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Negative feedback loop of cholesterol regulation is impaired in the livers of patients with Alagille syndrome

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ABSTRACT

Aim: To characterize cholesterol regulation in the liver of patients with Alagille syndrome (AGS).
Methods: Serum total cholesterol (TC) and total bile acid (TBA) levels were measured in 23 AGS patients. The expressions of genes involved in cholesterol regulation, including low-density lipoprotein receptor (*LDLR*), scavenger receptor class B type I (*SR-BI*), 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGCR*), cholesterol 7α-hydroxylase (*CYP7A1*), ATP-binding cassette transporter (*ABC) A1*, and *ABCG1/5/8*, were measured in liver tissues from five of these patients. Expression of regulators for these genes, including farnesoid X receptor/small heterodimer partner (*SHP*), liver X receptor α (*LXRα*) and mature Sterol regulatory element-binding protein 2 (SREBP2) was measured. The expression of mature SREBP2 protein was also examined.
Results: Serum TC and TBA levels were correlated in the AGS patients. Liver cholesterol was also increased compared with controls, and correlated with bile acid contents. *LDLR*, *SR-BI*, *HMGCR*, and *ABCGs* mRNA expression were upregulated, while *CYP7A1* mRNA expression was downregulated in AGS livers. *SHP* and *LXRα* mRNA expression was also increased, but maturation of SREBP2 was not suppressed in the patients.
Conclusions: The major upregulators of liver cholesterol might be increased in AGS patients, indicating an impaired negative feedback mechanism and accelerated liver cholesterol accumulation.

1. Introduction

Cholestatic disorders often accompany profound hypercholesterolemia [1–4]. Such distinct lipid abnormalities may unfavorably affect development and nutrition in childhood. Hypercholesterolemia generally predisposes patients to cardiovascular diseases, particularly atherosclerosis. It has been hypothesized that advanced liver damage leads to a considerable decrease in high-density lipoprotein (HDL), which is protective against atherogenesis. Decreased HDL in the face of hypercholesterolemia will likely engender atherosclerosis. Therefore, the adverse effects of increased plasma cholesterol have been extensively studied in patients with cholestatic disorders [5–7]. However, uniform results have not been obtained.

Alagille syndrome (AGS) is a rare hereditary cholestatic disorder primarily caused by mutations in *JAGGED1* (*JAG1*), a ligand of the Notch signaling pathway [8–13]. The cholestasis is thought to result from intrahepatic bile duct paucity. This disorder also presents various extrahepatic manifestations such as peripheral pulmonary stenosis, butterfly-like vertebrae, peculiar face, and ocular posterior embryotoxon [11–13]. In addition, children with AGS presenting cholestasis usually exhibit profound hypercholesterolemia and present with failure to thrive [14, 15]. However, limited information is available on the liver cholesterol regulatory system in this disease [3].

This study aimed to characterize cholesterol regulation in the liver of AGS patients by examining mRNA expression of major liver cholesterol regulatory proteins such as low-density lipoprotein receptor (*LDLR*), 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGCR*), and scavenger receptor class B type I (*SR-BI*). Concurrent expression of liver nuclear receptors such as farnesoid X receptor (*FXR*, *NR1H4*) and liver X receptor α (*LXRα*, *NR1H3*), which control liver cholesterol regulatory proteins, was also examined (Fig. 1) [16–19]. Furthermore, as a translational regulator of *HMGCR* and *LDLR*, mature sterol regulatory element-binding protein 2 (SREBP2) was examined [20–22]. Our results demonstrate a one-way positive feedback of cholesterol regulation in

Abbreviations: AGS, Alagille syndrome; BA, biliary atresia; TC, total cholesterol; TBA, total bile acids; LDLR, low-density lipoprotein receptor; HDL, high-density lipoprotein; SR-BI, scavenger receptor class B type I; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; FXR, farnesoid X receptor; LXRα, liver X receptor α; CYP7A1, cholesterol 7α-hydroxylase; SHP, small heterodimer partner; SREBP2, sterol regulatory element-binding protein 2.

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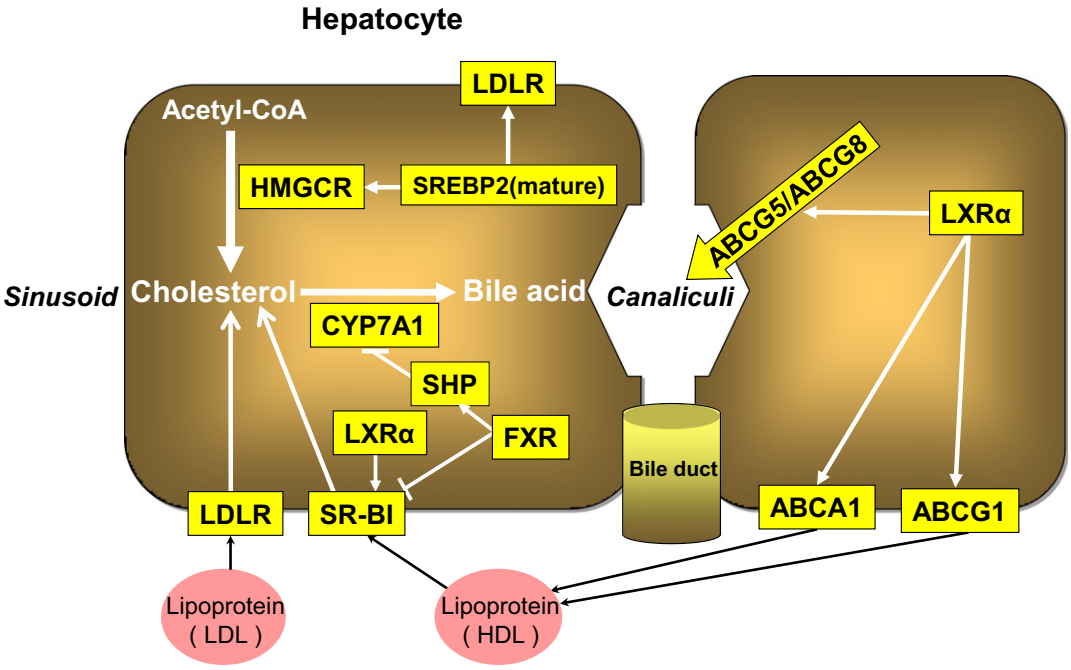

Fig. 1. The cholesterol regulatory system in hepatocyte. LDLR, low-density lipoprotein receptor; SR-BI, scavenger receptor class B type I; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; CYP7A1, cholesterol 7a-hydroxylase; FXR, farnesoid X receptor; SHP, small heterodimer partner; LXRα, Liver X receptor α; ABCA1, ATP-binding cassette transporters A1; ABCG1, ATP-binding cassette transporters G1; ABCG5/8, ATP-binding cassette transporters G5/8, SREBP2(mature); cleaved form of sterol regulatory element-binding protein 2.

cholestatic AGS livers, which results in higher liver cholesterol content and leads to profound hypercholesterolemia.

2. Patients and methods

2.1. Patients enrolled in the preliminary study to evaluate the impact of cholestasis on plasma cholesterol

Twenty-three AGS patients who had been followed at Osaka University Hospital, Osaka Medical Center and Research Institute for Maternal and Child Health, and Jichi Medical University (female/male, 10/13; age, 5 months–20 years) were enrolled in the preliminary study to explore the correlation between their serum total bile acid (TBA) and serum total cholesterol (TC) levels and evaluate the influence of cholestasis on serum cholesterol. Biochemical data were retrospectively collected from the patients' medical records.

AGS was diagnosed by the presence of cholestasis with specific liver histology demonstrating a paucity of interlobular bile ducts and the fulfillment of at least 3 of the following diagnostic criteria: cholestasis, heart disease, peculiar face, butterfly-like vertebrae, and posterior embryotoxon [11,12]. Genotyping of *JAG1* gene was performed to further confirm the diagnosis by direct sequencing of all exons.

2.2. AGS patients enrolled in the study of liver cholesterol regulation

Among the 23 AGS patients, five underwent living related liver transplantation because of liver failure and severe pruritus. Liver tissues were harvested from these patients at the time of liver transplantation, snap frozen in liquid nitrogen, and stored at −80 °C until analysis.

Six control liver tissues without cholestasis and dyslipidemia were also obtained from two patients with hemangioma, three patients with hepatoblastoma and a patient with ornithine transcarbamylase deficiency. Non-tumorous tissues away from the tumors were isolated from the liver obtained at the time of liver transplantation or lobectomy, and all of the isolated tissue showed normal histology. The background and biochemical data of each control patient were shown in Supplementary Tables 1 and 2. This study was approved by the relevant institutional medical ethics review boards. Informed consent was obtained from the parents of the enrolled children before study initiation.

2.3. Correlation between serum TC and TBA in AGS patients

To evaluate the influence of cholestasis on serum cholesterol levels, we examined the correlation between serum TC and serum TBA levels in the 23 AGS children.

Lipid and bile acids were extracted from the liver tissue according to the method described by Folch et al. [23] and Beher WT et al. [24]. TC and TBA levels were measured using the LabAssay cholesterol assay kit (Cayman Chemical, Ann Arbor, MI, USA) and the total bile acids assay kit (Diazyme Laboratories, Poway, CA, USA), respectively, according to the manufacturer's protocol.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

We examined the mRNA expression of proteins/enzymes and liver nuclear receptors involved in the liver cholesterol regulatory system (Fig. 1) [16–19]. Total hepatic RNA was isolated from frozen liver tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

One microgram of total RNA was reverse transcribed using the ReverTra Ace® qRT-PCR Kit (TOYOBO LIFE SCIENCE, Osaka, Japan). For the quantification of *CYP7A1* mRNA, we used Taqman® real-time quantitive PCR (Applied Biosystems, Foster City, CA, USA), and for other genes, we used SYBR® Green real-time quantitative PCR (Applied Biosystems, Foster City, CA, USA). All PCR procedures were performed using the ABI 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The relative mRNA levels were normalized to *GAPDH* expression, and the fold changes were determined using the $2^{-\Delta\Delta Ct}$ method. We performed melting curve analysis and confirmed that single amplicon with an expected melting temperature was generated in each reaction (supplementary figure). The primer sets used in qRT-PCR are listed in Table 3.

We evaluated the mRNA expression of lipoprotein receptors, including *LDLR* and scavenger receptor class B type I (*SR-BI*), which promote