EVALUATION OF RISK FOR ATHEROSCLEROSIS IN ALAGILLE SYNDROME AND PROGRESSIVE FAMILIAL INTRAHEPATIC CHOLESTASIS: TWO CONGENITAL CHOLESTATIC DISEASES WITH DIFFERENT LIPOPROTEIN METABOLISMS

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Objectives To evaluate the risk for atherosclerosis in Alagille syndrome (AGS) and progressive familial intrahepatic cholestasis (PFIC) on the basis of lipoprotein metabolism and by ultrasonography.

Study design Five patients with AGS and 5 with PFIC, ages 3 to 4 years, were enrolled. Intimal-medial thickness and wall stiffness of the common carotid artery were examined by ultrasonography. Serum levels of lipids and lipoproteins were determined. Further, the chemical composition of LDL and its ability to transform macrophages into foam cells were determined.

Results Intimal-medial thickness and wall stiffness were increased in patients with PFIC but not in patients with AGS. Total cholesterol, LDL cholesterol, HDL cholesterol, and lipoprotein X were remarkably increased in patients with AGS, whereas in patients with PFIC, an increase in triglyceride and a decrease in HDL cholesterol were the prominent findings. However, despite the normal LDL cholesterol level, oxidized LDL level was strikingly high in patients with PFIC. LDLs from patients with PFIC had high TG contents and exhibited high abilities to transform macrophages into foam cells.

Conclusions These findings suggest that patients with PFIC are at high risk for cardiovascular disorders involving atherosclerosis. *(J Pediatr 2005;146:329-35)*

Congenital cholestatic liver diseases that cause liver dysfunction in early life, including Alagille syndrome (AGS) and progressive familial intrahepatic cholestasis (PFIC), are usually accompanied by dyslipidemia.[1-4] Hypercholesterolemia almost always occurs in AGS and often becomes the most important problem in clinical practice, since liver disease may be mild or moderate.[2-7] However, there are only a limited number of reports describing the risk of coronary heart disease or atherosclerosis in cholestatic liver disease causing hypercholesterolemia.[8,9] On the other hand, PFIC is characterized by cholestasis without hypercholesterolemia.[10-12] It is expected that the risk for atherosclerosis would be low in PFIC because of normal or low total cholesterol level, although this has not been confirmed.[3,4,10-12] In the current study, we evaluated the risk for atherosclerosis in patients with AGS and PFIC by the wall stiffness (c-WS) and the intimal-medial thickness (c-IMT) of the common carotid artery and by lipoprotein metabolism.

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METHODS

Subjects

Five patients with AGS (patients 1 through 5) and 5 patients with PFIC (patients 6 through 10) with ages ranging from 3 to 4 years were enrolled in the current study. The

AGS	Alagille syndrome	MDA	Malondialdehyde
c-IMT	Intimal-medial thickness of common carotid artery	Ox LDL	Oxidized LDL
c-WS	Artery wall stiffness of common carotid artery	PFIC	Progressive familial intrahepatic cholestasis
FH	Familial hypercholesterolemia	TC	Total cholesterol
LpX	Lipoprotein X	TG	Triglycerides

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diagnosis of AGS was made by clinical manifestations and liver histology showing a paucity of interlobular bile ducts (Table I), whereas that of PFIC was confirmed by analysis of the *ATP8B1* gene responsible for PFIC-1 (patients 6 through 9) or *ATP binding cassette BII* responsible for PFIC-2 (patient 10), in addition to the clinical course and the liver histology.[12-14] As shown in Table I, serum levels of total bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were elevated slightly or mildly in all patients, but serum levels of total protein, albumin, and choline esterase, representative markers for protein synthesis of the liver, were normal. Further, liver histology did not show any findings suggestive of advanced stage of liver disease such as fibrosis leading to expanded portal area or massive necrosis. Oral administration of fat-soluble vitamins (A, D, E, and K) and ursodeoxycholic acid (10 to 20 mg/kg per day) were carried out for all patients, and blood levels of metabolites of respective vitamins were normal.

Determination of Total Cholesterol, Triglycerides, HDL Cholesterol, LDL Cholesterol, and Lipoprotein X

Serum levels of total cholesterol (TC) and triglyceride (TG) were determined by enzymatic methods, using commercial kits (Kyowa Medex Co, Tokyo, Japan). HDL cholesterol (HDL-C) was determined by using 13% polyethylene glycol (PEG) (PEG 6000, Wako Pure Chemicals Co, Osaka, Japan), as described previously.[15] LDL cholesterol (LDL-C) was determined by an enzyme immunoassay, using a commercial kit (LDL-C Daiichi, Daiich Co, Ltd, Tokyo, Japan). Lipoprotein X (LpX) was determined by selective immunoprecipitation, as described by Poittevin et al.[16] The values of age-matched control subjects were obtained from healthy 120 children 3 to 5 years of age (58 boys and 62 girls).

Determination of Malondialdehyde-Modified LDL

Oxidized LDL (Ox LDL), especially malondialdehyde (MDA)-LDL, plays a central role in development and progression of atherosclerosis.[17,18] Therefore, in the present study, serum MDA-LDL level was determined by an enzyme-linked immunosorbent assay, as described by Kotani et al.[17] In this method, a monoclonal antibody specific for MDA-protein (ML-25) and a monoclonal antibody specific for apolipoprotein B (AB16) were used.

Wall Stiffness and Intimal-Medial Thickness of Common Carotid Artery

The right common artery was examined for the subjects lying in the supine position, and all measurements were made 2 cm below the carotid bifurcation. A longitudinal 2-dimensional ultrasound image of the common carotid artery was scanned by an 8-MHz linear array transducer.[19-26] c-WS was defined as pulse pressure (mm Hg)/[the change in arterial diameter with each pulse (mm)/diastolic internal diameter (mm)] according to earlier reports.[19-21,26] c-IMT was measured by B-mode ultrasound, according to the method as described previously.[19,22-26] The age-matched control values were obtained from 56 healthy children with normal blood pressure (ages 3 to 4 years; 25 boys and 31 girls), of whom lipid levels were within the normal ranges that were deduced from 120 age-matched control subjects, as mentioned above. Further, the values of children with familial hypercholesterolemia (FH: 4 boys and 3 girls, ages 2 to 5 years) were also determined to investigate the association between TC or LDL-C level and such markers at this age. Their TC and LDL-C levels were considerably high, at 289 to 404 mg/dL and 137 to 295 mg/dL, respectively.

Separation of LDL by a Combination of Ultracentrifugation and Gel Infiltration and Determination of Chemical Composition

Fractionation of serum lipoprotein was performed by a combination of ultracentrifugation and gel filtration, as described previously.[15] The total lipoprotein fraction obtained by ultracentrifugation ($d < 1.225$ kg/L) was applied to a Sepharose CL4B column (1.5 × 92 cm) in 5 mmol/L Tris-HCl buffer (pH 7.4) containing 0.15 mol/L NaCl, 0.27 mmol/L EDTA-2Na, and 3 mmol/L NaN_3. The column was eluted at 6°C, and 3-mL fractions were collected under continuous monitoring of the absorbance at 280 nm. Lipoprotein subclass of LDL was collected and concentrated by an ultrafiltration membrane (Amicon XM-50). Thereafter, chemical composition of this lipoprotein fraction was determined. Protein was determined by the method of Lowry, modified by Markell et al,[27] using bovine serum albumin as standard. Control values were obtained from 20 healthy children (10 boys and 10 girls) ages 2 to 6 years.

Preparation of Oxidized LDL by $CuSO_4$

Ox LDL was prepared with LDL, designated as native LDL, isolated from serum of healthy volunteers (5 men and 5 women), ages 19 to 35 years, as described before.[28] In brief, LDL isolated by a combination of ultracentrifugation and gel filtration in the presence of 0.27 mmol/L EDTA-2Na was dialyzed against phosphate-buffered saline (PBS) at 4°C overnight to remove EDTA and then incubated with 10 μmol/L $CuSO_4$ at 37°C for 24 hours. The $CuSO_4$ was then removed from the LDL solution by dialyzing against PBS at 4°C. The resultant Ox LDL was used for experiments within 24 hours. Throughout the current study, Ox LDL derived from the same volunteer members was used as control in the following foam cell formation assay.

LDL-Mediated Foam Cell Formation

Peripheral mononuclear cells from a single healthy volunteer (male, 22 years of age) with no history of smoking, whose biochemical findings including serum lipid levels were entirely normal, were used as a source of macrophage preparation in the current study. The mononuclear cells were isolated by the Ficoll-Paque technique, as described elsewhere.[29] The cells were cultured at 37°C in a humidified 5% CO_2 environment and were allowed to adhere to walls of