the cholesterol crystal.

Another major finding was the effect of changing the bile salt composition on the CCG assay. Replacing 10% and 20% STC with STDC in the MB system did not significantly affect the CCG assay compared with that of the control MB (Figures 1 and 6), whereas the replacement of 30% STC with STDC in the MB system had a promoting effect. The elution profile of 10% of the MB system replaced with STDC resembled that of the control MB solution; the lipid distributions of the vesicles and micelles also did not differ significantly. This fact indicates that the dramatic force of the cholesterol pro-nucleating effect evoked by an altered composition of bile acids requires large amounts of hydrophobic bile acid. Bile acid species are also less effective than

the phosphatidylcholine species and biliary proteins in producing cholesterol nucleation. The proportion of deoxycholic acid (DCA) in bile has been implicated in the development of cholesterol-rich gallstones [15,16,28]. Thus studies have shown a linear relationship between the percentage of biliary DCA and the mol percentage of cholesterol in bile and the biliary cholesterol saturation index. DCA directly and indirectly affects cholesterol nucleation [15]. The hydrophobic detergent effects of DCA on the canalicular membrane may lead to the biliary hypersecretion of cholesterol. If an increased percentage of biliary DCA induces an increase in biliary cholesterol concentration that exceeds the capacity of the micelles to solubilize the excess cholesterol, this in turn would increase the molar concentration of cholesterol in the vesicles as well as the vesicular C/P ratio. DCA is also more hydrophobic than many other bile acids [28]. Thus if the micelles are rich in DCA, they will draw lipids from the vesicles. Since bile acids solubilize relatively more phospholipid than does cholesterol, the preferential transfer of phospholipid from the vesicles to DCA-rich micelles will produce vesicles having a high cholesterol concentration and a high C/P ratio. The indirect action of DCA on cholesterol nucleation occurs via the excretion of mucin. The concentration of DCA in bile correlates positively with both the mol percentage of cholesterol and the amount of arachidonic acid-rich phosphatidylcholine in bile [15]. This finding suggests that the secretion of mucin and its release by the gallbladder mucosa, which is presumably regulated by the prostanoid pathway, may be stimulated by arachidonic acid, a precursor of prostanoids, although we did not investigate this indirect action of DCA in the present *in vitro* study.

We showed that the nucleating-promoting potency of 100 µg/ml CABG was compatible with that of replacing 10% EYPC with DLPC in the MB solution. CSI 1.8 in the MB system produced a similar CCG curve to that of replacing 30% STC with STDC in the MB system. The ranking order of potency for the MB systems by the CCG assay was 100 µg/ml CABG > 10% replacement of DLPC > CSI 1.8 > control = 10% replacement of STDC. The phosphatidylcholine species, the bile acid species and the amount of cholesterol in the MB solution showed a dose-dependent effect on cholesterol crystallization. However, these substances did not significantly alter the vesicles or the crystal growth patterns, as observed by dimensional and morphological study.

In conclusion, a marked promotion of cholesterol crystallization and a significant increase in final crystal mass were similarly observed either by adding CABG or by replacing EYPC with DLPC in the MB solutions as compared with the addition of cholesterol or STDC. The dramatic force of the cholesterol pro-nucleating effect evoked by the alteration of the bile acid composition requires large amounts of hydrophobic bile acid, and bile acid species are less effective than phosphatidylcholine species and biliary proteins on pronucleation. These results suggest that hydrophilic phosphatidylcholine and pronucleating glycoproteins are relatively potent in the promotion of cholesterol crystallization. These factors are thus important in considering the nucleation time for native bile. That the promotion of cholesterol nucleation by an increase in the proportion of hydrophilic phosphatidylcholine and CSI (excess cholesterol), associated with an increase in the vesicular C/P ratio, indicates that these effector substances may destabilize the vesicles and stimulate the redistribution of cholesterol. In contrast, CABG exhibited a striking promoting activity that involved mechanisms differing from those described above.

The present study was supported, in part, by the Ministry of Education of the Japanese Government, awarded to S.T. (No. 08670597).

REFERENCES

- 1 Holzbach, R. T. (1990) *Hepatology* **12**, 155S–161S
- 2 Harvey, P. R. C., Upadhy, G. A. and Strasberg, S. M. (1991) *J. Biol. Chem.* **266**, 1–8
- 3 Halpern, Z., Dudley, M. A., Kibe, A., Lynn, M. P., Breuer, A. C. and Holzbach, R. T. (1986) *Gastroenterology* **90**, 875–885
- 4 Tazuma, S., Ochi, H., Teramen, K., Yamashita, Y., Horikawa, K., Miura, H., Hirano, N., Sasaki, M., Aihara, N., Hatsushika, S. et al. (1994) *Biochim. Biophys. Acta* **1215**, 74–78
- 5 Afdhal, N. H., Niu, N., Nunes, D. P., Bansil, R., Cao, X. X., Gantz, D., Small, D. M. and Offner, G. D. (1995) *Hepatology* **22**, 856–865
- 6 de Bruijn, M. A. C., Goldhoorn, B. G., Zijlstra, A. I. M., Tygat, G. N. J. and Groen, A. K. (1995) *Biochem. J.* **305**, 93–96
- 7 Yamashita, G., Ginanni, C. S., Secknus, R., Takabayashi, A., Williams, C., Hays, L., Chernosky, A. and Holzbach, R. T. (1995) *J. Lipid Res.* **36**, 1325–1333
- 8 Offner, G. D., Gong, D. and Afdhal, N. H. (1994) *Gastroenterology* **106**, 755–762
- 9 Abei, M., Nuutinen, H., Kawczak, P., Schwarzendrube, J., Pillay, S. P. and Holzbach, R. T. (1994) *Gastroenterology* **106**, 231–238
- 10 Groen, A. K., Noordam, C., Drapers, J. A. G., Egbers, P., Jansen, P. L. M. and Tygat, G. N. J. (1990) *Hepatology* **11**, 525–533
- 11 Groen, A. K. (1990) *Hepatology* **11**, 189S–194S
- 12 Jüngst, D., Lang, T., Huber, P., Lange, V. and Paumgartner, G. (1993) *J. Lipid Res.* **34**, 1457–1464
- 13 Ochi, H., Tazuma, S. and Kajiyama, G. (1996) *Biochem. J.* **318**, 139–144
- 14 Hatsushika, S., Tazuma, S. and Kajiyama, G. (1993) *Scand. J. Gastroenterol.* **28**, 131–136
- 15 Hussaini, S. H., Pereira, S. P., Murphy, G. M. and Dowling, R. H. (1995) *Hepatology* **22**, 1735–1744
- 16 Noshiro, H., Chijiwa, K., Makino, I., Nakano, K. and Hirota, I. (1995) *Gut* **36**, 121–125
- 17 Busch, N., Lammert, F., Marschall, H. U. and Matern, S. (1995) *J. Clin. Invest.* **96**, 3009–3015
- 18 Tao, S., Tazuma, S. and Kajiyama, G. (1993) *Biochim. Biophys. Acta* **1166**, 25–30
- 18a Nishioka, T., Tazuma, S., Yamashita, G. and Kajiyama, G. (1997) *Gastroenterology* **112**, A1347
- 19 Kibe, A., Holzbach, R. T., LaRusso, N. F. and Mao, S. J. T. (1984) *Science* **225**, 514–516
- 20 Carey, M. C. (1978) *J. Lipid Res.* **19**, 945–957
- 21 Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- 22 Holan, K. R., Holzbach, R. T., Hermann, R. E., Cooperman, A. M. and Claffey, W. Y. (1979) *Gastroenterology* **77**, 611–617
- 23 Busch, N., Tokumo, H. and Holzbach, R. T. (1990) *J. Lipid Res.* **31**, 1903–1909
- 24 Donovan, J. M. and Jackson, A. A. (1993) *J. Lipid Res.* **34**, 1121–1129
- 25 Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- 26 Fiske, C. H. and Subbarrow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
- 27 Halpern, Z., Moshkowitz, M., Laufer, H., Peled, Y. and Gilat, T. (1993) *Gut* **34**, 110–115
- 28 van de Heijning, B. J. M., Stolk, M. F. J., van Erpecum, K. J., Renooij, W., Groen, A. K. and van Berge-Henegouwen, G. P. (1994) *J. Lipid Res.* **35**, 1002–1011

Received 14 November 1997/4 February 1998; accepted 25 February 1998