Research Article [Araştırma Makalesi]

Determination of Apolipoprotein(a) Isoforms and Lipoprotein(a) in Children with Diabetes Mellitus Type 1

[Tip 1 Diyabetli Çocuklarda Apolipoprotein(a) İsoformları ve Lipoprotein(a) Tayini]

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ABSTRACT

Lipoprotein(a) is composed of apoB–100 and the unique protein apolipoprotein(a). Plasma levels of Lipoprotein(a) are determined largely by variation in the gene that encodes it. High plasma levels and small-sized apolipoprotein(a) isoforms are thought to be an independent risk factor for development of atherosclerosis. Children with diabetes mellitus are prone to early development of atherosclerosis. We investigated apolipoprotein(a) polymorphisms and lipoprotein(a) plasma concentrations in 60 normoalbuminuric children with diabetes mellitus type 1 and in 100 healthy children aged between 9 and 17 years. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used for separation of apolipoprotein(a) isoforms. Individuals expressed a single band (homozygotic), a double band (heterozygotic) or no band (null phenotype). In both groups single-banded phenotypes were more common than double-banded phenotypes: 50.0 % vs. 46.7 % for patients and 53.0 % vs. 43.0 % for controls. A higher prevalence of large size (≥22 Kringle IV repeats) apolipoprotein(a) isoforms, associated with lower lipoprotein(a) concentrations, was detected in the patients and in the controls. Most plasma lipoprotein(a) levels were in the low range. Lipoprotein(a) concentrations below the risk limit of 30 mg/dL were present in 90 % of subjects. The comparison of the two groups showed no significant difference between their mean and median plasma concentration of lipoprotein(a).

Key Words: Apolipoprotein(a), children, diabetes mellitus, lipoprotein(a), Sodium dodecyl sulphate polyacrylamide gel electrophoresis

ÖZET

Lipoprotein(a) apoB100 ve özel bir protein olan apolipoprotein(a)'dan oluşur. Lipoprotein(a)'nın plazma düzeyi kodlamayı yapan gendeki varyasyonlar tarafından belirlenir. Yüksek plazma düzeyleri ve küçük boyutlu apolipoprotein(a) isoformların arteroskleroz için bağımsız risk faktörleri olduğu düşünülmektedir. Diyabet Mellituslu çocuklar erken ateroskleroz gelişimine yatkındırlar. Yaşları 9 ve 17 arasında 60 normoalbuminurik, tip 1 diyabetli ve 100 sağlıklı çocukta apolipoprotein(a)ve lipoprotein(a) plazma düzeyleri incelenmiştir. Apolipoprotein(a) isoformlarını ayırmak için sodyum dodesil sülfat poliakrilamid jel elektroforezi kullanılmıştır. Bireylerde tek bant (homozigotik), çift bant (heterozigotik) veya hiç bant (null fenotip) gözlenmiştir. Her iki grupta da tek bantlı fenotipe çift bantlı fenotipten daha sık rastlanmıştır (Hasta grubunda % 50.0'ye % 46.67, kontrol gurubunda % 53'e % 43). Hasta ve kontrollerde büyük apolipoprotein(a) isoformlarının (≥22 Kringle IV tekrarlı) daha düşük lipoprotein(a) düzeyleri ile birlikte bulunma prevalansının yüksek olduğu gözlenmiştir. Çoğunlukla plazma lipoprotein(a) düzeylerinin düşük olduğu gözlenmiştir. Şahısların % 90'ında lipoprotein(a) düzeyleri 30 mg/dL'lik risk düzeyinin altındadır. İki grubun plazma lipoprotein(a) düzeyleri ortalama ve medyan karşılaştırması anlamlı bir fark göstermemiştir.

Anahtar Kelimeler: Apolipoprotein (a), çocuk, diabetes mellitus, lipoprotein (a), sodyum dodesil sülfat poliakrilamid jel elektroforezi

INTRODUCTION

Lipoprotein(a) [Lp(a)] has many properties in common with low-density lipoprotein (LDL), which is a well established atherogenic factor for coronary artery disease (CAD). Lp(a) contains the unique protein apolipoprotein(a) [apo(a)], linked by a disulfide bond to the apolipoprotein B100 characteristic of LDL (1,2). Apo(a) contains a 5' signal sequence, a 3' plasminogen (PLG)-like protease domain and ten different types of kringle IV domains (KIV–l to KIV–10) (3). Apo(a) shows a high degree of genetic polymorphisms, that results from differences in the number of KIV–2 repeats in the LPA gene located in 6q 26-27. Apo(a) size polymorphism was originally demonstrated at the protein level as variation in the apo(a) molecular weight which ranged from 250 to 800 kDa (4). Apo(a) isoforms are grouped into low (LMW) and high molecular weight (HMW) isoforms according to the number of KIV repeats in the apo(a) molecule. The apo(a) size (KIV repeat) polymorphism is associated with Lp(a) level, the number of KIV repeats being negatively correlated to Lp(a) level (4,5).

The KIV repeat polymorphism, determined either by genotyping or protein phenotyping, exhibits association with coronary heart disease (CHD) (6). Small apo(a) isoforms (<22 KIV repeats) are positively associated with premature development of atherosclerosis, particularly when accompanied with high plasma Lp(a) concentrations (>25–30 mg/dL) (6,7).

Atherosclerotic risk factors appear during childhood, and are then associated with atherosclerotic changes in vessel walls (8). Several authors have studied plasma Lp(a) level as a cardiovascular risk factor in childhood and its association other cardiovascular risk factors (8,9). Diabetes mellitus is such a risk factor since atherosclerosis occurs earlier and more frequently in diabetic than in non-diabetic subjects, and is the major cause of mortality in diabetic children (8,9).

In diabetic children, data on plasma Lp(a) levels are limited and contradictory. Studies suggest that the determination of both Lp(a) and apo(a) polymorphisms provides a more complete characterization of the risk for cardiovascular changes linked to the apo(a) gene than the evaluation of Lp(a) levels alone (10,11). We here report on the determination of apo(a) isoforms and Lp(a) concentration in children with diabetes mellitus type 1 and in healthy children in the Republic of Macedonia.

MATERIAL AND METHODS

Subjects

From Department of Endocrinology, University Children's Hospital, Skopje, sixty children (thirty boys and thirty girls) with diabetes mellitus type 1 who were free of complications (neuropathy, retinopathy or nephropathy or microalbuminuria) were studied. All children were with negative family history of cardiovascular diseases. One hundred healthy children (51 boys, 49 girls), recruited from the Department of Physiology and Anthropology, Medical Faculty, Skopje, served as a control group. All children were aged between 9 and 17 years. Parent's consent was compulsory since the age of children was 9-17 years.

Data collection

Blood samples were collected in fasting state. K_3EDTA plasma was prepared by low speed centrifugation at 1800 rpm for 15 min and stored at –80 °C as aliquots in segments of plastic tubes until assayed (12). All icteric or haemolytic blood samples were discarded.

Measurement of lipids, lipoproteins and apolipoproteins

Plasma total cholesterol (TC) and triglyceride (TG) concentrations were measured with enzymatic methods (Randox, Crumlin, UK) in fresh plasma samples within 24 hours.

An enzymatic HDL cholesterol determination was performed on the supernatant, after phospho-molibdate/magnesium precipitation of the lipoproteins that contained apolipoprotein B (LDL, VLDL). LDL cholesterol was calculated by Friedewald's formula (13). The concentration of Lp(a), apolipoproteins A-1 and B (apo A-1, apo B) was measured by immunonephelometry on a Behring Nephelometer Analyzer (Dade Behring Marburg GmbH, Marburg, Germany) at the Institute of Immunobiology and Human Genetics, Medical Faculty, Skopje. Albuminuria was checked in the first morning urine with Micral® test strips (Roche Diagnostics), which detect microalbumin levels as low as 20 mg/L. Excess antibody-gold conjugate is bound by immobilized albumin in a capture zone, so that the detection field is reached only by conjugate molecules charged with the urinary albumin.

Electrophoresis

Denaturing 3-15 % gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a vertical Bio-Rad Mini Protean II System (Bio-Rad Laboratories, Hercules, CA, USA) was used for separation of apo(a) isoforms. Two peristaltic pumps (Masterflex L/S, Cole Parmer Instrument Co., Vernon Hills, IL, USA) that were controlled by Cole - Parmer software, were used to convey the gradient gel characteristics. Twenty µL of plasma was mixed with 80 µL of reducing buffer (containing β-mercaptoethanol, bromphenol blue in glycerol, SDS) to give a total volume of 100 µL and boiled in a water bath for 5 min. Ten µL of treated plasma samples and 5 µL of the standard were resolved in 3-15 % gradient gels. A maximum of eight samples / gel was applied to avoid edge effects. Electrophoresis was performed in Tris-glycine buffer, for 90 min (until the dye front was just out of the gels), at 100 V, at room temperature.

Immunoblotting

After electrophoresis the proteins were transferred to nitrocellulose membranes (BA 83, 0.2 μm, NC.; S & S Protran, Dassel, Germany) by electroblotting using a Hoefer TE22 Transfer Unit (Amersham Pharmacia Biotech, Vienna, Austria), in Tris-glycine-methanol buffer, for 17 hours at 50 V. The apo(a) isoforms were visualized immunochemically with an Lp(a) phenotyping kit (Immuno Ag, Vienna, Austria) containing primary antibody [a polyclonal antihuman Lp(a) (sheep)] and secondary antibody [an alkaline phosphatase-conjugated antisheep Ig G (rabbit)] that was diluted with 5 % Blotto solution (non fat dry milk, Tween 20, 10 X PBS and antifoam A (Sigma Chemical Co., St Louis, MO, USA). The results were compared to standards of known apo(a) isoform size obtained from Immuno Ag, Vienna, Austria. Isoform size was expressed as number of kringle IV (KIV) repeats. Apo(a) isoforms were classified as being either of small or LMW (<22 KIV repeats) or of large or HMW (≥ 22 KIV repeats) size.

Sample preparations, electrophoresis and immunblotting have been already described (14,15).

Each sample of individual plasma contained one or two bands. The phenotype was defined as null when there were no detectable apo(a) bands. If two apo(a) isoforms were detectable, only the smaller apo(a) isoform was used for categorization (16,17,18). Gels were scanned with a Pharmacia LKB Ultra Scan XL laser (Pharmacia LKB Biotechnology, Uppsala, Sweden) controlled from a computer running the Image Master Software (Pharmacia Biotechnology, Uppsala, Sweden).

Statistical analysis

All statistical procedures were carried out using the STATWIN statistical software package (version 5.0 A, Statsoft Inc.1984-95; Tulsa, Oklahoma, USA). Data are presented as means and standard deviations (means ± SD) with $p < 0.05$ taken to indicate a significant difference. Because the distribution of values for Lp(a) was skewed, logarithmic transformations of these data were performed before statistical analysis. Medians were used to describe Lp(a) levels. Differences in the frequencies of apo(a) isoforms between two groups were assessed by the chi-square test for contingency tables. The level of significance was set at $p<0.05$.

RESULTS

Clinical characteristics of diabetic and healthy children are summarized in Table 1. Mean duration of the diabetes was 5.01 ± 2.99 years. None of these children had microalbuminuria.

Of the patients, 50 % exhibited one band (isoform) and were classified as homozygous, 46.67 % showed two bands and were classified as heterozygous and two patients (3.33 %) had the null phenotype (Table 2). Of the control subjects, 53 % were homozygous, 43 % were heterozygous and 4 % subjects had the null phenotype (Table 2). The distribution of phenotypes found in patients and controls was not significantly different ($p<0.7$).

Among the subjects in the study, we identified fifteen apo(a) isoforms. To avoid extensive subgrouping we grouped apo(a) phenotypes into LMW and HMW (Table 3). Those individuals who had only isoforms with more

Table 1. Clinical characteristics of the study population

Parameter	Diabetic children	Controls	p
Age (years)	12.92 ± 2.68	13.61 ± 2.28	0.196
BMI (kg/m²)	19.54 ± 3.12	20.47 ± 3.43	0.23
Total cholesterol (mmol/L)	3.88 ± 0.49	3.95 ± 0.66	0.463
HDL cholesterol (mmol/L)	1.21 ± 0.23	1.35 ± 0.28	<0.05
LDL cholesterol (mmol/L)	2.3 ± 0.45	2.21 ± 0.57	0.493
Triglyceride (mmol/L)	0.94 ± 1.05	0.76 ± 0.34	0.115
Glucose (mmol/L)	8.8 ± 2.08	4.58 ± 0.56	<0.0001
Lp(a) (mg/dL) median (range)	9.62 (0-58.2)	9.62 (0-48.3)	N.S.
Apolipoprotein A1 (mg/dL)	130.44 ± 16.37	134.71 ± 16.25	0.250
Apolipoprotein B (mg/dL)	97.76 ± 17.38	95.04 ± 16.81	0.475

* Lipid parameters, glucose and apolipoproteins are determined in plasma

Table 2. Frequency of apo(a) isoforms in diabetic children and in controls

	Diabetic children		Controls	
Isoform	n	%	n	%
Single	30	50	64	64
Double	28	46.67	32	32
Null	2	3.33	4	4
Total	60	100	100	100

n = represents the number of subjects

Table 3. Size of apo(a) isoforms and plasma Lp(a) levels in diabetic children and controls

	Diabetic children (n=60)		Controls (n=100)	
Apo(a) isoforms	n	Lp(a) (mg/dL) (mean ± SD)	n	Lp(a) (mg/dL) (mean ± SD)
LMW	8	42.39 ± 11.21	11	36.32 ± 5.2
HMW	50	10.75 ± 4.23	85	10.07 ± 3.41
null	2	0	4	0

n = represents the number of subjects

than 22 KIV repeats were classified as the HMW group and those with at least one isoform with less than 22 KIV repeats were classified as LMW.

A higher prevalence of HMW apo(a) was detected in both groups of children (Table 3). There was no significant difference in the frequency of LMW and HMW isoforms between the two groups ($p > 0.05$).

Both groups displayed significantly higher plasma Lp(a) concentrations with LMW apo(a) isoforms than that with HMW apo(a) isoforms ($p < 0.01$) (Table 3).
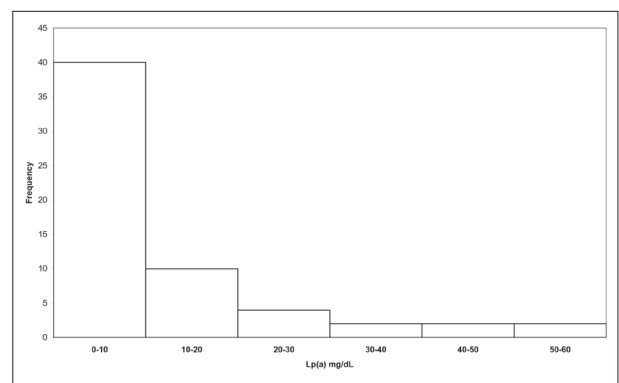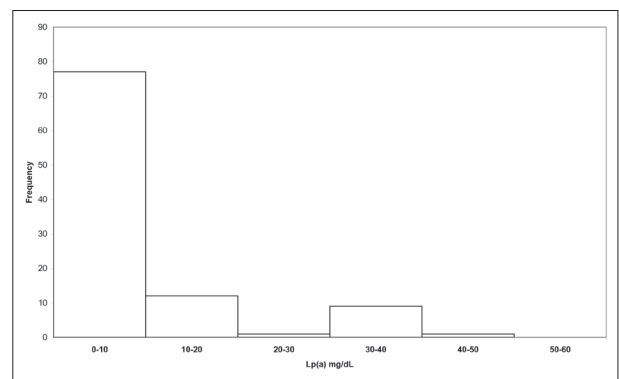
Figures 1 and 2 show the distribution of Lp(a) in the two groups. The distribution was positively skewed toward lower plasma levels, with the majority of subjects having a plasma Lp(a) concentration below the cut-off point of 30 mg/dL. 66.6 % of the diabetic children had Lp(a) levels of 10 mg/dL or less.

There was no difference in plasma Lp(a) concentrations between the two groups. In the controls, plasma Lp(a) concentrations ranged from 0 - 48.3 mg/dL with a mean value of 12.95 ± 9.0 mg/dL (not presented) and a median value of 9.62 mg/dL. In diabetic children plasma Lp(a) concentrations ranged from 0-58.2 mg/dL with a mean value of 14.97 ± 12.16 mg/dL (not presented) and a median value of 9.62 mg/dL.

We found a significant inverse correlation between the Mr of apo(a) isoforms and plasma levels of Lp (a) in patients ($r = - 0.441$, $p < 0.001$) and in healthy children ($r = - 0.426$, $p < 0.001$).

Figure 1. Frequency of plasma Lp(a) concentration in diabetic children

Figure 2. Frequency of plasma Lp(a) concentration in control group

DISCUSSION

The elevated cardiovascular risk of diabetic patients is only partially explained by the presence of conventional cardiovascular risk factors, such as glycemic control, lipid abnormalities, hypertension and visceral obesity (19,20). This has suggested that additional risk factors, such as genetic risk factors, may favour the increased cardiovascular morbidity and mortality observed in diabetic patients. Among the genetic risk factors,

Lp(a) and apo(a) polymorphism has been evaluated. As the process of atherosclerosis starts in childhood, the risk factors should be assessed and prevention of atherosclerosis should start in childhood. Studies analyzing the impact of Lp(a) on the development of CAD in diabetic patients gave controversial findings (21-24) which might be result of the insufficient number of included subjects in case-control studies, lack of information on apo(a) phenotype and urinary albumin excretion.

This is the first study on apo(a) isoforms and plasma Lp(a) concentration in Macedonian children with type 1 diabetes mellitus.

These parameters were compared in 60 diabetic children and 100 non-diabetic children; all were without family history of atherosclerosis and none had detectable albuminuria.

Several issues need to be taken into account when interpreting the results of this study. In this study SDS-PAGE followed by immunoblotting was used for separation and identification of apo(a) isoforms. The same technique was used in our previous study on healthy Macedonian adults (25). We confirmed that this modified Western blot technique is sensitive enough to detect low levels of apo(a) protein. Our data indicate that each apo(a) isoform we detected by our method was specified by a corresponding allele at the apo(a) locus, and the frequency distribution of apo(a) isoforms fit the expectations of the Hardy-Weinberg equilibrium.

In our previous study on healthy Macedonian children we showed that the carriers of single apo(a) isoform were more frequent among healthy Macedonian children (64 %), especially carriers of HMW S4 (42.65 %) (26).

The distribution of apo(a) isoforms found in our diabetic children was similar to the distribution among healthy Macedonian children. Our results demonstrate an increased prevalence of single-banded than of double-banded isoforms in diabetic children. These findings are in accordance with other studies reporting a prevalence of single-banded isoforms of 63.8 % to 67 % (10,11).

We were not able to find a significant difference in the percentage of LMW apo(a) isoforms between diabetics and the controls. HMW apo(a) isoforms were associated with decreased Lp(a) concentrations in both diabetic and non-diabetic subjects.

In agreement with Martinez et al. (27) our plasma Lp(a) levels did not differ significantly between the two groups. This agrees with the reports of similar Lp(a) values in adult patients with IDDM and in controls (28,29). On the contrary, Alsaeid et al.(30) and Levitzky et al.(31) found higher mean Lp(a) values in diabetics than in controls.

In accordance with previous findings (32,34), we observed a significant inverse correlation between the Lp(a) plasma concentration and the apo(a) isoform size.

Our observations do not support increased Lp(a) concentrations in young normoalbuminuric IDDM subjects. These results indicate that apo(a) isoforms and plasma Lp(a) concentration are primarily genetically determined, and are not affected by plasma lipid and apoprotein concentrations.

But, the identification of diabetic children with high Lp(a) levels and/or apo(a) isoforms of low MW may be very important in clinical practice. In these subjects the genetic predisposition for CAD due to apo(a) gene is added to the cardiovascular risk related to diabetes and associated cardiovascular risk factors. Although there is no practical method for lowering Lp(a) concentrations at this time, these patients could be more intensively treated with respect to reduction of modifiable risk factors for cardiovascular disease and diabetes complications.

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