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Cholesterol trafficking-related serum lipoprotein functions in children with cholesteryl ester storage disease

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ABSTRACT

Objective: Serum lipoproteins influence cell cholesterol content by delivering and removing cholesterol to/from cells, functions mainly exerted by LDL and HDL, respectively. Especially in the case of HDL, structure and composition are crucial for function, beyond serum levels. Cholesteryl ester storage disease (CESD) is caused by LIPA gene mutations and reduced activity of lysosomal acid lipase (LAL), the enzyme responsible for hydrolysis of cholesteryl esters and TG. CESD patients typically present dyslipidaemia, liver damage and premature atherosclerosis. The objective of this work was to evaluate serum HDL cholesterol efflux capacity (CEC) and serum cholesterol loading capacity (CLC) in CESD pediatric patients and to study lipoprotein qualitative modifications.
Methods: HDL CEC was evaluated by radioisotopic techniques, serum CLC was measured by a fluorimetric assay, HDL subclasses were determined by two-dimensional electrophoresis.
Results: CESD patients (n = 3) displayed on average increased LDL cholesterol (+163%; p = 0.019), TG (+203; p = 0.012), phospholipids (+40%; p = 0.024) and lower HDL cholesterol (−57%; p = 0.012) compared to controls (n = 9). CESD HDL CEC was impaired both as a whole (average reduction of 26%; p < 0.0001) and with respect to specific membrane cholesterol transporters (−23% for aqueous diffusion; p = 0.005; −32% for ABCA1-efflux; p = 0.0002; −60% for SR-BI-efflux; p < 0.0001; −42% for ABCG1-efflux p = 0.0003). A marked reduction in the pre-β HDL concentration (−69%; p = 0.012) was detected. Finally, CESD serum CLC was significantly increased (+21%; p = 0.0007). *Conclusion*: These new data demonstrate that the pro-atherogenic modifications of serum include disturbances in lipoprotein functions involved in cell cholesterol homeostasis occurring from very early age in CESD patients.

1. Introduction

Both alterations of lipid/lipoprotein metabolism and inflammatory events contribute to the formation of the atherosclerotic plaque, characterized by the accumulation of abnormal amounts of cholesterol and macrophages in the artery wall. Reverse cholesterol transport (RCT) may counteract the pathogenic events leading to the formation and development of atheroma, by promoting the HDL-mediated removal of cholesterol from the artery wall [1]. Mechanisms accounting for cell cholesterol efflux include aqueous diffusion (AD) processes, as well as active pathways mediated by the cholesterol transporters ATP-binding cassette A1 (ABCA1), ATP-binding cassette G1 (ABCG1) and Scavenger Receptor Class B Type I (SR-BI) [2].

The ability of HDL to promote cholesterol efflux from macrophages is thought to be important for the atheroprotective function of HDL [3] and several lines of evidence suggest that the efflux process is sensitive to HDL structure and composition rather than HDL-cholesterol (HDL-C) plasma levels. For example, it has been shown that the serum capacity to promote cholesterol efflux via

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ABCA1 strictly depends on the nascent (preβ) HDL plasma levels [4,5], normally accounting for a small fraction of total HDL. Subjects with the apo-AI_{Milano} mutation or LCAT deficiency have high levels of pre-β circulating particles and increased serum ability to induce macrophage cholesterol depletion despite very low total HDL-C levels [6,7]. In general, the various HDL subpopulations display a specific capacity to promote cholesterol efflux via each of the transporter-mediated pathways of cellular cholesterol efflux [8]. In addition, there is increasing evidence that the measure of HDL functionality in various populations may be a better predictor of coronary artery disease than the determination of absolute HDL-C levels [3,9].

As cell cholesterol efflux is the limiting step of RCT, an estimate of the entire process can be derived from serum cholesterol efflux capacity (CEC), which can be measured in an individual patient as a whole or evaluating the contribution of each specific efflux pathway. Several studies have shown that the ability of HDL to promote cholesterol efflux may be affected by various pathologic processes that induce their structural modifications [10,11].

Lysosomal acid lipase deficiency (LALD) is a rare disorder caused by mutations of *LIPA* gene which encodes for lysosomial acid lipase (LAL) [12], the enzyme responsible for acidic hydrolysis of cholesteryl esters and triglycerides delivered from lipoproteins to lysosomes [12]. Two phenotypes are recognized: Wolman disease, in which the complete absence of LAL activity results in death usually by the first year of life, and Cholesteryl Ester Storage Disease (CESD), in which a 5–10% LAL residual activity allows patients to survive usually to adulthood [13]. Children and adults typically present with some combination of dyslipidaemia, hepatomegaly, elevated transaminases, and microvesicular hepatosteatosis on biopsy. Liver damage with progression to fibrosis, cirrhosis and liver failure occur in a large proportion of patients. Elevated LDL cholesterol (LDL-C) levels and decreased HDL-C levels are common features of the CESD phenotype, and cardiovascular disease may manifest as early as childhood [13]. Total plasma cholesterol levels are often normal in infants with LALD but high triglycerides (TG) and very low density lipoprotein (VLDL) cholesterol levels have been reported in addition to low plasma HDL-C levels [14,15]. Children and adults with LALD often display type IIa or type IIb hyperlipidaemia [16–18], characterized by elevated total cholesterol, LDL-C, apoB and decreased HDL-C levels [19,20]. Atherosclerosis and premature cardiovascular disease have been described in this condition [20–23], and an aortic plaque was found in a child with LALD who died at the age of 9 [24].

Since the lipoprotein derangement in these patients already occurs in pediatric age [20,24] the objective of this work was to evaluate serum CEC as a whole and distinguishing between the SR-BI, ABCG1, ABCA1, and AD pathways in patients with CESD compared to age-matched control subjects. As macrophages cholesterol accumulation is the result of both efflux and influx processes [25,26], to evaluate the net effect of LAL deficiency on overall serum atherogenic properties, we also studied the modifications of serum macrophage cholesterol loading capacity (CLC), which is the overall serum ability to load macrophages with cholesterol, depending on the total lipoprotein profile in terms of both concentration and quality. The data on serum activity with respect to CEC and CLC processes may provide new information on the mechanisms of premature atherosclerosis in CESD [27,28].

2. Materials and methods

2.1. Patients

Three paediatric patients underwent clinical, biochemical, molecular and instrumental assessment to detect and characterize LALD. Further nine children of similar age and sex were studied as controls. The study was performed in accordance with the ethical principles set in the Declaration of Helsinki. Following ethical guidelines, patients and/or parents informed written consent was obtained using a form approved by the local Ethics Committee.

Blood samples for lipoprotein analysis were obtained after an overnight fasting. Plasma total cholesterol (TC) and unesterified cholesterol (UC), HDL-C, TG, apoA-I and apoB, aspartate transaminase (AST) and alanine aminotransferase (ALT) were measured by certified enzymatic or immunoturbidimetric techniques on a Roche diagnostics c311 autoanalyzer. LDL-C was calculated using the Friedewald's formula. Plasma levels of phospholipids (PL) were measured by a commercial enzymatic kit (BL chimica).

LAL activity was determined using the fluorimetric artificial substrate 4-methylumbelliferyl-palmitate in the presence of the inhibitor Lalistat 2 (Chemical Tools, South Bend, IN, USA) by Dried blood spot (DBS) test [29]. The LIPA gene mutation detection was performed by direct polymerase chain reaction (PCR) and direct sequencing [30]. For further details see Supplementary Material.

Instrumental techniques included abdominal ultrasound, hepatic magnetic resonance spectroscopy, carotid intimal-media thickness (cIMT) ultrasonography. Abdominal ultrasound was performed by multifrequency Convex waves (Philips iU22), hepatic magnetic resonance spectroscopy by a 1.5 T magnetic resonance imaging (MRI) scanner (Achieva, version 2.6, Philips Medical Systems, Einalhoven, The Netherlands). cIMT ultrasonography was done with a B-mode ultrasound device (General Electrics, LogiQ e) equipped with a 7.5 MHz linear array probe. cIMT was measured in far wall of left and right common carotids and the maximum value was considered.

A summary of patients' clinical signs, biochemical markers, LAL activity and the type of gene mutation is provided in Supplementary data (Table 1S).

2.2. HDL subclasses

The plasma concentration of HDL particles containing only apoA-I (LpA-I) and of particles containing both apoA-I and apoA-II (LpA-I:A-II) was determined by electroimmunodiffusion in agarose gel using a commercial kit (Sebia, Lisses, France) [31].

Preβ-HDL concentration was evaluated by nondenaturing two-dimensional (2D)-electrophoresis followed by immunodetection against human apoA-I [6]. Briefly, in the first dimension serum was run on a 0.5% agarose gel (Sebia, Lisses, France); agarose gel strips containing the separated lipoproteins were transferred to a 3–20% polyacrylamide gradient gel and run at 30 mA for 4 h. Fractionated HDL were then electroblotted onto a nitrocellulose membrane, detected with an anti-human apoA-I antibody (Calbiochem, Merck, Darmstadt, Germany) and visualized by enhanced chemiluminescence (GE Healthcare Biosciences, Uppsala, Sweden). Densitometric analysis was performed with a GS-690 Imaging Densitometer and the Multi-Analyst software (Bio-Rad Laboratories, Hercules, CA, USA). The content of preβ-HDL was calculated as percentage of total apoA-I signal; the absolute plasma preβ-HDL concentrations (in mg/dl) were calculated multiplying plasma apoA-I levels by the preβ-HDL percentage values. Each CESD serum was run in triplicate on three different gels.

2.3. Serum HDL cholesterol efflux capacity (CEC)

Serum CEC was measured in the three CESD patients and in all control subjects. In particular HDL CEC occurring trough the specific pathways was evaluated by using cell models expressing the transporters of interest and radioisotopic techniques as previously described [10]. For further details see Supplementary data.