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FILAMENTOUS CRYSTALLIZATION OF CHOLESTEROL AND ITS DEPENDENCE ON LECITHIN SPECIES IN BILE

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Abstract Cholesterol crystallization is a poorly understood obligatory step in cholesterol gallstone formation. We have studied early stages of cholesterol crystallization in a model bile system composed of an aqueous solution of taurocholate/cholesterol/lecithin (97.5/1.7/0.8 moles%). Nucleation of cholesterol crystals was induced by dilution of a micellar solution to achieve supersaturation. Following spontaneous vesicle formation, thin filamentous structures were observed by electron and light microscopy. The filaments were composed of a core of >95% cholesterol and a surface layer of <5% lecithin, which was preferentially enriched in hydrophobic molecular species. The filaments had a buoyant density of 1.03 g/ml, and revealed an x-ray diffraction pattern compatible with crystalline cholesterol monohydrate with an additional Bragg reflection at 4.9Å, suggesting a polymorph of cholesterol monohydrate, or early anhydrous crystallization. The filaments grew and transformed via intermediate spiral, helical and tubular microstructures to become classical plate-like cholesterol monohydrate crystals with a density of 1.045g/ml. The crystallization sequence was markedly influenced by substituting the natural lecithin species with unphysiologic synthetic species, and by incorporation of hydrophobic sterols into the model bile. These findings suggest that crystalline cholesterol in bile may not be completely mature or hydrated initially, but following nucleation undergoes a series of transformations to become thermodynamically stable monohydrate plates. The crystallization process is influenced and possibly controlled by a surface layer of biliary lecithins adsorbed by hydrophobic interactions.

INTRODUCTION

Cholesterol gallstones are composed of myriads of cholesterol monohydrate crystals held together by a glycoprotein-rich organic matrix[1]. Therefore, the process of cholesterol crystallization is obviously a pivotal, obligatory step in gallstone pathogenesis.

Cholesterol crystallization is believed to follow aggregation and possible fusion of cholesterol-rich phospholipid vesicles in supersaturated, lithogenic bile[2]. The details of the physical chemical events during nucleation and crystallization of biliary cholesterol are, however, incompletely understood[3,4]. The aim of the present study was to investigate the physical chemical phenomena occurring during the early stages of cholesterol crystallization in model bile.

MATERIALS AND METHODS

A model bile system, composed of cholesterol (Ch), egg yolk phosphatidylcholine (EYPC, lecithin) and taurocholate (TC) in a molar ratio of 1.7/0.8/97.5 was used for the study[5]. A coprecipitated and dried mixture of the three biliary lipids was solubilized in 0.15M NaCl to yield an isotropic, micellar solution with a total lipid concentration of 7g/dL and a Ch saturation index (CSI) of 90%[6]. Supersaturation (CSI=208%) and nucleation of cholesterol were induced by sixfold dilution of the micellar solution. The resulting crystallization process was followed by sequential analysis of the nucleating bile by time-lapse light and transmission electron microscopy, as well as quasielastic light scattering (QLS). Light and fluorescence microscopies were performed using polarizing and fluorescence microscopes, respectively, equipped with phase contrast optics (Carl Zeiss, Inc.). Digitally video-enhanced light microscopy was performed employing a video camera and a frame grabber[7]. QLS was performed on a home-built apparatus[8]. Precipitating crystals were analyzed by x-ray diffraction – conventional rotating anode[9], as well as synchrotron source (at Brookhaven National Laboratories, Upton, N.Y.) – using a wavelength of 1.36Å. Crystal densities were measured by sucrose gradient ultracentrifugation[10,11]. Lecithin molecular species were analyzed by HPLC[12].

RESULTS AND DISCUSSION

The nucleating bile exhibited a reproducible sequence of events, documented initially by QLS and subsequently by microscopy.

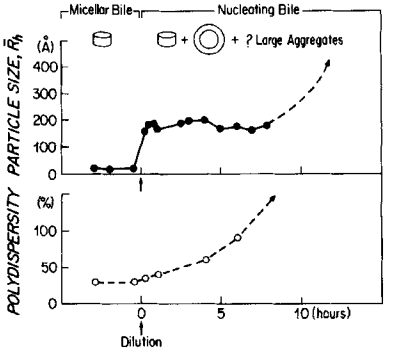

FIGURE 1 QLS of micellar and nucleating model bile. The upper panel shows

the mean hydrodynamic radius, and the lower panel the polydispersity, as functions of time before and after dilution (T=0).

By QLS the concentrated micellar bile disclosed a relatively homogeneous particle population with a mean hydrodynamic radius ($\bar{R}_h$) of 17Å (Fig 1). Upon dilution, a larger particle population ($\bar{R}_h$=212Å) compatible with unilamellar vesicles appeared. These remained of stable size for 6–8 hours. The polydispersity, however, increased continuously from the time of dilution, suggesting the emergence of additional, larger particles. Indeed, phase contrast light microscopy within 3–4 hours revealed the presence of thin filamentous structures (Fig 2a). By light and transmission electron microscopy, individual filaments extended to hundreds of μm in length and averaged 0.1μm in width. Over 2–5 days, filaments were replaced in sequence by spirals, helices, ribbons and tube-like crystals with a helical backbone, which eventually spawned plates at their ends (Fig 2b–d). The continuous transition of crystal habits was documented by video-enhanced time-lapse microscopy. When centrifugally separated (at 12 hours) and washed with water, chemical analysis showed that the filaments were composed of >95% cholesterol and <5% lecithin.

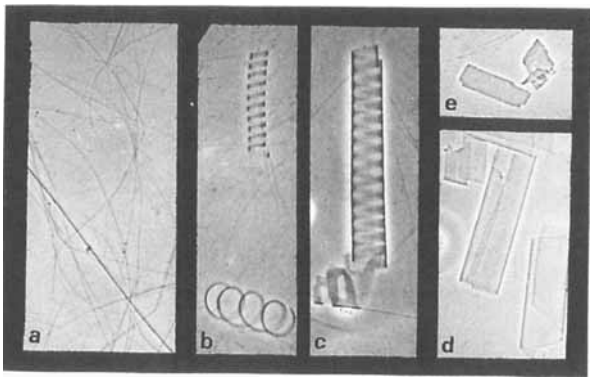

FIGURE 2 Reproducible crystallization sequence revealing early filaments (a; 4–12 hours) that transform via intermediate forms (b–d; 2–5 days) to classical cholesterol monohydrate plates (e; 10 days), respectively.

Rates of filament formation and transitions to thermodynamically stable plates accelerated curvilinearly with increases in temperature. At a temperature of 4, 37, and 60 °C, respectively, filament formation began at 30, 3, and 1 hours and plates were stable by >180, 10, and 2 days.

At 24 hours of incubation, biliary cholesterol crystals were isolated by ultracentrifugation and subjected to density gradient ultracentrifugation in 2–20% sucrose for 72 hours[10]. Two separate bands of crystals formed: filamentous crystals were confined to a band with a density of 1.02–1.03 g/ml, whereas plate-like crystals concentrated in a band at 1.04–1.05 g/ml. When a mixture of standard anhydrous and monohydrate

cholesterol crystals was subjected to density gradient ultracentrifugation under identical conditions, the anhydrous crystals were concentrated at 1.029 g/ml while monohydrate crystals at 1.045g/ml, consistent with the crystal densities reported in the literature[13,14].

To determine the molecular structure of the filaments we performed x-ray diffraction on filaments harvested by ultracentrifugation. The powder diffraction pattern of precipitated crystals (>90% filaments), harvested at 24 hours (plus 24 hours of exposure) disclosed three major peaks at Bragg's spacings of 34.1Å, 17.6Å, and 5.9Å, and was indistinguishable from that of standard cholesterol monohydrate crystals prepared by controlled crystallization from 95% ethanol. To obtain structural information at an earlier time point and to avoid possible crystal hydration or transformation during the prolonged x-ray exposure, we performed rapid (30 minute) synchrotron x-ray diffraction analysis early in the crystallization sequence. Figure 3 depicts the diffraction pattern of filaments harvested at 8–12 hours in comparison with diffraction patterns of standard cholesterol monohydrate and anhydrous crystals. While the monohydrate structure was in general confirmed, there was, however, an additional small diffraction peak at 4.9Å. Since this peak is not a feature of the monohydrate diffraction pattern and overlaps with only a minor peak of the anhydrous pattern, the finding suggests that the filaments contain some remnants of an anhydrous crystallization or are a polymorph of cholesterol monohydrate.

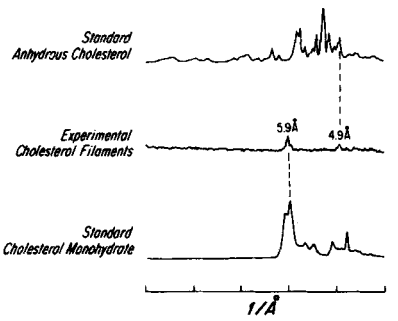

FIGURE 3 Synchrotron x-ray diffraction pattern of filaments (middle panel) harvested at 8–12 hours, compared with standard anhydrous (upper panel) and monohydrate (lower panel) cholesterol crystals.

When grown from model bile containing traces of a fluorescent phospholipid (rhodamine phosphatidylethanolamine), filaments were found by fluorescence microscopy to be covered by a surface layer of phospholipids. This surface layer could be washed off with a few μl of detergent solution (50mM taurodeoxycholate), without affecting the microscopic morphology of the filaments. This suggests that the phospholipid surface layer was hydrophobically adsorbed to the crystals. We then isolated the phospholipid surface layer from early filamentous cholesterol crystals by Folch extraction[15] after ultracentrifugal isolation of crystals from the nucleating model bile. The lecithin molecular species of this layer were then determined by HPLC (Fig 4).

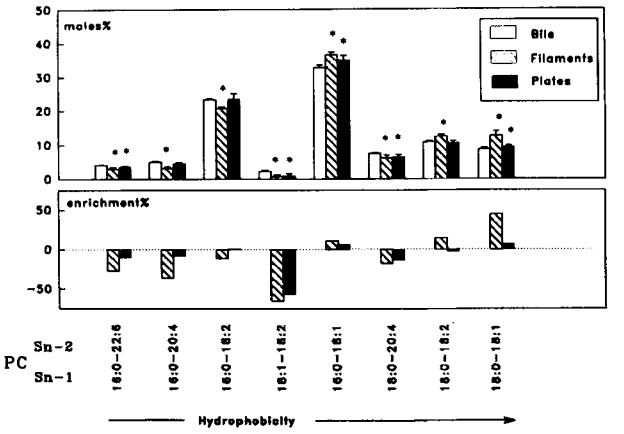

FIGURE 4 Distribution of EYPC molecular species on cholesterol crystals. Molar percentages (upper panel) and relative enrichments (lower panel) of 8 principal EYPC molecular species within the surface layer on early cholesterol filaments (hatched bars), on plate-like crystals at equilibrium (filled bars), and in the original model bile (open bars). Early filamentous cholesterol crystals are covered by a surface layer of lecithin enriched in molecular species with long saturated acyl chains, a finding typical of vesicular as opposed to micellar lecithins[16]. (* denotes $p<0.05$ compared to original bile.)

When compared to the whole original model bile solution, filaments were significantly enriched with lecithin molecular species having longer and more saturated acyl chains in both the sn-1 and the sn-2 positions. Conversely, filaments were depleted with species having shorter and more unsaturated acyl chains. The preferential enrichment with saturated species was less marked on plate-like crystals at equilibrium, although the distribution was still not identical to that of the original bile.

The influence of phospholipid species upon cholesterol crystallization was then studied by replacing EYPC in the model bile by other phospholipid species. Our experiments resulted in 3 different crystallization time-sequences and habit-transitions. First, when EYPC was substituted with either its principal molecular species or other naturally occuring lecithin mixtures, the crystallization process remained unchanged. Second, long unsaturated lecithins, such as 18:3-18:3-PC, resulted in rapid precipitaton of short filaments, which were transformed slowly into plates by an arborization pattern, without any intermediate helix formation. Third, synthetic saturated lecithins with long acyl chains (16 or 18 carbons long) resulted in slow precipitation, the duration of which increased with increasing acyl chain length. The initial precipitates were plate-like cholesterol monohydrate crystals; yet some filaments as well as tubular and helical microstructures appeared late in the crystallization sequence. An identical crystallization process was also observed when lecithin was replaced with other stiff-chain membrane lipids, such as sphingomyelin or cardiolipin. Taken together, these findings suggest that the surface layer of biliary phospholipids affects the earliest stages of cholesterol

crystallization by preferential binding of crystalline cholesterol (anhydrous more than monohydrate) to saturated phospholipids, possibly blocking early growth of initial filamentous cholesterol crystals.

We then examined the effects of partial substitution of cholesterol by other sterols, upon cholesterol crystallization in the model bile system. With increasing amounts of cholestanol, the saturated derivative of cholesterol, the crystallization process remained unchanged, i.e. identical to that with cholesterol alone. However, stigmasterol, a hydrophobic 27-carbon plant sterol, and doxyl-cholestane, a hydrophobic synthetic sterol, both resulted in persistence of filamentous crystals throughout the observation period. The emergence of plates, however, was not completely inhibited, and only minor amounts of intermediate structures appeared late in the crystallization sequence.

In summary, these experiments demonstrate that cholesterol crystallization from model bile is more complex than previously appreciated. In a dilute, bile salt-rich model bile, cholesterol nucleates as filamentous crystals, that have features suggesting an anhydrous origin or a monohydrate polymorph. The filamentous crystals transform via intermediate crystalline structures to become thermodynamically stable classic plate-like cholesterol monohydrate crystals. Early filamentous crystals are covered with a surface layer of lecithin, which is preferentially enriched with molecular species containing saturated acyl chains. The cholesterol crystallization sequence can be altered by substituting synthetic lecithin species, as well as by some other phospholipid classes. Finally, incorporation of hydrophobic sterols into model bile can prolong the metastability of filamentous cholesterol crystals. These findings provide some new insights into the complex process of biliary cholesterol crystallization, and will hopefully lead to a scientific basis for intervening with the earliest steps of cholesterol gallstone formation.

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