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Molecular pathophysiology and physical chemistry of cholesterol gallstones

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1. ABSTRACT

Cholesterol gallstones are one of the most prevalent and most costly digestive diseases in the developed countries. Although precipitation of cholesterol from supersaturated bile is the first irreversible physical-chemical step in cholesterol gallstone formation, hepatic hypersecretion of biliary cholesterol is the primary defect in the formation of cholesterol gallstones. The other common abnormalities of the hepatobiliary system in gallstone patients include accelerated cholesterol nucleation/crystallization, gallbladder hypomotility, hypersecretion and accumulation of mucins, high efficiency of intestinal cholesterol absorption and slow intestinal motility. Family and twin studies in humans as well as gallstone prevalence investigations in different strains of inbred mice have clearly demonstrated that a complex genetic basis could determine individual predisposition to develop cholesterol gallstones in response to environmental factors such as high dietary cholesterol. In this review, we summarize progress in understanding the molecular pathophysiology of cholesterol gallstone formation with particular reference to most recent advances in the physical-chemistry of bile and the physiology of biliary lipid secretion.

Figure 1. Macroscopic appearance of human gallbladder stones. Upper panel: series of pure spherical or oval shaped cholesterol stones with smooth (A) or morular (B) surface, and a tiny (C), medium (D) or large (E) dark pigment nucleus on the cut surface. A very large (F), almost pure cholesterol stone is shown, which discloses a conglomeration of stones. Middle panel: series of mixed cholesterol stones with spherical (G) or multifaceted (H and I) surface, and a large pigment nucleus (J) or a small cholesterol nucleus (K) surrounded by a pigment thick layer on the cut surface. Lower panel: appearance of pigment stones of different number, size and surface (L-O) with a small cholesterol content (of less than 20%) on the cut surface (P). Black horizontal lines are equal to 1 cm. Reproduced with permission from (2).

2. INTRODUCTION

Cholesterol cholelithiasis is one of the most prevalent diseases of the digestive system in the westernized countries (1,2). In fact, 20 million Americans (12% of adults) have gallstones, as well as each year, approximately one million new cases are discovered and half a million cholecystectomies are performed (3-5). About 75% of the gallstones in the United States and Europe are cholesterol gallstones (6), which are subclassified as either pure cholesterol or mixed stones that contain at least half cholesterol by weight (Figure 1). The remaining gallstones are pigment stones that contain mostly calcium bilirubinate, which are subclassied into two groups: black pigment stones (20%) and brown pigment stones (5%) (Figure 1). Epidemiological investigations, clinical studies, and basic research during the past 50 years have clarified substantially our understanding of the pathogenesis of cholesterol gallstones. Family and twin studies in humans as well as gallstone prevalence investigations in inbred mouse models have clearly demonstrated that a complex genetic basis could play a key role in determining individual predisposition to develop cholesterol gallstones in response to environmental factors such as high cholesterol diet (7-10). Recently, a powerful genetic technique, quantitative trait locus (QTL) analysis has been used to identify, localize, and analyze the effects of pathophysiologically relevant gallstone (*Lith*) genes in inbred mice (1,11,12). Some excellent review articles on genetic analysis of cholesterol gallstone formation as well as molecular physiology of hepatic lipid transporters and biliary lipid secretion have been published (13-21). In this review, we will summarize recent progress in understanding the biochemistry of biliary lipids as well as physical-chemical basis of biliary cholesterol hypersecretion and cholesterol crystallization. These studies have provided many novel insights into the complex pathophysiological mechanisms that are involved in the formation of cholesterol gallstones.

3. PHYSICAL-CHEMISTRY OF BILE

3.1. Chemical compositions of bile

In bile, cholesterol, phospholipids and bile salts are three major lipid species and bile pigments are minor solutes. Cholesterol is present solely as the nonesterified alcohol, which accounts for approximately 95% of the total sterols in bile. The remaining 5% of the sterols are cholesterol precursors and dietary sterols from plant, animal and shellfish sources. As a major phospholipid in bile, lecithin (phosphatidylcholine) is an insoluble, swelling amphiphile with a hydrophilic, zwitterionic phosphocholine head group and hydrophobic tails including two long fatty acyl chains. Biliary lecithin is derived from the least hydrophobic hepatic lecithins and typically contains a saturated C-16 acyl chain in the sn-1 position and an unsaturated C-18 or C-20 acyl chain in the sn-2 position. The common bile salts possess a steroid nucleus of four fused hydrocarbon rings with polar hydroxyl functions and an aliphatic side chain conjugated in amide lindage with glycine or taurine. Because the ionized carboxylate or sulfonate group on the side chain renders bile salts water soluble, they are classified as soluble amphiphiles. The common bile salts differ in the number and orientation of the hydroxyl groups on the steroid nucleus. Bile salts synthesized from cholesterol in the liver are primary bile salts, which in humans are cholate, with three hydroxyl groups, and chenodeoxycholate, with two hydroxyl groups. Secondary bile salts are made from primary bile salts by the intestinal bacteria. Deoxycholate, with two hydroxyl groups, and lithocholate, with a single hydroxyl group, are formed from cholate and chenodeoxycholate, respectively. "Tertiary" bile salts are the result of modification of secondary bile salts by intestinal flora or hepatocytes. These are the sulfate ester of lithocholate and ursodeoxycholate, the 7beta-epimer of chenodeoxycholate. Bile pigments are minor solutes and account for approximate 0.5% of total lipids by weight in bile. They are mainly bilirubin conjugates with traces of porphyrins and unconjugated bilirubin. In humans, bile pigments include bilirubin mono- and diglucuronides predominating: invariably present are other mono- and diconjugates of xylose, glucose, and glucuronic acid as homo- and heteroconjugates.

Besides lipids, proteins and elements are found in bile. Albumin appears to be the most abundant protein in bile, followed by immunoglobulins G and M, apolipoproteins AI, AII, B, CI, and CII, transferring, alpha$_2$-macroglobin. Other proteins that have been identified but not quantitated in bile include epidermal growth factor, insulin, haptoglobin, cholecystokinin, lysosomal hydrolase, and amylase. Some of these proteins

are known to bind cholesterol and bile salts and have been implicated in affecting cholesterol nucleation/crystallization and precipitation in supersaturated bile. Elements detected in bile include sodium, phosphorus, potassium, calcium, copper, zinc, iron, manganese, molybdenum, magnesium, and strontium. Calcium ions bind to bile salt micelle and may affect Ca^{2+} anion salt precipitation, which may also influence cholesterol precipitation.

3.1.1. Cholesterol

Cholesterol is the principal sterol in bile and gallstones of humans, which accounts for up to 95% of sterols in both bile and gallstones. In addition, the non-cholesterol sterols are found in normal human bile and their pattern and proportions are broad and highly dependent on diet. In general, on a regular (nonshellfish) diet, the concentrations of non-cholesterol sterols in bile are less than 5%, and their pattern and proportions are cholestanol (1.5%), sitosterol (1.2%), campesterol (0.7%), lathosterol (0.6%), 24-methylene cholesterol (0.1%), stigmasterol (0.1%), brassicasterol (0.1%) and isofucosterol (0.03%). If a high shellfish diet is consumed, shellfish sterols in bile would be increased and consist of 5-10% of total sterols. Because of cocrystallization of certain non-cholesterol sterols with cholesterol as well as increased dietary intake of vegetable oils, the possible effects of non-cholesterol sterols on the precipitation of cholesterol from bile for the formation of gallstones need to be further investigated. Of note, the concentrations of cholesteryl esters are negligible in bile, but in gallstones they account for less than 0.02% of total sterols.

3.1.2. Phospholipids

The major phospholipids in human bile are lecithins (phosphatidylcholines), accounting for more than 95% of total phospholipids. The remainder is composed of cephalins (phosphatidylethanolamines) and a trace amount of sphingomyelin. The phospholipids comprise 15-25% of total lipids in bile. Similar to all naturally occurring phospholipids, biliary lecithins are a complex mixture of molecular species. The sn-1 position is esterified by the saturated fatty acyl chains 16:0 (~75%) and 18:0 (<20%), with small amounts of monounsaturated sn-1 16:1 or 18:0 comprising the remainder. The sn-2 position is esterified by unsaturated fatty acyl species, with 18:2, 18:1 and 20:4 fatty acids predominating. The major molecular species of lecithins in human bile are 16:0-18:2 (40-60%), 16:0-18:1 (5-25%), 18:0-18:2 (1-16%) and 16:0-20:4 (1-10%). Lecithins are principally synthesized in the endoplasmic reticulum of the liver from diacylglycerol by way of the cytidine diphosphate-choline pathway. Although a large variation in hepatic outputs of biliary bile salts, the proportion of lecithins to other phospholipid classes in bile is essentially constant. Of note, when bile becomes cholesterol-supersaturated, oleoyl and arachidonyl lecithins are increased and linoleoyl lecithins are reduced.

3.1.3. Bile salts

Bile salts comprise approximately two thirds of the solute mass of normal human bile by weight and are a family of closely related acidic sterols that are synthesized from cholesterol in the liver. The hydrophilic (polar) areas of bile salts are the hydroxyl groups and conjugation side chain of either glycine or taurine and their hydrophobic (nonpolar) area is the ringed steroid nucleus. Due to the possession of both hydrophilic and hydrophobic surfaces, bile salts are highly soluble, detergent-like amphiphilic molecules. Their high aqueous solubility is due to their capacity to self-assemble into micelles when a critical micellar concentration (CMC) is exceeded. Under normal physiological conditions, the CMC values for common bile salts are between 1 and 20 microM, which is dependent on the species of bile salts, the ionic strength and composition, and the types and concentrations of other lipids present in solution. Because bile is concentrated gradually within the biliary tree, bile salt concentrations steadily exceed their CMCs so that bile salts can form simple micelles in bile. Of note, micelles of bile salts can solubilize other types of lipids such cholesterol and phospholipids by forming mixed micelles in bile. The potency of bile salts as detergents depends critically upon the distribution and orientation of hydroxyl groups around the steroid nucleus of the molecule, which is usually conferred on its hydrophobicity. Bile salt hydrophobicity can be quantified by high performance liquid chromatography (HPLC) and used to predict the biological effects of individual bile salts (22). In addition, the physical-chemical properties of bile salts depend upon the nature and ionization state of functional groups on the side chain. In general, the glycine conjugate is more hydrophobic than the taurine conjugate. In human bile, more than 95% of bile salts are 5beta,C-24 hydroxylated acidic steroids amide-linked to taurine or glycine in an approximate ratio of 1:3. The primary bile salts are hepatic catabolic products of cholesterol and are composed of cholate (a trihydroxy bile salt) and chenodeoxycholate (a dihydroxy bile salt). Whereas, the secondary bile salts are derived from the primary bile salt species by the action of intestinal bacteria in the ileum and colon, being composed of deoxycholate, ursodeoxycholate, and lithocholate. The most important of these reactions is 7alpha-dehydroxylation of primary bile salts to produce deoxycholate from cholate, and lithocholate from chenodoxycholate. Another important secondary reaction is the 7alpha-dehydrogenation of chenodeoxycholate to form 7alpha-oxo-lithocholate. This bile salt does not accumulate in bile, but is metabolized to a “tertiary’ bile salts by hepatic or bacterial reduction to form chenodeoxycholate (mainly in the liver) or its 7beta-epimer, ursodeoxycholate (primarily by colonic bacteria).

3.2. Physical states of biliary lipids

Because bile is an aqueous solution and cholesterol is virtually insoluble in water, the mechanisms for the solubilization of cholesterol in bile are complex. Lecithins are also insoluble in water. So, they must travel together with bile salts for transport in bile. Both micelles and vesicles are the two main types of macromolecular aggregates in bile. It has been known that bile salts, either as simple solutions or as mixtures together with lecithin and cholesterol, can form micellar solutions. A micelle is a colloidal aggregation of molecules of an amphiphthic compound (i.e., bile salts) in which the hydrophobic portion of each molecule faces inward and the hydrophilic

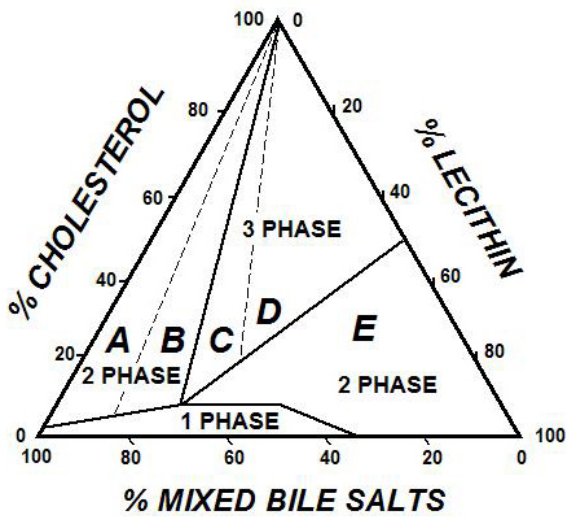

Figure 2. Equilibrium phase diagram of cholesterol-lecithin-mixed bile salt system (37°C, 0.15 M NaCl, pH 7.0, total lipid concentration 7.5 g/dL) showing positions and configuration of crystallization regions. The components are expressed in moles percent. The one-phase micellar zone at bottom is enclosed by a solid curved line. Above it, two solid lines divide the two-phase zones from a central three-phase zone. Based upon the solid and liquid crystallization sequences present in the bile, the left two-phase and central three-phase regions are divided by dashed lines into regions A to E. The number of phases given represents the equilibrium state. They are cholesterol monohydrate crystals and saturated micelles for crystallization regions A and B. Cholesterol monohydrate crystals, saturated micelles and liquid crystals for regions C and D, and liquid crystals of variable compositions and saturated micelles for region E. Of note is that decreases in temperature (37°C→4°C), total lipid concentration (7.5 g/dL→2.5 g/dL), and bile salt hydrophobicity (3alpha,12alpha→3alpha,7alpha→3alpha,7alpha,12alpha→3alpha,7beta hydroxylated taurine conjugates) progressively shift all crystallization pathways to lower phospholipid contents, as well as retard crystallization and reduce micellar cholesterol solubilities. These changes generate a series of new condensed phase diagrams with enlarged region E. Reproduced with permission from (24).

groups point outward. The cholesterol molecule is solubilized within the hydrophobic center of the micelle. Furthermore, biliary vesicles could also act as a cholesterol solubilizer in human bile, which are spherical membrane bilayers that contained mainly lecithin and cholesterol with only traces of bile salts. Vesicles are unilamellar (i.e., a single bilayer that encircles an aqueous core) or multilamellar (i.e., contain multiple concentric spherical bilayers). The precise compositions and proportions of micelles and vesicles are determined essentially by the concentrations of biliary lipids, which vary considerably within the biliary tree and gallbladder. In dilute biles (meaning a total lipid concentration less than 3 g/dL), vesicles are extremely stable, neither aggregating, fusing, nor nucleating cholesterol. Despite cholesterol monohydrate crystals are one of the equilibrium phases, they form very slowly in dilute bile. However, in concentrated gallbladder biles (~10 g/dL), vesicle instability is significantly increased and cholesterol precipitation is greatly accelerated.

Studying the molecular nature of the biliary lipid aggregates in bile of typical physiological compositions would help us in understanding the physical state of cholesterol-unsaturated and supersaturated biles in terms of the distribution of biliary lipid aggregates. Unsaturated bile is inferred from that cholesterol is solubilized in thermodynamically stable simple and mixed micelles, whereas "supersaturated" bile is inferred from that cholesterol is solubilized by the micellar particles and dispersed in unilamellar vesicles. Concerning relative lipid compositions and physical-chemical states of metastable model bile plotted on triangular coordinates (23,24), micellar bile falls within a micellar zone of the model bile system, whereas micellar plus vesicular bile falls outside this zone (Figure 2). The proportional distance outside the micellar zone directed along an axis joined to the cholesterol apex is often calculated as the cholesterol saturation index (CSI) or lithogenic index (25). Thus, the degree of saturation of bile with cholesterol can be quantitated. A saturation index for a sample of bile can be estimated directly from the diagram or can be calculated by using a formula. The saturation index is the ratio of the actual amount of cholesterol present in a bile sample to the maximal amount of cholesterol that can be dissolved in it. Bile that has a saturation index of 1 is "saturated"; less than 1 is "unsaturated"; and greater 1 one is "supersaturated". The degree of saturation can also be expressed as a percentage of saturation by multiplying the saturation index by 100. For example, at the boundary of the micellar zone, bile is saturated, and the CSI is 100%. Supersaturated bile has a CSI above 100% and unsaturated bile has a CSI below 100%. The CSI values are also very useful in predicting both the proportions of lipid particles as well as the metastable and equilibrium physical-chemical states in bile.

3.2.1. Biliary micelles

The molecules of bile salts have both hydrophilic and hydrophobic areas with the property of amphiphilicity so that they are soluble in aqueous solutions to varying degrees, depending on the number and characteristics of hydroxyl groups and side chains as well as the composition of the particular aqueous solution. Bile salt monomers can aggregate spontaneously to form simple micelles when their concentration exceeds the CMC. These simple micelles (~3 nm in diameter) are small, thermodynamically stable aggregates that can solubilize cholesterol. They are shaped like disks, which has been characterized in physiologically relevant model biles and in native biles. The formation of simple micelles of bile salts alone depends mainly on their concentrations. Thus, micelles form at, and not below, a CMC of bile salts in bile, which is about 2 mmol/L. Also, simple micelles are capable of solubilizing and incorporating phospholipids, which enables the micelles, then referred to as mixed micelles, to solubilize at least triple the amount of cholesterol

solubilized by simple micelles. Mixed micelles (~4-8 nm in diameter) are large, thermodynamically stable aggregates that are composed of bile salts, cholesterol, and phospholipids. Their size varies depending on the relative proportion of bile salts and phospholipids. The shape of a mixed micelle is that of a lipid bilayer with the hydrophilic groups of the bile salts and phospholipids aligned on the "outside" of the bilayer, interfacing with the aqueous bile, and the hydrophobic groups on the "inside". Therefore, cholesterol molecules can be solubilized on the inside of the bilayer away from the aqueous areas on the outside. The amount of cholesterol that can be solubilized depends on the relative proportions of bile salts and maximal solubility occurs when the molar ratio of phospholipids to bile salts is between 0.2 and 0.3. Furthermore, solubility of cholesterol in mixed micelles is enhanced when the concentration of total lipids (bile salts, phospholipids, and cholesterol) in bile is increased.

3.2.2. Biliary vesicles, nonmicellar carriers of cholesterol in bile

Clinical observations have found that: (i) some subjects without gallstones or crystals have bile that is supersaturated; (ii) there is a difference in metastability between patients with and without cholesterol gallstones; and (iii) some patients with cholesterol gallstones have cholesterol solubility greatly exceeding the limits of metastability (23, 26). These findings suggest that besides micellar systems of bile acids, other mechanisms must exist for the solubilization of cholesterol in bile. By using techniques such as quasi-elastic light-scattering spectroscopy and electron microscopy to examine model and native biles (27-33), a more complex mechanism of cholesterol solubilizaiton in bile has been defined. It has been found that gallbladder biles from many healthy individuals are supersaturated with cholesterol, indicating that cholesterol concentrations exceeded what could be solubilized by micellar particles. Biliary vesicles were first reported in 1983 and this finding clarified substantially our understanding of cholesterol solubility in bile, including metastability (23, 34), liquid crystal formation (35, 36), and cholesterol nucleation and crystallization in bile (37-39). Vesicles are unilamellar spherical structures, and contain phospholipids and cholesterol and little, if any, bile salts. Thus, vesicles (~40 to 100 nm in diameter) are substantially larger than either simple or mixed micelles, but much smaller than liquid crystals (~500 nm in diameter) that are composed of multilamellar spherical structures. Because vesicles are present in large quantities in hepatic bile, they are presumably secreted by the hepatocyte. As a result of the time-dependent physical-chemistry of postcanalicular events during bile formation and because of very slow disappearance of vesicles by micellar dissolution, unilamellar vesicles are often detected in all freshly collected cholesterol-unsaturated biles and are physically indistinguishable from those in supersaturated biles. Moreover, dilute hepatic biles, in which cholesterol gallstones never formed, are always supersaturated with cholesterol in a high proportion of people because vesicles are the particle responsible for solubilizing biliary cholesterol in excess of what could be solubilized in mixed micelles. Consistent with the absence of cholesterol crystallization in hepatic biles, cholesterol-rich vesicles proved to be remarkably stable in dilute biles. The unilamellar vesicles can fuse and form large multilamellar vesicles (also known as liposomes or liquid crystals). It has been suggested that crystals of cholesterol may nucleate from multilamellar vesicles in concentrated gallbladder bile.

3.2.3. Co-existence and interconversion of micelles and vesicles in bile

Although vesicles are relatively static structures, the dynamics of vesicles, in terms of size, composition, and interaction with each another and with micelles, is influenced by several factors such as bile concentrations and the relative ratios of cholesterol, phospholipids, and bile salts in bile. The relative ratios of three lipids are influenced by the fasting or feeding state through alterations in bile secretion rates. For example, during fasting, biliary bile salt output is relatively low. Consequently, the ratio of cholesterol to bile salts is increased and cholesterol is carried more in vesicles than in micelles. In contrast, during meals, biliary bile salt output is higher and more cholesterol is solubilized in micelles. In addition, when the concentration of bile salts is relatively low, especially in dilute hepatic bile, vesicles are relatively stable and only some vesicles could convert to micelles. In contrast, vesicles may transform or convert completely to mixed micelles with increasing bile salt concentrations in concentrated gallbladder bile. Because relatively more phospholipids than cholesterol can be transferred from vesicles to mixed micelles, the residual vesicles are remodeled, which may be enriched in cholesterol relative to phospholipids. If the remaining vesicles have a relatively low cholesterol/phospholipid ratio (less than 1), they are relatively stable. However, if the cholesterol/phospholipid ratio in vesicles is greater than 1, vesicles become increasingly unstable. These cholesterol-rich vesicles may transfer some cholesterol to less cholesterol-rich vesicles or to micelles, or may fuse or aggregate to form larger (~500 nm in diameter) multilamellar vesicles (i.e., liposomes or liquid crystals). These liquid crystals are visible by polarizing light microscopy as lipid circular droplets with typical birefringence in the shape of a Maltese cross (Figure 3). Liquid crystals are inherently unstable and could form solid plate-like cholesterol monohydrate crystals (Figure 3), which is termed cholesterol nucleation. As a result, nucleation of cholesterol monohydrate crystal results in a decrease in the amount of cholesterol contained in vesicles but not of cholesterol in micelles, supporting the concept that vesicles serve as the primary source of cholesterol for nucleation.

Although bile is rarely at equilibrium, the physical states of biliary lipids at equilibrium have provided the basis of physical-chemistry for describing biliary lipid aggregation *in vivo*. Because bile is gradually concentrated within the biliary tree, bile salt concentrations approach their CMC values. When this occurs, bile salts begin to modify the structure of phospholipid-rich vesicles that are secreted into bile by the hepatocyte. These interactions indicate the start of a complex series of molecular rearrangements that ultimately form simple and

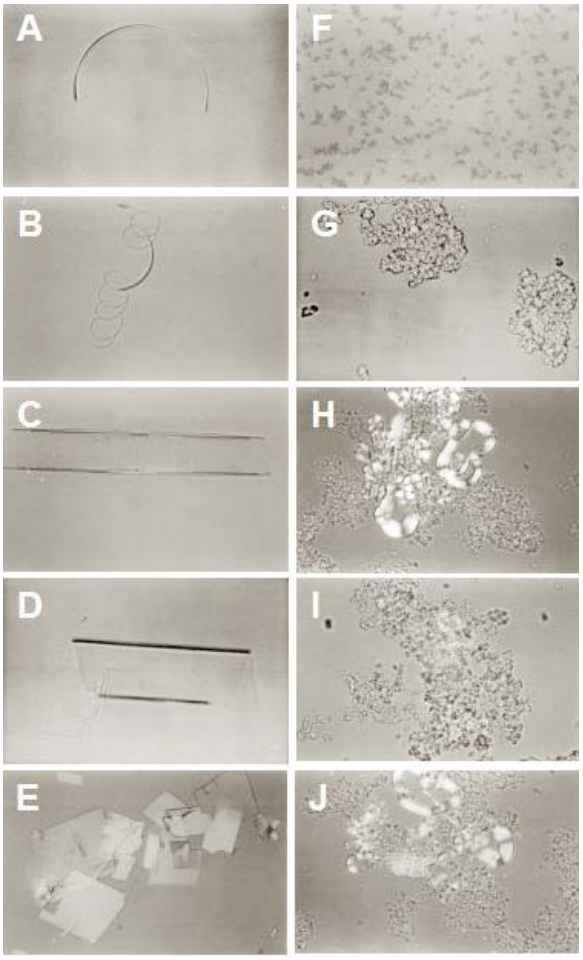

Figure 3. Habits of liquid crystals and solid plate-like cholesterol monohydrate crystals observed by polarizing light microscopy: (A) arc-like crystal; (B) irregular right-handed helical crystal; (C) tubular crystal with helical stripes; (D) tube-like crystal fracturing at ends to produce plate-like cholesterol monohydrate crystals; (E) typical cholesterol monohydrate crystals, with 79.2 and 100.8 angles, and often a notched corner; (F) small non-birefringent liquid crystals (labeled "small"); (G) aggregated non-birefringent liquid crystals (labeled "aggregated"); (H) typical fused liquid crystals (labeled "fused") with Maltese-cross birefringence and focal conic textures; (I) cholesterol monohydrate crystals emerging from an aggregated liquid crystal; and (J) same, from fused liquid crystals. All magnifications x 800. Reproduced with permission from (24).

mixed micelles. In human bile supersaturated with cholesterol, there are two pathways for forming cholesterol-rich vesicles from phospholipid-rich vesicles that are assembled at the canalicular membrane. Because bile salts solubilize phospholipids more efficiently than cholesterol, cholesterol-rich vesicles may form when bile salts preferentially extract phospholipid molecules directly from phospholipid-rich vesicles. The second possibility that has been observed in model bile systems is rapid dissolution of phospholipid-rich vesicles by bile salts. This alteration produces unstable mixed micelles with excess cholesterol. Consequently, structural rearrangements of these unstable micellar particles result in the formation of cholesterol-rich vesicles.

4. PHYSIOLOGY OF BILIARY LIPID SECRETION

4.1. Source of lipids secreted in bile

Under low or no dietary cholesterol conditions, bile contains newly synthesized cholesterol from the liver and preformed cholesterol that reaches the liver from several different ways. Approximately 20% of the cholesterol in bile come from *de novo* hepatic biosynthesis and 80% are from pools of preformed cholesterol within the liver. *De novo* cholesterol synthesis in the liver uses acetate as a substrate and is regulated mainly by a rate-limited enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. This enzyme can be up- or down-regulated depending on the overall cholesterol balance in the liver. An increase in the activity of this rate-limiting enzyme could lead to excessive cholesterol secretion in bile. The major sources of preformed cholesterol are hepatic uptake of plasma lipoproteins (mainly HDL, LDL, and VLDL through their receptors on the basolateral membrane of hepatocyte). Consistent with their central role in reverse cholesterol transport, HDL particles are the main lipoprotein source of cholesterol that is targeted for biliary secretion. Furthermore, the metabolic determinants of the supply of hepatic cholesterol molecules that can be recruited for biliary secretion depend upon cholesterol input-output balance and its catabolism in the liver. Input is related to the amount of cholesterol (both unesterified and esterified) taken up by the liver from plasma lipoproteins (LDL>IDL>HDL>chylomicron remnants) plus *de novo* hepatic cholesterol synthesis. Output is related to the amount of cholesterol disposed of within the liver in conversion to cholesterol ester (to form new VLDL plus ester storage) minus the amount of cholesterol converted to primary bile salts. An appreciable fraction of cholesterol in bile also may be derived from the diet via apolipoprotein E-dependent delivery of chylomicron remnants to the liver. Under conditions of a diet with high cholesterol, dietary cholesterol reaches the liver through the intestinal lymphatic pathways as of chylomicrons, and then, chylomicron remnants after chylomicrons are hydrolyzed by plasma lipoprotein lipase and hepatic lipase. Newly synthesized cholesterol in the liver is reduced and consists of only approximately 5% of biliary cholesterol. Overall, the liver can systematically regulate the total amount of cholesterol within it, and any excess cholesterol is handled efficiently.

Although biliary phospholipids are derived from the cell membranes of hepatocytes, their compositions differ markedly. The membranes of hepatocyte contain phosphatidylcholine (lecithins), phosphatidylethanolamines, phosphatidylinositols, phosphatidylserines and sphingomyelins. The major source of phosphatidylcholine molecules destined for secretion into bile is hepatic synthesis. However, a fraction of biliary phosphatidylcholines may

also originate in the phospholipid coat of HDL particles. Approximately 11 g of phospholipids are secreted into bile per day in humans.

More than 95% of bile salt molecules, which are secreted into bile, have returned to the liver through the enterohepatic circulation by absorption mostly from the distal ileum due to active transport. As a result, newly synthesized bile salts in the liver contributes only a small fraction (<5%) to biliary secretion, which compensate for bile salts that escape intestinal absorption and lost in feces. The fecal excretion of bile salts is increased under conditions in which the enterohepatic circulation of bile salts is partially or completely interrupted by surgery, disease states, or drugs (e.g., bile salt-binding resins such as cholestyramine). Complete interruption of the enterohepatic circulation results in up-regulation of bile salt synthesis, which could restore bile salt secretion rates to approximately 25% of their native values. Two sources of cholesterol in the smooth endoplasmic reticulum can serve for *de novo* bile salt synthesis, including cholesterol molecules that are newly synthesized in the smooth endoplasmic reticulum and that are preformed outside the smooth endoplasmic reticulum. The first step in this process is catalysed by the enzyme called cholesterol 7alpha-hydroxylase. It has been found that in the basal state, *de novo* bile salt synthesis principally utilizes newly synthesized cholesterol as substrate in humans. However, because long-term therapy of competitive HMG-CoA reductase inhibitors (statins) could impair *de novo* cholesterol biosynthesis to a significantly lesser extent, it is likely that preformed cholesterol from plasma lipoprotein origin could substitute for newly synthesized cholesterol.

4.2. Hepatic secretion of biliary lipids

Physical evidence has found that bile salts stimulate secretion of vesicles and unilamellar vesicles are always detected in freshly collected hepatic biles. When cultured under specified conditions, rat hepatocytes form couplets with isolated “bile canaliculi” at interfaces between adjoining cells. Using laser light-scattering techniques, vesicle formation has been observed within these “bile canaliculi” after exposure to bile salts. Finally, rapid fixation techniques and electronic microscopy have provided direct morphologic evidence for vesicle formation at the outer surface of the canalicular membrane. It has been proposed that most, if not all, bile salts enter canalicular spaces as monomers and that biliary phospholipids and cholesterol enter as unilamellar vesicles. Unilamellar vesicles have been visualized and their sizes have been determined within canalicular spaces of rat liver by transmission electron microscopy and quasi-elastic light-scattering spectroscopy. The radii of these vesicles by both methods are in general agreement and vary in a range of 60 to 80 nm. Furthermore, to quantify the changing proportions of micelles to vesicles, the bile formation path in a cholesterol-unsaturated fresh rat bile is investigated by dynamic light scattering spectroscopy. It is found that these vesicles are apparently of more variable size, usually 40 to 120 nm in radius. The physical-chemical responses of biliary lipids are not instantaneous, and bile that is collected form hepatic ducts as well as from gallbladder is still undergoing equilibration. More recently, it is found that the principal driving forces for biliary lipid secretion are ATP-dependent membrane transporters that are located on the canalicular membrane of the hepatocyte.

Although the mechanisms by which cholesterol is incorporated into biliary vesicles are not yet elucidated, recent evidence from the study of molecular genetics of sitosterolemia shows that efflux of biliary cholesterol from the canalicular membrane could be protein-mediated. Two plasma membrane proteins, ATP-binding cassette (ABC) transporters ABCG5 and ABCG8, promote cellular efflux of cholesterol and its significance for bile formation has been examined in genetically modified mice (40-44). Overexpression of ABCG5 and ABCG8 in the liver increases the cholesterol content of gallbladder bile. In addition, scavenger receptor class B type I (SR-BI) is localized in sinusoidal, and possibly, in canalicular membranes of the hepatocyte. In transgenic and knockout mice, biliary secretion of cholesterol varies in proportion to hepatic expression of SR-BI and the established contribution of SR-BI to sinusoidal uptake of HDL cholesterol that is destined for secretion into bile. Furthermore, cholesterol contents of vesicles may be determined simply by the degree to which cholesterol partitions into phosphatidylcholine-rich microdomains in the canalicular membrane of hepatocyte. Because of the high affinity of sphingomyelin for cholesterol, microdomains form in membranes with sufficient amounts of cholesterol and sphingomyelin on the canalicular membrane (45). In addition, another possibility is that bile salts could regulate partitioning of cholesterol into nascent vesicles. Because bile salts reduce the affinity of cholesterol for sphingomyelin, they could induce migration of cholesterol into phosphatidylcholine-rich microdomains. More recently, it is found that despite a reduced gallstone prevalence rate, the formation of gallstones is still observed in ABCG5/G8 double knockout mice as well as in ABCG5 or ABCG8 single knockout mice challenged to the lithogenic diet. These findings strongly suggest an ABCG5/G8-independent pathway for hepatic secretion of biliary cholesterol and its role in the formation of cholesterol gallstones.

It has been found that hepatic secretion of biliary phospholipids is completely inhibited in mice with deletion of the *Abcb4* gene (46), a P-glycoprotein member of the multi-drug resistance gene family. It has been proposed that ABCB4 could be responsible for translocation or “flip” of phosphatidylcholines from the endoplasmic (inner) to ectoplasmic (outer) leaflet of the canalicular membrane bilayer, and the action of ABCB4 may form phosphatidylcholine-rich microdomains within the outer membrane leaflet. Bile salts may partition preferentially into these areas to destabilize the membrane and release phosphatidylcholine-rich vesicles, because detergent-like bile salt molecules within the canalicular space could interact with canalicular membrane. A number of diverse observations indicate that bile salts promote vesicular secretion of biliary cholesterol and phosphatidylcholines, in spite of that the ectoplasmic leaflet of the canalicular membrane is cholesterol- and sphingomyelin-rich and is

relatively resistant to penetration by bile salts. Biliary secretion of organic anions in laboratory animals does not influence bile salt secretion, but does inhibit secretion of phospholipid and cholesterol into bile, because organic anions bind bile salts within bile canaliculi and prevent interactions with the canalicular membrane. Indeed, mutations of the *ABCB4* gene in humans are the molecular defect underlying type 3 progressive familial intrahepatic cholestasis (PFIC3).

Biliary bile salts consist of those that are newly synthesized in the liver and those undergoing enterohepatic cycling. The precise mechanism of bile salt secretion is not known, although it probably involves a transport protein, ABCB11, a bile salt export pump (47-49). It is not known that the molecular mechanism by which bile salt secretion is coupled to cholesterol and phospholipid secretion, although hepatic secretion of bile salts directly affects cholesterol-phospholipid vesicle secretion. The relationship between bile salt secretion and cholesterol secretion is curvilinear. At low bile salt secretion rates, generally less than 10 micromol/h/kg, more cholesterol is secreted per molecule of bile salt than at higher rates. Although bile salt secretion rates are not usually low in normal subjects, they diminish during prolonged fasting, during the overnight period, and with substantial bile salt losses such as with a biliary fistula or ileal resection when the liver cannot compensate sufficiently with increased bile salt synthesis. At high bile salt secretion rates - for example, during and after eating - biliary saturation is less than during interprandial periods.

5. PATHOPHYSIOLOGY OF CHOLESTEROL GALLSTONE FORMATION

There is now a general agreement that hepatic hypersecretion of biliary cholesterol is the primum movens in the pathogenesis of cholesterol gallstone formation. Relative cholesterol hypersecretion into bile may or may not be accompanied by normal, high, or low secretion rates of biliary bile salts or phospholipids. By definition, bile supersaturated with cholesterol contains cholesterol that cannot be solubilized *at equilibrium* by bile salts and phospholipids. Cholesterol supersaturation could result from (i) hepatic hypersecretion of biliary cholesterol; (ii) decreased rates of bile salt or phospholipid secretion into bile; or (iii) a combination of cholesterol hypersecretion with hyposecretion of the solubilizing lipids. With the passage of time and in the presence of a heterogeneous pro-nucleating agent, usually mucin gel, cholesterol supersaturation leads to precipitation of cholesterol monohydrate crystals from a phase-separated liquid-crystalline phase in gallbladder bile, followed by agglomeration and growth of the crystals into mature and macroscopic stones. Many biliary proteins besides mucin gel have been proposed as either pro-nucleating or anti-nucleating factors influencing cholesterol nucleation/crystallization in bile; however, their *in vivo* pathophysiological role, if any, in the pathogenesis of cholesterol gallstone formation remains unclear. Furthermore, proteolysis of soluble biliary glycoproteins does not influence the speed of cholesterol monohydrate crystal detection time either in normal or abnormal gallbladder and hepatic biles, suggesting that soluble biliary proteins may not be an important pathophysiological factor affecting cholesterol crystallization. Gallbladder motility is compromised quite early in gallstone formation, most likely because of gallbladder absorption of cholesterol molecules from supersaturated bile and their incorporation into sarcolemmal membranes. The absorbed cholesterol may induce signal-transduction decoupling in the gallbladder smooth muscle, presumably because of stiffening of the lipid membranes. As a result, the gallbladder becomes "sluggish", i.e., shows enlarged fasting volume and does not appropriately respond to cholecystokinin (CCK) stimulation during meals. Increased postprandial residual volume, a marker of incomplete emptying and gallbladder stasis, leads to increased enterohepatic cycling of the bile salt pool. This results in greater bacterial catabolism of bile salts and augmented hepatic cholesterol hypersecretion as well as more rapid nucleation/crystallization in gallbladder bile.

5.1. Phase diagrams and cholesterol solubility in bile

Bile salts and phospholipids are the principal agents of solubilization of cholesterol in bile. In the 1960s, Small and colleagues defined the maximal solubility (saturation) limits for cholesterol in model bile systems with varying proportions of cholesterol, phospholipids, bile salts, and water together in a quaternary system (50-54). The relative proportions of three lipids in bile play a critical role in determining the maximal solubility of cholesterol (23,52). When the relative proportions (molar percentages) of three lipids at a fixed total lipid concentration are plotted in a triangular coordinate, they are used to express the solubility of cholesterol for any given solute concentration. In addition, the number and type of phases determined when the system reaches equilibrium, are plotted on these triangular phase diagrams. In the 1970s, these findings were further confirmed in native biles of humans and animals. Therefore, the composition of any bile sample can be represented by a single point on such a triangular coordinate.

The triangular coordinate diagram also illustrates the physical phases of cholesterol in bile. For example, the phase diagram shown in Figure 2 is specific for a total lipid concentration of 7.5 g/dL, which is typically in human gallbladder biles. For hepatic biles, with a typical total lipid concentration of 3 g/dL, the phase boundaries would be different, with a smaller micellar zone. This effect of total lipid content on cholesterol solubilization in the micellar zone explains why hepatic bile tends to be more supersaturated than gallbladder bile in the same patient. Because hepatic bile contains cholesterol-phospholipid vesicles that are relatively stable, solid cholesterol monohydrate crystals are less likely to occur in hepatic bile than gallbladder bile. In addition, phase diagrams are specific in terms of defining solubility not just for different proportions of lipids but also for different total lipid concentrations. Within the micellar zone, bile is in a visually clear stable solution, which is termed "unsaturated" because it is capable of solubilizing more cholesterol. At the boundary line of the micellar zone, bile

is termed "saturated" because all the solubilizing capacity for cholesterol is utilized; no further cholesterol can be carried in micelles. Outside the micellar zone, bile is termed "supersaturated" because excess cholesterol cannot be solubilized within micelles. Under these circumstances, cholesterol exists in more than one phase (micelles, liquid crystals, and solid monohydrate crystals) and the solution is visually cloudy. Obviously, relatively stable unilamellar cholesterol-phospholipid vesicles solubilize a significant proportion of cholesterol outside the micellar zone. The term metastable zone refers to the area in the phase diagram (above but near the micellar zone) where bile is supersaturated with cholesterol but may not form solid cholesterol crystals even after many days. The diagram also suggests that when the quantity of cholesterol in bile exceeds that which can be solubilized by the available bile salts and phospholipids, the cholesterol crystallizes out of solution.

Equilibrium phase diagrams are also used to predict the phases in which cholesterol crystals can be found at equilibrium. Although the equilibration process starts after bile secretion from the hepatocyte and continues in the biliary tree, the evolution to cholesterol monohydrate crystals only occurs in the gallbladder. Cholesterol-unsaturated bile is not at thermodynamic equilibrium especially in hepatic bile, in which all cholesterol can be solubilized in the coexistence of simple and mixed micelles and therefore relative lipid compositions lying in the micellar zone of the appropriate phase diagram. In contrast, cholesterol supersaturated bile that cannot be completely solubilized by the coexistence of simple and mixed micelles, has relative lipid compositions lying outside the micellar zone of the metastable phase diagram. Of note, high degrees of vesicle cholesterol concentrations and high total lipid concentrations are two principal factors that work together in producing the solid crystalline phase. Therefore, with typical physiological lipid ratios, at equilibrium, cholesterol monohydrate crystals are present with saturated simple plus mixed micelles, or with saturated micelles plus vesicles that have become multilamellar liquid crystals. The final physical chemical state is also determined by bile salt to lecithin ratio as well as overall hydrophilic-hydrophobic balance of both bile salt and lecithin species.

5.2. Classification of defects leading to supersaturation

On the basis of the mechanisms for the saturation of bile with cholesterol, the recognized defects leading to supersaturation can be classified into four types.

5.2.1. Type 1

Excessive secretion of biliary cholesterol. Excessive cholesterol is secreted by the hepatocyte into bile, which causes supersaturated bile despite biliary secretion rates and normal pool sizes of bile salts. It is often observed in obese patients with gallstones.

5.2.2. Type 2

This defect is often found in nonobese patients with gallstones. The secretion of cholesterol is normal in these patients. The possible explanation is that relatively low rates of recirculating bile salts to the liver are adequate to depress the hepatic synthesis of bile salts, i.e., oversensitive hepatic response to feedback of bile salts. During the development of this inappropriate hepatic response to the feedback of bile salts, more bile salts are lost from the enterohepatic circulation than are synthesized, although the rate of synthesis is normal or low. Consequently, these alterations result in a reduced pool size, hepatic return, and secretion rate of bile salts. In some of these patients, the size of the pool may be normal, but the secretion rate of bile salts is reduced because of diminished enterohepatic circulations of the pool.

5.2.3. Type 3

Excessive loss of bile salts. Excessive loss of bile acids occurs in patients with ileectomy, diseases of the ileum (e.g., Crohn's disease), or surgical bypass of the ileum. These changes result in a reduced return of bile salts to the liver, but the maximal rate of normal hepatic synthesis of bile salts cannot make up for the significant loss. Therefore, the pool and the hepatic secretion of bile salts are decreased, inducing a supersaturated bile despite a normal secretion of cholesterol.

5.2.4. Type 4

Mixed defect. This defect, a combination of types 1 and 2, has been found in both American Indians and Caucasians with gallstones. In these patients, an oversensitive hepatic response to the feedback of bile salts (causing decreased biliary secretion of bile salts), combined with an increased biliary secretion of cholesterol, produce supersaturated bile. The decreased hepatic synthesis of bile salts (when present) and increased biliary secretion of cholesterol in these patients may be the result of diminished 7-hydroxylation of cholesterol.

5.3. Nucleation/crystallization of solid cholesterol monohydrate crystals from supersaturated bile

Cholesterol nucleation/crystallization from gallbladder biles in patients with cholesterol gallstones is conventionally defined as the earliest day that plate-like cholesterol crystals are detected by polarizing light microscopy in a sample of bile previously rendered crystal-free ("isotropic") (37). Rapid *in vitro* nucleation/crystallization of cholesterol monohydrate crystals from the "isotropic phase" of gallbladder bile discriminates lithogenic gallbladder biles of cholesterol gallstone patients from cholesterol-supersaturated biles of controls (55-58). In understanding the kinetics, characteristics and metastable intermediates in the phase transitions of bile, the phase diagram for cholesterol, phospholipids and bile salts has been utilized as a template to study the regions wherein different sequences of metastable intermediates occur. Recently, five crystallization pathways have been identified in model biles as functions of bile salt to phospholipid ratio, total lipid concentration, bile salt species (hydrophilic/hydrophobic series), temperature, and cholesterol saturation indices (24). Furthermore, these crystallization pathways are detected and confirmed in fresh human and mouse biles (10,59-61). As shown in Figure 4, for cholesterol-phospholipid-mixed bile salt model systems, five distinct crystallization pathways (A-E) have been defined, each illustrating a

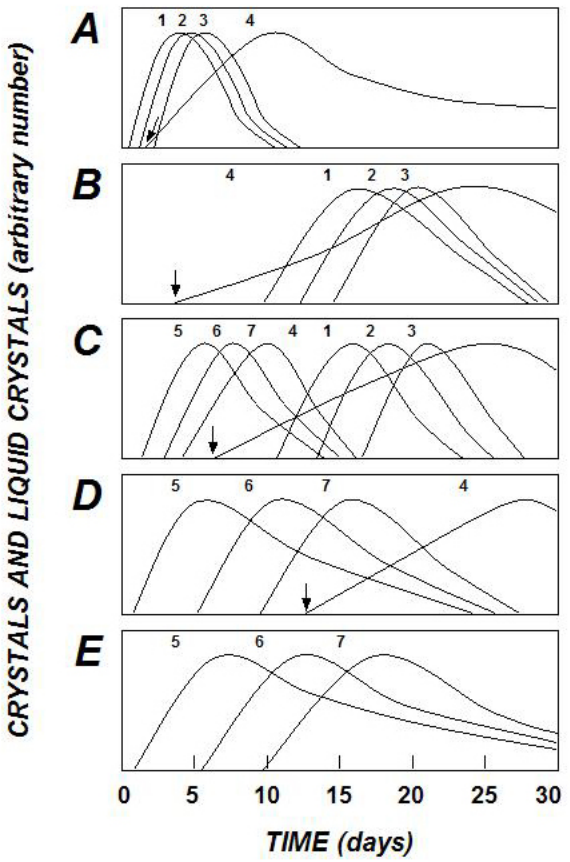

Figure 4. Crystallization sequences are subdivided into pathways A, B, C, D, and E based upon the principal habits observed in a cholesterol-lecithin-mixed bile salt system (37°C, 0.15 M NaCl, pH 7.0, total lipid concentration 7.5 g/dL). The vertical axes represent arbitrary numbers of crystals and liquid crystals per high power microscopic field, and all are normalized to the same maximum. Consecutive numbers represent (1) arc (most likely anhydrous cholesterol), (2) helical, (3) tubular, and (4) plate-like cholesterol monohydrate crystals, and (5) small, (6) aggregated, and (7) fused liquid crystals, respectively. The detection times of cholesterol monohydrate crystals (arrows on baseline) are retarded progressively between pathways A and D; cholesterol monohydrate crystals do not appear in pathway E. Because of repeated sampling, relative number of cholesterol monohydrate crystals and liquid crystals are decreased artifactually at later time points. When frequent sampling is not carried out, microscopic liquid crystals are invariably present at 30 days in pathways C to E but are absent from pathways A and B. When temperature, total lipid concentration, and bile salt hydrophobicity are decreased, the curves of anhydrous cholesterol crystals (numbers 1 to 3) and cholesterol monohydrate crystals (number 4) move to the right, whereas those of liquid crystals (numbers 5 to 7) shift to the left. Reproduced with permission from (24).

different sequence of phase transitions, including an anhydrous cholesterol pathway and a liquid crystalline pathway to the formation of solid cholesterol monohydrate crystals (24,62). Interestingly, transient arc-like crystals appear in some pathways and these are consistent with crystalline 'anhydrous' cholesterol (24,62-65). It is unknown why anhydrous cholesterol crystals should precipitate in an aqueous environment but it is characteristic of crystallization pathways that seem to originate from unilamellar as opposed to multilamellar vesicles. It appears that in these pathways the critical nucleus may be a unilamellar vesicle containing liquid anhydrous cholesterol molecules in its core, possibly reflecting internal nucleation. So, these early vesicular 'nuclei' may have already initiated the nucleation cascade by the time bile enters the gallbladder. The current paradigm for nucleation/crystallization, based principally on video-enhanced polarized light microscopy, suggests that biliary vesicles must fuse or at least aggregate for the formation of the crystalline cholesterol monohydrate phase (38,39). Since cholesterol nucleation/crystallization is apparently initiated in vesicles, vesicle stability determines the stability of bile. Unstable vesicles can fuse, aggregate, and grow into multilamellar liquid crystalline structures (liposomes) in which cholesterol crystallizes out of solution. Furthermore, evidence from quasi-elastic light-scattering spectroscopy shows that nucleation of solid cholesterol crystals could occur directly from supersaturated micelles in conjugated deoxycholate-rich biles *in vitro* without an intervening vesicle or liquid crystalline phase. This may occur because of the unusual phase equilibria relationships of the ternary systems composed with very hydrophobic bile salts.

Figures 2 and 4 illustrate that with the lowest phospholipid contents (region A), arc-like crystals with habit and density (d=1.030 g/mL) consistent with anhydrous cholesterol appear first and evolve via helical and tubular crystals to form plate-like cholesterol monohydrate crystals (d=1.045 g/mL). With higher phospholipids (region B), cholesterol monohydrate crystals appear earlier than arc and other transitional crystals. With typical physiological phospholipid contents (region C), early liquid crystals (d=1.020 g/mL) are followed by cholesterol monohydrate crystals and subsequently, arc and other intermediate crystals appear. With still higher phospholipid contents (region D), liquid crystals are followed by cholesterol monohydrate crystals only. At the highest phospholipid mole fractions (region E), liquid crystals are stable and no solid crystals form. Decreases in temperature (37°C→4°C), total lipid concentration (7.5 g/dL→2.5 g/dL), and bile salt hydrophobicity (3alpha,12alpha→3alpha7alpha→3alpha,7alpha,12alpha→ 3alpha,7beta hydroxylated taurine conjugates) progressively shift all crystallization pathways to lower phospholipid contents, as well as retard crystallization and reduce micellar cholesterol solubilization. It has been found that cholesterol crystallization pathways and sequences in human gallbladder biles are identical to model biles matched for appropriate physical-chemical conditions, and in a physiological state, three of the five sequences observed in model biles are found in human and mouse gallbladder biles. Most notably, the kinetics of all these phase transitions are faster in lithogenic human biles compared with identically patterned model biles, most likely due, in part, to the combined influences of increased

levels of cholesterol, secondary bile salts, and mucin glycoproteins. In addition, biliary lipid, electrolyte, and protein factors are important in stabilizing supersaturated bile.

Nonprotein factors that retard cholesterol nucleation/crystallization are as follows: (i) total lipid concentrations less than 3 g/dL; (ii) reduced hydrophobicity of the bile salt pool; (iii) low bile salt-to-lecithin ratios; (iv) low cholesterol-to-lecithin ratios in vesicles; and (v) low total calcium ions. The converse of these conditions accelerates nucleation/crystallization. Apart from mucin glycoproteins, the true identity, quantitative importance, and *in vivo* roles of putative pro- and anti-nucleating proteins in human biles are much less certain. Mucin glycoproteins are secreted continuously by gallbladders and in great excess during cholesterol lithogenesis (see paragraphs 5.4.2, 5.4.3, and 5.4 for details).

5.3.1. Nucleation time (cholesterol crystal detection time)

Clinical investigations reveal that biles from patients with cholesterol gallstones and from controls are often supersaturated with cholesterol and that the degree of cholesterol supersaturation is not a reliable predictor of gallstones. For identical CSI, there is a clear-cut discrimination in nucleation times between bile in patients with cholesterol gallstones and control patients. Nucleation time is the time until the first appearance of plate-like cholesterol monohydrate crystals in isotropic bile incubated *in vitro* at 37°C (37). This measurement provided a reliable means of distinguishing biles from cholesterol gallstone patients and controls. Moreover, differences in nucleation times suggest that human biles may contain factors that determine gallstone formation by inhibiting or accelerating the appearance of cholesterol crystals. Of note, growth of cholesterol crystals is an event distinct from nucleation. In practice, however, the precise moment of nucleation cannot be distinguished clearly from growth of cholesterol crystals in bile. Despite this limitation, combined measurements of nucleation and crystal growth (i.e., cholesterol crystal detection time) have been standardized, quantified, and automated. These have proven useful in the search for factors that influence cholesterol crystallization in human bile.

The precise molecular mechanisms of cholesterol nucleation remain incompletely understood and the study of the nucleating process is limited by available laboratory techniques. Of note, many of the conditions are associated with this process. The process of nucleation may be homogeneous or heterogeneous. Homogeneous nucleation occurs in the labile zone of supersaturation (above the metastable zone), where cholesterol crystals rapidly form without the need for promoters of nucleation. In the initial process of homogeneous nucleation of cholesterol crystals from supersaturated bile, vesicles separate from the mixed micellar system. This partitioning probably is due to stratification of the particles according to their density in gallbladder bile. Then, these vesicles fuse and aggregate until multilamellar vesicles form. Finally, the multilamellar vesicles fuse and aggregate, which could eventually result in the formation of solid cholesterol crystals. This process occurs slowly but can be accelerated by pro-nucleating agents. Bile from the gallbladder of most patients with cholesterol gallstones has a composition that falls with in the metastable zone and, therefore, is prone to heterogeneous nucleation. The observations from a video-enhanced imaging study suggest that before cholesterol nucleation, cholesterol-rich unilamellar vesicles aggregate to form multilamellar vesicles (38,39). It is likely that vesicles represent the main source of cholesterol for nucleation, as supported by biochemical analyses and high-resolution cryoelectron microscopy. These observations shows that an early step in this process may be the fusion of unilamellar cholesterol-rich vesicles and liquid crystals are an intermediate stage in this process. When the resulting cholesterol monohydrate crystal, with its typical notched shape, becomes large in appropriate size, they can be detected with conventional or polarizing light microscopy. Although current laboratory techniques do not allow direct study of the earliest stages of cholesterol nucleation/crystallization, further refinements in technology may permit more precise elucidation of these intermediate steps. The term "pro-nucleating factor" has used to describe a factor that promotes the precipitation of cholesterol crystals from bile and shortens the nucleation time (56,66,67). An "anti-nucleating factor" has the opposite effect and retards nucleation, increasing the nucleation time. Pro-nucleating and anti-nucleating factors may influence this process at one or more points in the process of starting the nucleus with cholesterol molecules in bile (68), with intermediate steps of lipid transfer and vesicle disruption or fusion, and finally the appearance of microscopically detectable cholesterol monohydrate crystals.

Recent detailed analyses of cholesterol crystallization in model and native biles suggest that there are two distinct mechanisms for cholesterol nucleation (24), as illustrated in Figures 2-4. In biles with relatively high concentrations of lecithin, aggregation and fusion of cholesterol-rich vesicles results in multilamellar vesicles that produce cholesterol monohydrate crystals, which are the "building blocks" of cholesterol gallstones. At lower concentrations of lecithin, cholesterol-rich vesicles become unstable and may burst or collapse inwards. Crystals formed by this mechanism are composed mainly of anhydrous cholesterol in the form of ark-like crystals, and eventually, these anhydrous crystals become hydrated to form cholesterol monohydrate crystals.

5.3.2. Pro-nucleating factors

More rapid crystallization of cholesterol in biles of patients with gallstones implied that lithogenic bile may contain components (i.e., pro-nucleating agents) that accelerate crystallization or that normal biles may contain components (i.e., anti-nucleating agents) that inhibit crystallization (69). Bile may contain both accelerators and inhibitors of crystallization and their imbalances could induce rapid crystallization in gallbladder biles of patients with cholesterol gallstones.

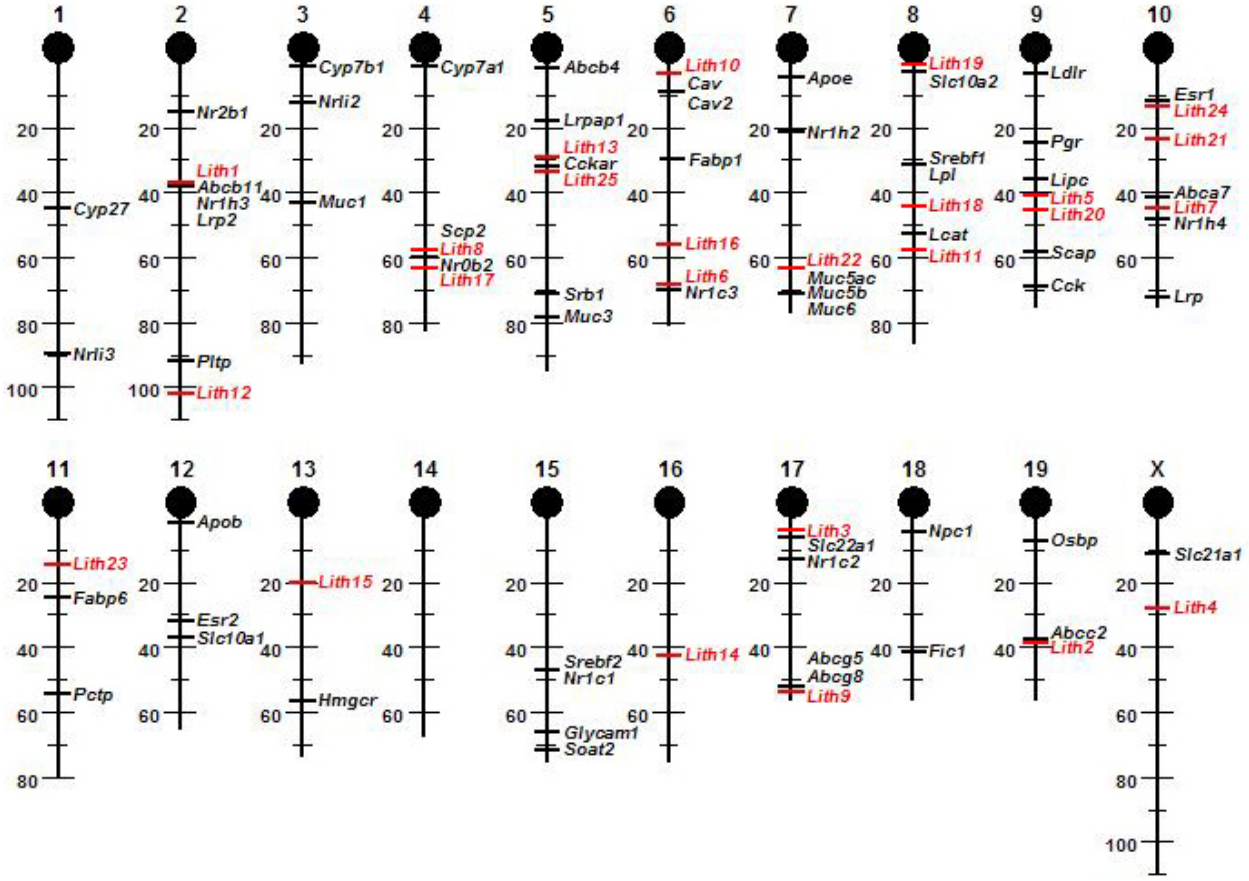

Figure 5. Composite map of quantitative trait loci (QTLs) for *Lith* genes, as well as candidate gallstone genes for cholesterol gallstone formation on chromosomes representing the entire mouse genome. A vertical line represents each chromosome, with the centromere at the top; genetic distances from the centromere (horizontal black lines) are indicated to the left of the chromosomes in centimorgans (cM). Chromosomes are drawn to scale, based on the estimated cM position of the most distally mapped locus taken from Mouse Genome Database. Gallstone QTLs (*Lith* genes) are represented by horizontal red lines, as well as candidate gene locations are indicated by horizontal black lines with the gene symbols to the right (see Table 1 for list of gene symbols and names).

Mucin was the first biliary protein shown to promote cholesterol crystallization (70). Subsequently, it was found that many glycoproteins that bind reversibly to concanavalin A-Sepharose also speed up cholesterol crystallization (71). These include aminopeptidase N, immunoglobulins, alpha1-acid glycoprotein, phospholipase C, fibronecitin, and haptoglobin. Other pro-nucleating agents include the amphipathic anionic polypeptide fraction/calcium-binding protein, albumin-lipid complexes, and group II phospholipase A_2. Non-protein components of bile also expedite cholesterol crystallization. A low-density particle composed principally of lipids is a potent promoter of crystallization. Calcium bound to micelles and vesicles in bile may accelerate cholesterol crystallization by promoting fusion of cholesterol-rich vesicles. The precipitation of calcium salts in biles that are supersaturated with calcium salts and cholesterol may lead to rapid crystallization of cholesterol, an effect that is enhanced by the presence of mucins. Rapidity of cholesterol crystal formation also varies in proportion to the deoxycholate content of bile (24,72). This effect probably contributes to the influence of this hydrophobic bile salt on the equilibrium phase relationships of biliary lipids. The degree of cholesterol supersaturation of bile *per se* may also be a determinant of rapid crystallization of cholesterol (60,73). Although evidence supports the significance of various pro- and anti-nucleating proteins for gallstone formation, some of their effects are either non-specific or unphysiological. As technologies improve for the study of cholesterol nucleation and crystal growth in bile, the contributions of promoters and inhibitors of cholesterol crystallization to gallstone formation should become clearer. In addition, serial spectrophotometry has been used to measure both cholesterol nucleation and the rate of crystal growth (74). In general, as biliary CSI increases, crystal growth begins earlier and the growth is faster.

5.3.3. Anti-nucleating factors

Although investigation of potential anti-nucleating factors has produced some information but not as much as is available about pro-nucleating factors, several inhibitors of cholesterol crystallization have been identified (75-77). These include apolipoproteins AI and AII, a 120-kD glycoprotein, a 15-kD protein, and secretory

Table 1. The candidate gallstone genes in the mouse

Gene symbol	Gene Name
Liver	
Lipid membrane transporters	
Abcg5 and *Abcg8*	ATP-binding cassette sterol transporters g5 and g8
Abcb4 (Mdr2)	Phosphatidylcholine transporter (multiple drug resistance 2)
Abcb11 (Bsep, Spgp)	Bile salt export pump (Sister of p-glycoprotein)
Abcc2 (Cmoat, Mrp2)	Canalicular multispecific organic anion transporter (multidrug resistance-related protein 2)
Slc10a1 (Ntcp)	Sodium/taurocholate cotransporting polypeptide
Slc21a1 (Oatp1)	Organic anion transporting polypeptide 1
Slc22a1 (Oct1, Orct)	Organic cation transporter 1
Fic1	Familial intrahepatic cholestasis type 1
Lipid regulatory enzymes	
Cyp7a1	Cholesterol 7alpha-hydroxylase
Cyp7b1	Oxysterol 7alpha-hydroxylase
Cyp27	Sterol 27-hydroxylase
Hmgcr	3-Hydroxy-3-methyglutaryl-coenzyme A reductase
Soat2 (Acat2)	Sterol *O*-acyltransferase 2 (acyl-coenzyme A:cholesterol acyltransferase 2)
Intracellular lipid transporters	
Akr1c3	3alpha-Hydroxysteroid dehydrogenase
Cav	Caveolin 1
Cav2	Caveolin 2
Fabp1	Fatty acid-binding protein 1, liver
Npc1	Niemann-Pick type C1
Osbp	Oxysterol-binding protein
Pctp	Phosphatidylcholine transfer protein
Scp2	Sterol carrier protein 2
Lipid regulatory transcription factors	
Nr2b1 (Rxralpha)	Retinoid X receptor alpha
Nr1h3 (Lxralpha)	Liver X receptor alpha
Nr1h2 (Lxrbeta)	Liver X receptor beta
Nr1h4 (Fxr)	Farnesoid X receptor
Nr1c1 (Ppara)	Peroxisomal proliferator activated receptor alpha
Nr1c2 (Ppard)	Peroxisomal proliferator activated receptor delta
Nr1c3 (Pparg)	Peroxisomal proliferator activated receptor gamma
Nr0b2 (Shp1)	Small heterodimer partner 1
Nrli3 (Car)	Constitutive androstane receptor
Srebf1	Sterol regulatory element binding transcription factor 1
Srebf2	Sterol regulatory element binding transcription factor 2
Scap	SREBF cleavage activating protein
Esr1 (Eralpha)	Estrogen receptor alpha
Esr2 (Erbeta)	Estrogen receptor beta
Lipoprotein receptors and related genes	
Apob	Apolipoprotein B
Apoe	Apolipoprotein E
Lrp	LDLR-related protein
Lrp2 (Gp330)	LDLR-related protein 2 (Megalin, Glycoprotein 330)
Ldlr	Low density lipoprotein receptor
Srb1	Scavenger receptor class B type I
Lpl	Lipoprotein lipase
Hpl	Hepatic lipase
Lcat	Lecithin-cholesterol acyltransferase
Lrpap1	LRP-associated protein 1
Pltp	Phospholipid transfer protein
Gallbladder	
Hormone receptors	
Cck	Cholecystokinin
Cck1r (Cckar)	Cholecystokinin-1 receptor (CCK-A receptor)
Esr1 (Eralpha)	Estrogen receptor alpha
Esr2 (Erbeta)	Estrogen receptor beta
Pgr	Progesterone receptor
Mucin	
Muc1	Mucin 1
Muc3	Mucin 3
Muc4	Mucin 4
Muc5ac	Mucin 5ac
Muc5b	Mucin 5b
Muc6	Mucin 6
Muc7	Mucin 7
Lipid membrane transporters	
Abcg5 and *Abcg8*	ATP-binding cassette sterol transporters g5 and g8
Srb1	Scavenger receptor class B type I
Lipid regulatory enzymes	

Hmgcr	3-Hydroxy-3-methyglutaryl-coenzyme A reductase
Soat2 (Acat2)	Sterol *O*-acyltransferase 2 (acyl-coenzyme A:cholesterol
	acyltransferase 2)
Small intestine	
Lipid membrane transporters	
Abcg5 and *Abcg8*	ATP-binding cassette sterol transporters g5 and g8
Srb1	Scavenger receptor class B type I
Slc10a2 (Ibat)	Ileal (apical) sodium/bile salt transporter
Lipid regulatory enzymes	
Hmgcr	3-Hydroxy-3-methyglutaryl-coenzyme A reductase
Soat2 (Acat2)	Sterol *O*-acyltransferase 2 (acyl-coenzyme A:cholesterol acyltransferase 2)
Intracellular lipid transporters	
Cav	Caveolin 1
Cav2	Caveolin 2
Fabp6 (Illbp)	Fatty acid-binding protein 6, ileal
Osbp	Oxysterol-binding protein
Scp2	Sterol carrier protein 2
Lipid regulatory transcription factors	
Nr2b1 (Rxralpha)	Retinoid X receptor alpha
Nr1h3 (Lxralpha)	Liver X receptor alpha
Nr1h2 (Lxrbeta)	Liver X receptor beta
Nr1h4 (Fxr)	Farnesoid X receptor
Nr1c1 (Ppara)	Peroxisomal proliferator activated receptor α
Nr1c2 (Ppard)	Peroxisomal proliferator activated receptor δ
Nr1c3 (Pparg)	Peroxisomal proliferator activated receptor γ
Esr1 (Eralpha)	Estrogen receptor alpha
Esr2 (Erbeta)	Estrogen receptor beta

Former gene symbols and names are given in the parentheses, See Figure 5 for candidate gene locations on mouse chromosomes. Reproduced with permission from (1).

immunoglobulin A and its heavy and light chains. Apolipoproteins A-I and A-II could prolong the nucleation time of supersaturated model bile (75). Apolipoproteins A-I and A-II are present in a fraction of human bile that inhibited cholesterol nucleation. Because precholecystectomy treatment for 3 months with ursodeoxycholic acid (UDCA) can prolong the nucleation time of bile from patients with cholesterol gallstones, it has been proposed that UDCA could be an anti-nucleating factor (24,78-80). It may exert its effect by stabilizing vesicles, perhaps by enhancing the incorporation of apolipoprotein A-I into (or onto) the vesicles. In addition, a potential anti-nucleating factor from normal human gallbladder bile is detected by lectin affinity chromatography and HPLC-ion-exchange chromatography and found to be a slightly acidic glycoprotein with an apparent molecular size of 120 kDa. The protein may inhibit crystal growth by attaching to the most rapidly growing microdomains on a crystal face and interfering with further solute attachment. Video-enhanced microscopy studies suggestd that spiral and edge dislocations on cholesterol monohydrate crystals represent areas of rapid growth. Nevertheless, although it is not known whether only one or several anti-nucleating factors exist and how they may inhibit the initiation of cholesterol crystal formation, it has been proposed that unilamellar vesicles may be the key site of action and further studies are requested.

5.4. Gallbladder mucin

The epithelial cells of the gallbladder mucosa secrete mucin that normally serves as a protective layer over the mucosa and is considered as a part of the unstirred water layer. Mucin or mucin glycoproteins are large molecules consisting of a protein core and many carbohydrate side chains. An important property of mucin is its ability to form a gel phase in higher concentrations; the gel has greatly increased viscosity compared with the sol phase. Gallbladder mucins, a heterogeneous family of *O*-linked glycoproteins, are divided into two classes: epithelial and gel-forming mucins. It has been proposed that the epithelial mucins produced by mucin gene 1 (*Muc1*), *Muc3*, and *Muc4* do not seem to form aggregates, and are integral membrane glycoproteins located on the apical surface of epithelial cells. The gel-forming mucins, *Muc2*, *Muc5ac*, and *Muc5b*, secreted by specialized gallbladder mucin-producing cells, provide a protective coating to the underlying mucosa. They form disulfide-stabilized oligo- or polymers, a phenomenon that accounts for their viscoelastic properties. Mucins from different organs vary in carbohydrate side chain and protein composition and charge but generally have similar properties. Mucins have hydrophilic domains to which many water molecules bind. Mucins have an overall charge and are capable of binding other charged species such as calcium. Hydrophobic domains in the mucin molecule (on the nonglycosylated regions of the polypeptide core) allow binding of lipids such as cholesterol, phospholipids, and bilirubin. Accumulated evidence suggests that gallbladder mucins play an important role in the early stages of cholesterol gallstone formation, and are a potent pro-nucleating/crystallizing agent for accelerating cholesterol crystallization in native and model biles (60,70,81-83). Indeed, hypersecretion of gallbladder mucins is a prerequisite for gallstone formation, and increased amounts of gallbladder mucins are consistently observed in gallbladder bile of several animal models of gallstones (10,70). Also, mucins are found within cholesterol gallstones where they act as a matrix for stone growth. It has been found that the mucins in gallstones extend from the amorphous center to the periphery in either a radial or laminated fashion. Also, mucins are a major component of sludge in the gallbladder, and sludge has been suggested to

be a precursor of gallstones (84,85). Therefore, two roles in the formation of gallstones have been proposed for mucins: (i) as a pro-nucleating/crystallizing agent for the nucleation/crystallization of cholesterol from saturated bile, and (ii) as a scaffolding for the deposition of crystals during growth of stones. The synthesis of mucous glycoproteins that are secreted by the epithelium of the gallbladder and biliary ducts could be regulated by mucosal prostaglandins that are derived from arachidonic acid-containing biliary phospholipids (86). It has been proposed that in the formation of gallstones, the gallbladder hypersecretes mucins, which is perhaps stimulated by some component of saturated bile. Then, the carbohydrate groups of the polymers of mucins avidly bind water to form gels. Moreover, the hydrophobic polypeptides in the core of mucous glycoproteins can bind the bilirubin and calcium in bile. The resulting water-insoluble complex of mucous glycoproteins and calcium bilirubinate provides a surface for nucleation of cholesterol monohydrate crystals and a framework of matrix for the growth of stones. Despite evidence that mucin overexproduction is critical in the pathogenesis of gallstones, the mechanisms triggering mucin production during gallstone formation are still unclear. Furthermore, mucin secretion and accumulation in the gallbladder is determined by multiple mucin genes. Although the regulation of gallbladder mucin secretion and accumulation and its role in gallstone pathogenesis have been intensively studied *in vivo* and *in vitro*, no information is available on how individual mucin genes contribute to cholesterol gallstone formation and whether the epithelial mucins influence susceptibility to cholesterol gallstone formation. More recently, it is found that reduced MUC1 mucin in the gallbladders of mice with disrupted *Muc1* gene could decrease susceptibility to cholesterol gallstone formation (87). Also, gene expression of the gallbladder *Muc5ac*, a gel-forming mucin gene, is significantly reduced in Muc1 knockout mice in response to the lithogenic diet. As a result, cholesterol crystallization and the development of gallstone formation are significantly retarded. It suggests that there may be gene-gene interactions between the *Muc1* and *Muc5ac* that might affect mucin secretion and accumulation in the gallbladder, and it remains unclear how the knockout of the gallbladder *Muc1* gene or the lack of the membrane-associated mucin determined by the *Muc1* gene on the apical surface of epithelial cells in the gallbladder influences the expression and function of the *Muc5ac* gene. A recent study by QTL mapping in a backcross between SWR/J and AKR/J strains shows that polymorphisms in the *Muc3* gene or its promoter may be linked to gallbladder accumulation of mucin gel and affect cholesterol gallstone formation (88). These results suggest that the epithelial mucin genes may influence gallbladder mucin accumulation by regulating expression and function of the gel-forming mucin genes. Furthermore, increased gallbladder epithelial MUC1 mucin enhances cholelithogenesis mostly by promoting gallbladder cholesterol absorption and impairing gallbladder motility in mice transgenic for the human *MUC1* gene, a lithogenic mechanism completely different from the gel-forming mucins (61). Therefore, these findings support the concept that inhibiting the secretion and accumulation of not only the gel-forming mucins but also the epithelial mucins in the gallbladder may completely prevent the formation of cholesterol gallstones. Taken together, studying Muc1 knockout and MUC1 transgenic mice opens avenues to explore whether the epithelial mucins contribute to cholesterol cholelithogenesis and constitutes the basic framework for investigating how individual mucin genes influence the various cholesterol gallstone phenotypes (61,87).

5.5. Gallbladder factors

5.5.1. Gallbladder motility

Under normal physiological conditions, frequent gallbladder contractions occur throughout the day. Between meals, the gallbladder stores hepatic bile (average fasting volume around 25-30 mL in healthy subjects) (89); while following the meal, the gallbladder discharges a variable amount of bile depending on the degree of neuro-hormonal response. A combined method of cholescintinography and ultrasonography has observed that after a meal the gallbladder empties immediately and refills repeatedly. In contrast, increased fasting gallbladder volume as well as incomplete emptying and high residual gallbladder volumes are often observed in cholesterol gallstone patients, regardless of whether they have tiny or large stones or just lithogenic bile. In this group of cholesterol gallstone patients with motility defects, gallbladder wall inflammation is usually mild and cannot account, *per se*, for the impaired dynamics of the gallbladder. Furthermore, the poor interdigestive gallbladder filling is consistent with delivery of a greater percentage of lithogenic bile from the liver directly into the small intestine, augmenting the enterohepatic artifacts of increased recycling and bile salt hydrophobicity. This suggests that both emptying and filling of the gallbladder are affected in patients with hypomotility (90-92). Clinical investigations further confirm that gallbladder hypomotility is an abnormality principally associated with the formation of cholesterol gallstones, although an intermediate degree of gallbladder motility defect, in the absence of enlarged fasting gallbladder and any gallbladder inflammation, has been found also in patients with pigment stones (93). In cholesterol gallstone patients, the impaired gallbladder motility persists in the stone-free gallbladder following successful extracorporeal shock-wave lithotripsy and oral bile acid dissolution therapy (94, 95). It has been found that the degree of impairment of gallbladder emptying increases in proportion to the cholesterol content of gallbladder bile, even in healthy subjects without gallstones. These findings suggest that excess cholesterol molecules in the gallbladder wall could act as myotoxic agents. *In vitro* studies comparing gallbladder function in cholesterol gallstone patients versus control subjects show abnormalities in binding of agonists, e.g., CCK, to plasma membrane CCK-1 receptors, alterations in contraction of isolated smooth muscle cells, and decreased contractility of isolated smooth muscle strips and whole gallbladder preparation. In particular, there is an impairment of signal transduction in response to binding of agonists. Defects in contractility associated with cholesterol gallstones are reversible at an early stage and are attributable primarily to excess accumulation of biliary cholesterol in membranes of gallbladder smooth muscle cells. This mechanism appears

to explain why gallbladder emptying is impaired before gallstone formation in animal models at a time when bile is only supersaturated with cholesterol. Similar effects of cholesterol on plasma membranes may cause diminished relaxation of the gallbladder, which is associated with cholesterol gallstone disease (96). In addition, the intracellular mechanisms for smooth muscle contraction in human gallbladder muscle cells from the gallbladders with cholesterol gallstones seem to be intact. These findings strongly support the hypothesis that the absorption of cholesterol from the gallbladder lumen is associated with gallbladder smooth muscle dysfunction. This alteration may induce stiffening of sarcoplasma membranes secondary to increased membrane cholesterol contents. As a result, when CCK binds to its receptor on smooth muscle cells of the lithogenic gallbladder, G-proteins are not activated and gallbladder motility is impaired (97-100). If a high degree of chronic or acute-on-chronic inflammation in the gallbladder wall has intervened, restoration of the gallbladder motility defect might not take place.

It has been proposed that gallbladder hypomotility could precede gallstone formation. The stasis induced by the hypofunctioning gallbladder provides the time necessary to accommodate nucleation of crystals and growth of gallstones within the mucin gel in the gallbladder (101, 102). In addition, the viscous mucin gel that forms within the gallbladder may contribute to hypomotility by mechanically impairing gallbladder emptying, possibly at the level of the cystic duct. Especially, sludge contains calcium, pigment, bile acids, and glycoproteins and probably serves as a nidus for nucleation of cholesterol or precipitation of calcium bilirubinate. It has found the high incidence of cholelithiasis in patients receiving long-term total parenteral nutrition (TPN), underscoring the importance of gallbladder stasis in the formation of gallstones (103). For example, 49% of patients with Crohn's disease who are receiving TPN have gallstones (Crohn's disease alone causes gallstones in 27% of patients). During TPN, the gallbladder does not empty because the stimulus (ingestion of meals) for the release of CCK is essentially eliminated. Consequently, bile stagnates and sludge develops in the gallbladder, which enhances the formation of gallstones. In contrast, it has been shown that daily intravenous administration of CCK can prevent gallbladder dysmotility completely and eliminate the inevitable risk of "biliary sludge" and gallstone formation. In addition, slow emptying and increased volume of the gallbladder, as measured by ultrasonography, occur during pregnancy and during administration of oral contraceptives, two conditions that predispose to the formation of gallstones (104-106).

5.5.2. Gallbladder concentrating function

Although concentration of bile within the gallbladder increases cholesterol solubility, it promotes nucleation/crystallization of cholesterol, suggesting that enhanced concentration of bile is a contributing factor for gallstone formation (24, 107, 108). In addition to concentrating bile, the normal gallbladder also acidifies bile. Acidification increases the solubility in bile of calcium salts (e.g., bilirubinate and carbonate) that may be promoters of nucleation/crystallization. Thus, defective acidification has been proposed to have a role in the formation of gallstones. Because the concentrating function of the gallbladder is retained, the total concentration of lipids in bile increases. This has an important effect on the equilibrium phase diagram for the biliary lipids. In more dilute hepatic biles, the dashed phase boundaries are positions much further to the left. Therefore, although hepatic biles are supersaturated with cholesterol, cholesterol crystallization does not occur because the bile compositions fall within the two-phase region in which mixed micelles co-exist at equilibrium with vesicles (24). More recently, it is found that an association between decreased gallbladder concentrating function and reduced expression levels of some Aquaporins (AQP1 and AQP8) suggests the involvement of these water channels in gallbladder water transport and could play a role in cholesterol crystallization and gallstone formation in mice challenged to the lithogenic diet (109, 110).

5.5.3. Gallbladder lipid absorption

Differential absorption of cholesterol, phospholipids and bile salts by the gallbladder reduces cholesterol saturation of bile in normal subjects; however, the gallbladder epithelium of patients with cholesterol gallstones loses the capacity for selective absorption of biliary cholesterol and phospholipids (111-113). Impaired lipid absorption may contribute to gallstone formation by sustaining cholesterol supersaturation of bile during storage. The physical-chemical fate of cholesterol absorbed by the gallbladder may be similar to that which occurs during the development of the atherosclerotic plaque. It seems that cholesterol molecules are absorbed continuously by the gallbladder mucosa from supersaturated bile (114). Most likely, the unesterified cholesterol molecules diffuse rapidly to the muscularis propria as the gallbladder lacks an intervening submucosa and muscularis mucosae. Since the gallbladder apparently does not synthesize lipoproteins for export cholesterol to plasma, excess unesterified cholesterol molecules are removable from gallbladder mucosa and muscle only by esterification and storage or back diffusion into bile (96). In the lithogenic state, back diffusion of cholesterol molecules into bile is blocked because gallbladder bile is continuously saturated despite normal diurnal excursions. So, gallbladder mucosal ACAT esterfies most, but no all, cholesterol molecules. Therefore, similar to the atherosclerotic plaque, mucosal and muscle membranes apparently become markedly saturated with cholesterol and co-exist with stored cholesteryl ester droplets. In addition, excess cholesterol molecules absorbed from the lithogenic bile may also be direct stimulants to proliferative and inflammatory changes in the gallbladder's mucosa and lamina propria. In prairie dogs fed a 1-2% cholesterol diet, polymorphonuclear and mononuclear leucocytes infiltrate the lamina propria at 2 weeks (115). Acute and chronic inflammatory changes are accompanied by a doubling in mucosal blood flow, also directed related to lithogenic bile in the gallbladder's lumen (116). It is unclear what factor in lighogenic bile is the precise antigenic trigger that produces these inflammatory responses. Nevertheless, all of these changes occur before microscopic stones are detected. *In vitro* studies show that

in gallstone patients and cholesterol-fed prairie dogs, gallbladder muscle dysfunction is hallmarked by a doubling of the normal ratio of cholesterol to phospholipid in sarcolemmae. This level can be restored to normal when isolated muscle cells are incubated with cholesterol-free liposomes. It is most likely, therefore, that the unesterified cholesterol molecules become intercalated within the membrane bilayer of muscle cells, which may alter the physical state of phospholipid molecules as reflected by their increased rigidity. Consequently, gallbladder motility function is impaired because signal transduction in response to CCK is diminished markedly.

5.6. Intestinal factors

Epidemiological investigations and clinical studies have found that the incidence of cholesterol gallstones in North America and European countries with the "Western" diet containing high cholesterol is significantly higher than that in the developing nations. In Japan, cholesterol gallstones once were rare, but over the past 50 years with the adoption of Western eating habits, the incidence has increased markedly. It has found an association between the increasing incidence of cholesterol gallstones in China and a "westernization" of the traditional Chinese diet; that is, excessive consumption of high cholesterol diets (117). However, studies on the effect of dietary cholesterol on biliary lipid metabolism in humans gave conflicting results, showing that high dietary cholesterol either increases or does not affect cholesterol saturation of bile. It is found recently that high efficiency of intestinal cholesterol absorption correlates positively and significantly with the prevalence of cholesterol gallstones in inbred mice, and gallstone-susceptible C57L mice display significantly higher intestinal cholesterol absorption than resistant AKR mice (9). Taken together, these studies suggest that high dietary cholesterol and high efficiency of intestinal cholesterol absorption are two independent risk factors for cholesterol gallstone formation. Differential metabolism of chylomicron remnant cholesterol between C57L and AKR mice clearly plays a crucial role in lithogenic bile formation, and the cholesterol molecules absorbed from the small intestine provides an important source for biliary cholesterol hypersecretion in mice challenged to the lithogenic diet (9). Also, altered intestinal motility can have a role in gallstone formation. Delayed or impaired small intestinal transit time is associated with enhanced intestinal cholesterol absorption, biliary cholesterol secretion and gallstone prevalence (118). Moreover, bile salts secreted into the duodenum are reabsorbed in the ileum by active transport and returned to the liver where they are secreted into bile. Slow large intestinal transit can increase the rates of deoxycholic acid formation; indeed, the association of impaired large intestinal motility with increased biliary deoxycholate levels are found in some patients with cholesterol gallstones. Evidence for a causal relation between impaired intestinal motility, deoxycholate formation and bile lithogenicity comes from studies in humans and mice. Clinical studies found that acromegalic patients with treatment of octreotide (a known risk factor for cholesterol gallstone disease) display prolonged colonic transit, high levels of biliary deoxycholate concentration, and biliary cholesterol precipitation (72,119-122). In addition, higher levels of biliary deoxycholate are associated with increased amounts of Gram-positive anaerobic bacteria and increased activity of 7alpha-dehydroxylase in the cecum of patients with cholesterol gallstones versus controls. Moreover, biliary deoxycholate and cholesterol concentration can be lowered by antibiotic treatment that reduces fecal 7alpha-dehydroxylation activity. Compared with resistant mice, gallstone-susceptible mice also have high levels of biliary deoxycholate, which is associated with cholesterol supersaturation and gallstone formation (123). It has been proposed that chronic intestinal infection is a potential factor in cholesterol gallstone pathogenesis. A mouse study shows that distal intestinal infection with a variety of enterohepatic *Helicobacter* species, but not *Helicobacter pylori*, is essential for nucleation/crystallization of cholesterol from a supersaturated bile (124,125). These *Helicobacter* species have been identified in the bile and gallbladder tissue from Chilean patients with chronic cholecystitis. It is imperative to further investigate whether chronic enteritis has a direct pathogenic role in the formation of cholesterol gallstones.

Moreover, patients with Crohn's disease, as well those who underwent bowel resection or total colectomy, have cholesterol supersaturated bile, which are prone to cholesterol crystal precipitation and cholesterol gallstone formation (126). It is possible that the enterohepatic circulation of bile salts is impaired in these patients so that biliary bile salt secretion is greatly reduced so that the solubilization of cholesterol in bile is significantly decreased. In addition, Crohn's disease might lead to impaired enterohepatic cycling of bilirubin, increased biliary bilirubin levels, and precipitation of calcium bilirubinate (127,128). The latter may work as a nidus to promote cholesterol nucleation/crystallization.

5.7. Growth of gallstones

Nucleation/crystallization of cholesterol crystals usually is considered the critical stage in the formation of cholesterol gallstones; however, the findings from patients who have crystals but no gallstones in the gallbladder suggest that growth of cholesterol crystals into gallstones does not always follow nucleation/crystallization. It is possible that the stone growth could represent the critical stage in the formation of gallstones due to delayed emptying of the gallbladder. When multiple gallstones are in the gallbladder, they often are equal in size, indicating that nucleation/crystallization for this family of stones occurs simultaneously and that the stones grow at the same rate. Stones of unequal size could be a symbol of different generations. The presence of calcium salts and pigments within the center of most cholesterol gallstones suggests a requirement for a central nidus. Cholesterol crystals could be assembled about this nidus. The formation of a nidus and subsequent stone growth could be determined by mucins, other biliary proteins, and the cholesterol saturation of bile. Growth of stones is most likely a discontinuous process that is punctuated by deposition of rings of calcium bilirubinate and calcium carbonate.

Because cholesterol crystals often aggregate randomly in amorphous groupings as well as to layer radially and concentrically, cholesterol gallstones consist of radially or horizontally oriented cholesterol crystals imbedded within an organic matrix. Scanning electron microscopy also reveals randomly aggregated crystals of cholesterol in the center of gallstones. In addition, the amorphous material in the center of stones contains bilirubin, bile acids, mucous glycoproteins, calcium, carbonate, phosphate, copper, and sulfur, which might have provided a nidus for cholesterol nucleation/crystallization. In the outer portion of stones, the crystals are oritented perpendicularly to the surface (129). Throughout the formation of gallstones, mucins could provide a matrix on which the growth of gallstones occurs. Furthermore, concentric pigmented rings separate layers of cholesterol crystals that make quite different axial orientations. The chemical composition of these rings often resembles the center of gallstones. It is highly likely that the rings may reflect cyclic deposition of calcium bilirubinate, other calcium salts, and glycoproteins. It should be emphasized that biliary sludge is an important co-factor for the formation of gallstones. Sludge is originally described as an ultrasonographic finding. Subsequent analysis reveals that sludge is a sediment consisting of cholesterol monohydrate crystals or bilirubinate granules trapped in a mucus gel. The formation of sludge is a key precursor of cholelithiasis because it provides a viscous, gel-like microenvironment for assembly of stones (84,85). Nonetheless, biliary sludge progresses to gallstone formation in only 5-15% of patients over a 3-year period. When an etiology is transient (e.g., pregnancy and total parenteral nutrition), sludge often disappears upon removing its cause (130). As studied by using carbon isotope dating, the growth rate of gallstones is approximately 2.6±1.4 mm/year. The time from stone formation to development of symptoms (in patients who developed symptoms) is roughly 8.0±5.1 years. Other work using serial OCG shows a stone growth rate of 0.6 mm/year. In spite of these observations, many aspects of the process of stone growth remain poorly understood. For example, it is not clear why gallstones in the gallbladder are small (1 to 15 mm) and numerous (more than 10,000), large (6 to 10 cm) and solitary, as well as how mucins, biliary proteins, bile pigments, and inorganic salts are deposited in stones together with cholesterol crystals.

6. FUTURE DIRECTIONS

At least four defects must occur simultaneously for nucleation and crystallization of cholesterol monohydrate crystals in bile. These are unphysiological supersaturation with cholesterol, accelerated cholesterol nucleation/crystallization, gallbladder hypomotility, and increased amounts of cholesterol from the intestine. Growth of crystals to form gallstones is a consequence of both gallbladder mucin hypersecretion and gel formation with incomplete evacuation by the gallbladder. Evidence for variations in incidence rates of gallstones among geographically and ethnically diverse populations as well as family and twin studies strongly support a genetic susceptibility to the formation of cholesterol gallstones in humans. Inbred mice represent a powerful model for genetic analysis of cholesterol gallstones. Current technologies result in a rapid localization of both specific genes and phenotypic traits to a precise position on mouse chromosomes. On the basis of observations that certain inbred strains of mice form gallstones when challenged to the lithogenic diet whereas other strains are resistant, gallstone susceptibility in mice, as identified by QTL analysis, has been linked to 25 *Lith* loci on mouse chromosomes so far. The first gallstone gene, *Lith 1* is the major gallstone gene in mice and is located in a narrowly restricted region on chromosome 2 and the successful positional cloning of *Lith1* will foster the identification of other *Lith* genes at a molecular level and the investigation of lithogenic mechanisms of individual *Lith* genes. Furthermore, a current "gallstone map" has listed all known genetic loci that confer gallstone susceptibility as well as candidate genes in inbred mice. A detailed understanding of molecular genetics of gallstones in mice will facilitate the search for *LITH* genes in humans. Rapid advances in techniques for genetic analysis and the availability of the nucleotide sequence of the human genome will lead to the identification and characterization of *LITH* genes in the coming years. Similar to atherosclerosis, the risk of cholesterol gallstone disease increases with age, obesity, type 2 diabete, dyslipidaemia (hypertriglyceridaemia and low HDL serum cholesterol), hyperinsulinaemia, and sedentary lifestyle. All these conditions are risk factors for the metabolic syndrome, of which cholesterol gallstone disease is deemed as just another complication (2,131).

7. CONCLUSIONS

Growing physical-chemical, biochemical, genetic and molecular biological evidence strongly suggests that (i) the crystallization and precipitation of plate-like cholesterol monohydrate crystals to the formation of cholesterol gallstones in the human is multifactorial; (ii) the primary pathogenic factor is hepatic hypersecretion of cholesterol into bile; and (iii) no mode of inheritance fitting to the Mendelian pattern could be demonstrated in most cases. Up until now, 25 Lith loci in inbred mice with different susceptibility to gallstones have been identified by QTL analysis (Figure 5 and Table 1). The identification of such genes responsible for Lith QTL regions by a positional cloning approach will be coming out in the near future, which should lead to the discovery of pathophysiological effects of individual Lith genes and yield new insights into the molecular mechanisms that potentially influence the formation of cholesterol gallstones. As there is exceptionally close homology between mouse and human genomes, these studies will opens the door for searching for the orthologous human LITH genes and for exploring their cholelithogenic effects in humans.

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Abbreviations: ABC: ATP-binding cassette transporters; AQP: aquaporin; CCK: cholecystokinin; CMC: critical micellar concentration; CSI: cholesterol saturation index; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; HPLC: high performance liquid chromatography; MUC: mucin gene; PFIC3: type 3 progressive familial intrahepatic cholestasis; QTL: quantitative trait locus; TPN: total parenteral nutrition; UDCA: ursodeoxycholic acid.

Key Words Bile Flow, Bile Salt, Biliary Lipid Secretion, Cholesterol, Crystallization, Lipid Transporter, Liquid Crystal, Nucleation, Phase Diagram, Phospholipid, Micelle, Vesicle, Review

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