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Publication date
1997

Published in
Journal of Lipid Research

Citation for published version (APA):
Lipoprotein[a] is not present in the plasma of patients with some peroxisomal disorders

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Abstract Peroxisomal disorders arise either from defects in the biogenesis of peroxisomes or from the defective synthesis of one or more peroxisomal enzymes. These defects result in metabolic disturbances in peroxisomal β-oxidation of various fatty acids and derivatives and/or in the biosynthesis of ether lipids. In the current study, lipoprotein levels were determined in plasma samples from patients diagnosed with one of four different peroxisomal disorders. While low density lipoprotein (LDL) levels were found to be within the normal range, lipoprotein[a] (Lp[a]) could not be detected by enzyme-linked immunosorbent assay (ELISA) in plasma from patients with cerebro-hepato-renal (Zellweger) syndrome (ZS) and rhizomelic chondrodysplasia punctata (RCDP). Conversely, Lp[a] was clearly present in control plasma obtained from healthy newborns and from patients affected with one of two other peroxisomal disorders, X-linked adrenoleukodystrophy (X-ALD) and Refsum disease (RD) as determined by ELISA. The lack of Lp[a] in plasma of patients with ZS may result from defective secretion of apolipoprotein[a] (apo[a]) (the distinguishing protein component of Lp[a]), as apo[a] mRNA transcripts were clearly present in ZS livers as assessed by PCR, and intracellular apo[a] protein was detected in total liver homogenates from ZS patients as determined by Western blot analysis. Furthermore, LDL present in the plasma of ZS patients was able to associate with recombinant apo[a] in an in vitro Lp[a] assembly assay. —van der Hoek, Y. Y., R. J. A. Wanders, A. E. van den Ende, H. G. Kraft, B. R. Gabel, J. J. P. Kastelein, and M. L. Koschinsky. Lipoprotein[a] is not present in the plasma of patients with some peroxisomal disorders. J. Lipid Res. 1997. 38: 1612–1619.

Lipoprotein[a] (Lp[a]) was first reported over 25 years ago as a genetic trait present in human plasma (1). Strong evidence exists that Lp[a] levels greater than 30 mg/dl are an independent risk factor for coronary heart disease, myocardial infarction, and infarct artery patency (2–8). However, the mechanism by which Lp[a] expedites the atherosclerotic process is poorly understood at present. Lp[a] resembles low density lipoprotein (LDL) in both lipid content and the presence of apolipoprotein B-100 (apoB-100), but is distinguished by the presence of the large glycoprotein apolipoprotein[a] (apo[a]) (2, 3), which is covalently linked to the apoB-100 moiety by a single disulfide bridge (9, 10).

Apo[a] consists of multiple tandem repeats of a sequence closely resembling plasminogen kringle IV, followed by sequences that exhibit a high degree of similarity to the kringle V and protease regions of plasminogen (11, 12). Apo[a] is synthesized in the liver (13) and exhibits genetically determined size polymorphism, with individual isoforms ranging in apparent molecular weight from ~300,000 to ~800,000 (14). Plasma levels of Lp[a] vary over 1000-fold in the human population, ranging from less than 0.1 to greater than 100 mg/dl, and are generally inversely correlated with apo[a] protein isoform size (2, 15). More than 30 different apo[a] isoforms have been reported, and have been shown to arise from variation in the number of kringle IV repeats in the apo[a] gene (16, 17).

The apo[a] gene has been estimated to account for greater than 90% of the variation in plasma Lp[a] concentrations in the Caucasian population (18). The number of apo[a] kringle IV repeats was found to account for 69% of the variation in this population, with

Supplementary key words apolipoprotein[a] • peroxisomes • Zellweger syndrome • apolipoprotein B-100 • apolipoprotein A-I • secretion

Abbreviations: Lp[a], lipoprotein[a]; LDL, low density lipoprotein; apoB-100, apolipoprotein B-100; apo[a], apolipoprotein[a]; apoA-I, apolipoprotein A-I; ZS, cerebro-hepato-renal (Zellweger) syndrome; RCDP, rhizomelic chondrodysplasia punctata; X-ALD, X-linked adrenoleukodystrophy; RD, Refsum disease; PFGE, pulsed-field gel electrophoresis.

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approximately 22% of the variation under the control of as yet undefined cis-acting sequences in the apo[a] gene and the remainder due to polygenic factors (18). At present, however, the identity of these polygenic factors is unknown; the influence of variations in apo[a] gene transcription and mRNA stability (due to hormonal factors, for example), in apo[a] secretion from the liver, and in clearance of Lp[a] from the circulation remains unclear.

Peroxisomes catalyze a number of essential metabolic functions such as β-oxidation of very long chain fatty acids (VLCPA), ether phospholipid synthesis, glycolate metabolism, cholesterol/dolichol metabolism, and phytanic acid catabolism (19, 20). The importance of peroxisomes is exemplified by the existence of inherited metabolic diseases in which one or more of these peroxisomal functions is impaired. Peroxisomal disorders are usually divided into three groups, depending on whether there is singular or more general loss of peroxisomal functions. Despite the fact that peroxisomes are involved in cholesterol biosynthesis (21–23) and bile acid biosynthesis (24), lipid and lipoprotein metabolism in these disorders has not been investigated in detail. There are, however, a few reports of abnormal lipoprotein levels in patients suffering from Refsum disease (25, 26).

In the present study, we have investigated lipoprotein levels in patients with peroxisomal disorders. It was determined that Lp[a] is not present in the plasma of patients with either cerebro-hepato-renal (Zellweger) syndrome or rhizomelic chondrodysplasia punctata. In the former disorder the lack of plasma Lp[a] likely results from defective secretion of apo[a] from the liver.

MATERIALS AND METHODS

Subjects

Plasma samples were obtained from patients with classical Zellweger syndrome (cerebro-hepato-renal (Zellweger) syndrome; ZS), X-linked adrenoleukodystrophy (X-ALD), rhizomelic chondrodysplasia punctata (RCDP), Refsum disease (RD), and from healthy controls for use in the present study. Liver biopsies were obtained from two patients with ZS and from two healthy liver transplant donors (with consent from legal authorities and the patients and/or donor families). In all cases, diagnoses of specific peroxisomal disorders were based on clinical criteria, in addition to a complete analysis of peroxisomal functions in fibroblasts isolated from the various patients (see ref. 20 for a review). The experimental protocol was approved by the Institutional Review Board at the Academic Medical Center in Amsterdam and written informed consent was obtained from all participants or immediate family members.

Lipoprotein measurement

Lp[a] levels in all patients were determined by enzyme-linked immunosorbent assay (ELISA; Terumo, Elkton, MD). The ELISA was calibrated using Lp[a] standards provided in the Terumo kit, and the sensitivity of the ELISA was reported by the manufacturer to be less than or equal to 0.8 mg/dl Lp[a] (expressed in terms of total lipoprotein mass). ApoB-100 and apoA-I were measured by a nephelometric procedure (27). The levels of apoA-I and apoB-100 in ZS, RCDP, and X-ALD were compared to those in healthy controls using the paired t-test (SYSTAT software; SYSTAT Inc., Evanston, IL).

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed as described previously (28) using fibroblasts obtained from ZS patients. Briefly, fibroblasts were thawed and embedded in agarose plugs. After proteinase K treatment, the plugs were incubated twice with 40 U of KpnI in buffer supplied by the manufacturer (Boehringer Mannheim, Germany). The PFGE was performed in 1.2% LE agarose (Seakem, Rockland, ME) in 0.5 × Tris-acetate-EDTA buffer (TAE) at 14°C, using the Chef Mapper System apparatus (Bio-Rad, Richmond, CA). After electrophoresis, DNA fragments were nicked and transferred to Hybond N nylon membrane (Amersham, UK). The filter was hybridized to a digoxigenin (DIG)-labeled 342 bp PstI fragment derived from the apo[a] cDNA and apo[a] bands were detected using a DIG chemiluminescence kit (Boehringer-Mannheim, Germany).

PCR analysis of liver cDNA

Liver samples (approximately 100 mg) from two ZS patients and from two healthy liver transplant donors (see above) were used for poly(A)+ RNA isolation with the FastTrack mRNA isolation kit (Invitrogen Corporation, San Diego, CA). RNA was quantitated by spectrophotometry and stored at −70°C. First-strand cDNA was prepared from 1 μg of poly(A)+ liver RNA using a cDNA synthesis kit (Amersham). Oligonucleotide primers were synthesized that correspond to apo[a] kringle IV type 9 (5′-GATTCTTGGAAGCACCGTG-3′) and apo[a] kringle V (5′-CTGCTTGCCCCACGCC-3′) (17). Polymerase chain reactions (PCR) were performed using an automated thermal cycler (Techne) as previously described (17). The reaction conditions were as follows: 30 cycles of denaturation at 96°C for 40 s, annealing at 55°C for 1 min,
and extension at 72°C for 1 min. The amplified product 

**Immunoblotting of liver homogenates**

Liver samples (approximately 1.5 g) from two ZS patients and a control individual were homogenized in 10 ml ice-cold buffer (20 mM MOPS, pH 7.4, 0.25% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemicals, St. Louis, MO). 5 mM EDTA, 10 µg/ml leupeptin (Boehringer Mannheim, Germany)). The suspension was centrifuged for 20 min at 8000 g and the supernatant was diluted 1:1 in Laemmli sample buffer (29). The samples were boiled for 5 min and a total of 40 µl of the sample was subjected to electrophoresis under reducing conditions on a 1.5% agarose gel (containing 0.1% SDS) for 8 h at 100 V. The resolved proteins (corresponding to the total liver homogenate) were then transferred (16 h at 25 V) to nitrocellulose (Schleicher and Schuell) (30). The membrane was blocked with 3% bovine serum albumin (BSA) in NET buffer (0.15 M NaCl, 5 mM EDTA, 0.05% Triton X-100) and incubated with an anti-apo[a] monoclonal antibody (Boehringer Mannheim, Germany) (1:1000 dilution in NET buffer). Apo[a] proteins were visualized by chemiluminescence (Boehringer Mannheim, Germany).

**In vitro association of r-apo[a] and LDL**

In vitro assays for Lp[a] formation were performed as described previously (10). In these studies, plasma from two patients with ZS (as well as one control individual) was incubated with conditioned medium harvested from the cell line 293/apo[a]24 which had been metabolically labelled with [35S]Cys. As previously described, this cell stably expresses a 17-kringle form of recombinant apo[a] (r-apo[a]) and does not express endogenous apo[a] (31). After a 30-min incubation at 37°C, samples were immunoprecipitated overnight at 4°C using 10–20 µg of an apo[a]-specific monoclonal antibody (2G7; ref. 32). Protein A-Sepharose (Pharmacia LKB Biotechnology Inc., Sweden) was then added for an additional 3 h. The Sepharose was pelleted by brief centrifugation at 16,000 g and washed three times using RIPA buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 20 mM EDTA, 1% sodium deoxycholate, 0.1% SDS) containing 0.5 M NaCl. After a final wash with TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), the immunoprecipitates were boiled for 5 min in 50 µl Laemml sample buffer (29) under non-reducing conditions. Solubilized proteins were separated by SDS-PAGE using a 2.5–15% polyacrylamide gradient gel.

**Lipoprotein profiles of patients with peroxisomal disorders**

ApoB-100, apoA-I, and Lp[a] levels in the plasma of healthy newborns and patients affected by different peroxisomal disorders were measured as described in Materials and Methods; the results are shown in Table 1. Lp[a] was not detected in all 15 patients with classical ZS and in all 5 patients with RCDP, while Lp[a] was present in the plasma of all patients with X-ALD. Refsum disease is an extremely rare disorder, and therefore Lp[a] was measured in the only patient available (a 45-year-old man) and was determined to be 8 mg/dl. In contrast to newborns with ZS, Lp[a] was detectable in the plasma of each of 50 healthy newborns (10 weeks of age) used as control subjects; the mean Lp[a] level in the control samples was determined to be 7.8 ± 7.6 mg/dl. The apoA-I levels in ZS and X-ALD patients were significantly lower ($P < 0.0005$ and $P = 0.002$ respectively) than those in healthy controls. The apoB-100 levels were significantly lower in patients with RCDP ($P = 0.002$) than those in healthy control patients.

**Analysis of the apo[a] gene in ZS patients**

It has been shown that a general inverse correlation exists between apo[a] gene size and corresponding plasma Lp[a] concentration (15). Therefore, we determined the sizes of the apo[a] gene present in 8 of the 15 ZS patients using pulsed-field gel electrophoresis (PFGE) in order to rule out the presence of unusually large apo[a] gene sizes in these patients which could account for their lack of detectable plasma Lp[a]. The average size of the KIV type 2 (major repeat kringle) domain in these individuals was 20; based on this allele size, corresponding detectable plasma Lp[a] levels would be expected (15). It is interesting to note that one patient possessed an apo[a] allele containing 8 copies of the KIV type 2 repeat sequence; as was the case with the other ZS patients tested, this individual also did not possess detectable plasma Lp[a].

**Detection of apo[a] mRNA transcripts in ZS liver by PCR analysis**

In order to investigate whether the apo[a] gene is expressed in patients with ZS, poly(A)+ RNA isolated from the livers of two patients with ZS (as well as from two control individuals) was subjected to PCR analysis.

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**Table 1.** Lipid Research. Volume 38, 1997
Western blot analysis of total liver homogenates

A PCR product of the expected size (590 bp) was observed in both ZS patients as well as in the control samples obtained from healthy liver transplant donors (Fig. 1B). To confirm that the amplified product was apo[a] (and not derived from the plasminogen transcript), the PCR product was digested with AvrII, whose recognition sequence is present only in apo[a] kringle IV type 10 and is absent in plasminogen kringle IV. Digestion of the 590 bp PCR product resulted in the expected band sizes (471 and 119 bp; Fig. 1C).

Western blot analysis of total liver homogenates

Western blot analysis was performed on total liver homogenates to determine whether the apo[a] mRNA present in the liver of ZS patients is translated into protein. For this study we used liver samples from two patients with ZS (Z1 and Z2), in addition to a transplanted liver sample from a healthy individual. Results of the Western blot (Fig. 2) indicate that two apo[a]-specific bands (corresponding to S4 isoform sizes (>~700,000)) are present in the liver of both ZS patients as well as in the control liver sample. PFGE analysis of the apo[a] genes for these individuals is consistent with the apo[a] isoforms seen in the corresponding liver homogenates (both Z1 and Z2 individuals were determined by PFGE to have apo[a] alleles containing between 20 and 30 copies of the major repeat kringle (data not shown).

Ability of LDL present in the plasma of Zellweger patients to associate with apo[a] in vitro

It is possible that the lack of detectable plasma Lp[a] in ZS patients is a result of perturbations in the lipid composition of the LDL particle in these individuals. Such a possibility is suggested by the characteristic alter-
Alterations in lipid composition of LDL in these patients could impair the ability of LDL to associate with apo[a] to form Lp[a] complexes in the plasma of ZS patients. In order to test this hypothesis, conditioned medium was harvested from metabolically labeled 293/a24 cells (stably expressing a 17-kringle form of r-apo[a]; ref. 31) and incubated with plasma collected from two patients with ZS (Z1 and Z2) or from healthy controls. Samples were immunoprecipitated with an anti-apo[a] monoclonal antibody and analyzed by SDSPAGE under non-reducing conditions. In samples containing plasma from both ZS and control patients, a high molecular weight complex (corresponding to recombinant Lp[a]) was clearly present (Fig. 3). These data indicate that LDL present in the plasma of patients with ZS is capable of associating extracellularly with apo[a] to form Lp[a] particles.

DISCUSSION

The cerebro-hepato-renal (Zellweger) syndrome (ZS) is an autosomal recessive disorder caused by mutations in different genes involved in the biogenesis of peroxisomes. ZS is clinically characterized by a typical craniofacial dysmorphism, hepatomegaly, severe hypotonia and psychomotor retardation, failure to thrive, and usually an early death (80% of the patients die within the first 6 months of life) (33). In ZS, disturbances in bile acid biosynthesis, very long chain fatty acid (VLCPA) oxidation, and ether phospholipid biosynthesis have been reported (19, 20). All ZS patients examined in this study demonstrated these typical clinical and biochemical features.

The current study was based on our initial observation that patients with ZS possessed no detectable plasma Lp[a]. In order to measure Lp[a] levels, we used a commercially available ELISA kit. Although it has been demonstrated that Lp[a] levels are generally low at birth (34, 35), Lp[a] was clearly detectable in the plasma of all healthy newborn controls examined. Lp[a] assays with a sensitivity comparable to that used in the present study suggest that very few “Lp[a]-negative plasmas” exist (36, 37). Therefore, our findings provide strong evidence that Lp[a] is absent in the plasma of patients with ZS. Interestingly, Lp[a] was detectable upon ELISA analysis of plasma from a patient with a mild variant of ZS (38) (data not shown).

ApoA-I levels were significantly lower in patients with ZS than in healthy newborn controls, while apoB-100 levels were not significantly different between these two groups. The levels of these apolipoproteins in some ZS patients may be low due to the malnutrition and diarrhoea that characterizes this disorder. In this context, hypolipidemia has been observed in other gastrointestinal disturbances (39). Taken together, these data imply that the lack of Lp[a] in patients with ZS is not a result of gross abnormalities in LDL and HDL metabolism.

An inverse correlation has been demonstrated between apo[a] isoform size and plasma Lp[a] concentration (2, 15). Therefore, PFGE analysis was performed on genomic DNA isolated from 8 of the 15 ZS patients.
to exclude the possibility that these individuals possessed unusually large apo[a] isoforms. The results of this analysis indicated that alleles in these individuals contained an average of 20 copies of the KIV type 2 sequence which is not unusually large; detectable plasma Lp[a] levels are commonly reported corresponding to apo[a] alleles in this size range (15).

To determine whether the apo[a] gene is transcribed in the livers of patients with ZS, poly(A)+ RNA was isolated from liver biopsy tissue of unaffected and affected newborns. PCR analysis of these RNA samples using apo[a]-specific primers flanking kringle IV type 10 demonstrated that apo[a] transcripts are present in liver samples from both ZS patients, as well as healthy controls (Fig. 1).

To determine whether the apo[a] transcripts present in the livers of ZS patients are translated into apo[a] protein, Western blot analysis of total liver homogenates from either control or ZS patients was performed. The results as shown in Fig. 2 indicate that there is detectable apo[a] protein in liver homogenates of both ZS patients tested as well as in the control sample. For each sample (including the control homogenate sample), 2 apo[a]-specific bands in the S4 size range were observed. The lower bands may correspond to incompletely glycosylated intracellular forms of apo[a]; these latter species have been previously described in human hepatoma cells (10) and baboon hepatocytes (40).

Taken together, the results of the PCR and Western blot analyses indicate that there is no obvious impairment of apo[a] gene expression or protein synthesis in ZS patients. Therefore, we hypothesized that the composition of LDL of ZS patients may be altered due to perturbations in lipid metabolism resulting from impaired cholesterol biosynthesis and β-oxidation of VLCFA. It is possible that as a result of these alterations, the nature of the interaction between apoB-100 and the lipid component of LDL may be modified in ZS patients such that moieties on apoB-100 required for coupling with apo[a] (such as the free cysteine involved in disulfide bond formation) are concealed. In order to test this possibility, we assessed the ability of LDL present in the plasma of ZS patients to covalently associate in vitro with a 17-kringle form of recombinant apo[a]. The results presented in Fig. 3 clearly demonstrate that LDL from ZS patients can become incorporated in Lp[a] particles to an extent similar to LDL from the plasma of a healthy control individual.

In order to further define the biochemical defect in ZS that might be responsible for the lack of plasma Lp[a] in these patients, it was of interest to examine Lp[a] levels in patients with three other peroxisomal disorders: X-linked adrenoleukodystrophy (X-ALD), rhizomelic chondrodysplasia punctata (RCDP), and Refsum disease (RD). Although peroxisomal disorders are very rare, we were able to obtain a small group of patients with X-ALD and RCDP for analysis of plasma Lp[a] concentrations. Lp[a] was clearly present in patients with X-ALD (ranging from <1 mg/dl to 9.5 mg/dl in the seven patients studied; see Table 1). However, in patients with RCDP, plasma Lp[a] was not detectable (n = 5). Interestingly, ZS and RCDP share a number of biochemical features such as the inability to synthesize ether phospholipids and the inability to oxidize phytic acid. However, phytic acid oxidation is also deficient in patients with Refsum disease, a rare disorder in which patients survive into adulthood. In the only patient available for our study, Lp[a] was clearly detectable in the plasma of this subject (see Table 1). As such, these data suggest that there is no functional link between plasma Lp[a] and phytic acid oxidation. Therefore, it is possible that the lack of plasma Lp[a] in patients with ZS and RCDP results from deficiency in ether phospholipid biosynthesis.

Ether phospholipids contain an ether bond rather than an ester bond at the sn-1 position of the fatty acid chain. The two key enzymes in ether phospholipid synthesis are dihydroxyacetone phosphate acyl transferase (DHAP-AT) and alkyl dihydroxyacetone phosphate synthase (alkyl DHAP synthase), both of which are predominantly (if not exclusively) located in peroxisomes (19, 20). Patients with ZS lack peroxisomes and therefore are deficient in ether phospholipid biosynthesis (19). In general, stimulatable cells (macrophages, neutrophils, muscle, brain and neural tissue) contain high levels of ether phospholipids. In neutrophils, for example, 65% of the ethanolamine phospholipid is the ether phospholipid plasmenyl ethanolamine. In liver cells, 20% of the phospholipids contain the ether bond (41, 42). Functional roles for ether phospholipids are not known, although proposed functions include signal transduction (43), prostaglandin production and/or arachidonic acid metabolism (44), protection against active oxygen species such as singlet oxygen (45), and protein secretion (46). Thus it is possible that these phospholipids are involved in secretion of apo[a], which is an extremely large glycoprotein ranging in apparent molecular weight from ~300,000 to ~800,000.

In summary, we have demonstrated that Lp[a] is not present in the plasma of RCDP and ZS patients; both apo[a] mRNA transcript and apo[a] protein are clearly detectable in the liver of ZS patients. These data are consistent with the hypothesis that the lack of Lp[a] in ZS patients (and possibly in patients with RCDP) is due to impaired secretion of apo[a]. However, it is also possible that the inability to detect Lp[a] in the plasma of patients with ZS and RCDP reflects altered metabolism (i.e., enhanced catabolism) of Lp[a] in these individu-
als. The biochemical basis of the inability of apo[a] to be secreted in ZS patients, or the potentially enhanced clearance of Lp[a] in these patients, may be related to the deficiency in synthesis of ether phospholipids which is characteristic of this disorder. This study represents the first report of a class of disorders characterized by the absence of detectable plasma Lp[a]. Further studies will be required to elucidate the biochemical abnormalities underlying the absence of plasma Lp[a] in these individuals, as well as potential physiological consequences arising from the absence of this lipoprotein.

This work was supported by a grant (to M.L.K.) from the Medical Research Council of Canada.

References