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SERUM sdLDL-C AND CELLULAR SREBP2-DEPENDENT CHOLESTEROL LEVELS; IS THERE A CHALLENGE ON TARGETING PCSK9?

NIVOI sdLDL-C U SERUMU I SREBP2-ZAVISNOG HOLESTEROLA U ĆELIJAMA; DA LI PCSK9 PREDSTAVLJA IZAZOV KAO META?

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Summary

Background: Serum small dense LDL-cholesterol (sdLDL-C) value is suggested to be an important risk factor for atherosclerosis. Since sdLDL-C changes may be related to PCSK9 and SREBP-2 functions, the aim of this study was to investigate correlations between sdLDL-C, circulating PCSK9, SREBP-2 expression and some lipid parameters in serum and buffy coat fraction of healthy subjects.
Methods: One hundred and twenty-four subjects were randomly included in the study. The lipid profile was measured using routine laboratory methods. The serum sdLDL-C level was calculated by a heparin-related precipitation technique. The cellular LDL-C/protein and cholesterol/protein values were measured after lysing of cells with methanol/chloroform binary solvent. The circulating PCSK9 level was measured using ELISA technique. The SREBP-2 expression level was estimated using the RT-qPCR technique.
Results: Data showed significant correlations between LDL-C, TG and sdLDL-C levels (r=0.34, p=0.001; r=0.2, p=0.04). The circulating PCSK9 level was correlated to LDL-C (r=0.29, p=0.04), but not to sdLDL-C (r=-0.08, p=0.57). Also, cellular LDL-C value was not related to serum LDL-C level (r=-0.12, p=0.39). Furthermore, there was an inverse correlation between cellular LDL-C/protein value and estimated *de novo* cholesterol/protein value (r=-0.5, p=0.001). Similar results were observed for cellular LDL-C/protein value and SREBP-2 expression level (r=-0.52, p=0.004).

Kratak sadržaj

Uvod: Vrednosti malih gustih čestica LDL-holesterola (sdLDL-C) predstavljene su kao važan faktor rizika za aterosklerozu. Pošto promene u sdLDL-C mogu biti povezane sa funkcijom PCSK9 i SREBP-2, cilj ove studije bio je da se istraže korelacije između sdLDL-C, PCSK9 u cirkulaciji, ekspresije SREBP-2 i nekih lipidnih parametara u serumu i frakciji leukocita zdravih ispitanika.
Metode: U studiju je nasumično uključeno 124 ispitanika. Lipidni profili izmereni su rutinskim laboratorijskim metodama. Nivo sdLDL-C u serumu izračunat je heparin precipitacionom tehnikom. Vrednosti ćelijskog LDL-C/proteina i holesterola/proteina merene su posle liziranja ćelija binarnim rastvorom metanola/hloroforma. Nivo PCSK9 u cirkulaciji meren je tehnikom ELISA. Nivo ekspresije SREBP-2 određen je tehnikom RT-qPCR.
Rezultati: Podaci su pokazali značajne korelacije između nivoa LDL-C, TG i sdLDL-C (r=0,34, p=0,01; r=0,2, p=0,04). Nivo PCSK9 u cirkulaciji korelisao je sa LDL-C (r=0,29, p=0,04), ali ne i sa sdLDL-C (r=-0,08, p=0,57). Takođe, vrednost ćelijskog LDL-C nije bila povezana sa nivoom LDL-C u serumu (r=-0,12, p=0,39). Štaviše, otkrivena je inverzna korelacija između vrednosti ćelijskog LDL-C/proteina i vrednosti određenog *de novo* holesterola/proteina (r=-0,5, p=0,001). Slični rezultati su uočeni za vrednost ćelijskog LDL-C/proteina i nivo ekspresije SREBP-2 (r=-0,52, p=0,004).

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Conclusions: We concluded that the serum sdLDL-C value is not related to circulating PCSK9. Furthermore, SREBP-2 regulatory system was able to elevate the cellular cholesterol level after reducing LDL influx. We suggest to investigate the cellular sdLDL fate and lipid synthesis pathways in PCSK9-targeting studies.

Keywords: sdLDL-C, LDL-C, PCSK9, SREBP-2

Zaključak: Zaključili smo da vrednost sdLDL-C u serumu nije povezana sa PCSK9 u cirkulaciji. Štaviše, regulacioni sistem SREBP-2 bio je sposoban da podigne nivo ćelijskog holesterola posle smanjenja dotoka LDL. Savetujemo da se istraži sudbina ćelijskog sdLDL kao i putanje sinteze lipida u studijama koje će se fokusirati na PCSK9.

Ključne reči: sdLDL-C, LCL-C, PCSK9, SREBP-2

Introduction

It is well known that inflammatory events trigger atherogenic processes in order to develop atherosclerotic plaques. Furthermore, the plaque growth and development relate individually to classic risk factors and local molecular events (1). Circulating LDL particles are reported as a strong risk factor for atherosclerosis. These particles are able to pass through the vessel endothelial layer and to become modified by extracellular catalytic and oxidant agents (minimally modified LDL (mmLDL) and oxidized LDL (Ox-LDL) particles) in subendothelial space. The modified particles are the subject for formation of foam cells and central core of atherosclerotic plaque. Moreover, the components of modified LDL particles, as chemotactic agents, can improve the inflammatory events and plaque remodeling (2, 3).

Several studies showed that cases with the normal serum LDL levels develop CVD (4, 5). Numerous studies also showed that the physical characteristics of LDL particles such as size, density, lipid and protein contents rather than simply LDL concentration are more important factors for coronary vascular disease (CVD) risk. The small dense LDL (sdLDL) particles are the subfractions with density range 1.034–1.066 g/mL that are thought to be a more important atherogenic factor than the large particles (namely large buoyant LDL, lbLDL). They contain less cholesterol values and lower affinity for the LDL receptor (LDLR). Furthermore, they are characterized as having a longer half-life undergoing greater susceptibility for oxidation reactions (6).

On the other hand, the molecular studies showed that cholesterol homeostasis is dependent upon the proprotein convertase subtilin/kexin type 9 (PCSK9) function. The PCSK9 expression is mostly found in the liver and is secreted into circulation. It acts as a plasma chaperone to internalize LDLR into the lysosome (7). Furthermore, the sterol regulation element binding protein 2 (SREBP-2) regulatory system relates to the PCSK9 and LDLR expression levels so that they can inversely affect serum LDL level and cholesterol synthesis (8, 9). Here, we investigated correlations between PCSK9, SREBP-2 expression, sdLDL-C, total cholesterol, total triglyceride and LDL-C values.

Methods and Materials

Subjects

One hundred twenty-four healthy adults aged <83 years with BMI 29.78±3.73 kg/m^2 were randomly recruited from people referred into health centers (North of Tehran) for routine checkup. Based on medical history, the subjects had no clinical problems (diabetes, myocardial infarction, lupus and immune defects). None of the subjects had taken medications and supplements for at least the previous three months. University ethics committee approved the study and informed consent was obtained from all subjects.

Samples

The sera were obtained from whole blood samples (2500 rpm, 900 s). Furthermore, buffy coat fractions were partially separated from the EDTA-containing whole blood samples (5 mL) and aliquots were preserved at –80 °C.

Biochemical measurements

Lipid profile. Serum total cholesterol (TC), total triglyceride (TG) and HDL-C levels were measured by routine laboratory techniques (Pars Azmoon kit, Pars Azmoon Inc., Tehran, Iran). Serum LDL-C value was directly measured by the enzyme colorimetric method after omitting HDL, VLDL and chylomicron lipoproteins (Pars Azmoon kit, Pars Azmoon Inc., Tehran, Iran).

Serum small dense LDL-C (sdLDL-C). Heparin-Mg^{+2} (150 U/mL, 90 mmol/L) and Heparin-Mn^{+2} (40 U/mL, 30 mmol/L) solution (0.1 mL) was added to serum (0.1 mL) and incubated at room temperature (600 s). Then, it was transferred into cold-room (900 s) and centrifuged (1500 rpm, 900 s) to separate the supernatant containing sdLDL particles (10). The supernatant sdLDL-C and cholesterol values were directly determined by Pars Azmoon kits.

Cellular cholesterol and LDL-C. The EDTA-containing whole blood samples were centrifuged (2500 rpm, 1200 s) and their plasma were poured out. The buffy coats (300 μL) were partially separated and their red blood cells were lysed by RBC lysis buffer (500 μL; 8.26 g ammonium chloride (NH_4Cl), 1 g

potassium bicarbonate ($KHCO_3$), 0.037 g EDTA dissolved in 1 L ddH_2O, pH 8). The cell plates were rinsed and centrifuged at least three times with RBC lysis buffer (10000 rpm, 60 s). Then, they were lysed by methanol/chloroform (2:1) binary solvent based on Bligh and Dyer method (11). The bottom fractions were separated and KCL solution (0.88%, diluted with methanol) was added to each sample and centrifuged at 2400 rpm for 600 s. The supernatants were evaporated inside the chemical hood (24 hours). The protein (g/L, Bradford method (12)), total cholesterol (mmol/L) and LDL-C values were measured after adding 50 μL of methanol.

De novo cholesterol. The cellular LDL-C value was subtracted from cellular total cholesterol value to estimate other cholesterol sources suggested as *de novo* cholesterol of cellular fraction (estimated *de novo* cholesterol value = Cellular total cholesterol value – Cellular LDL-C value).

Circulating PCSK9. The PCSK9 ELISA kit based on the standard sandwich enzyme-linked immunosorbent assay was used and applied according to the manufacturer's instructions (Abnova, cat. no: KA1072). The PCSK9 values were determined with standard linear curve.

RNA. Total RNA was isolated from buffy coat (RNA isolation kit, Arya tous). Buffy coat sample (150 μL) was transferred into a tube containing lysis solution (750 μL). Then, chloroform (150 μL) was added and centrifuged at 13000 rpm for 600 s. The upper phase was separated and rinsed with ethanol (70%) on a spin column and finally RNA was prepared from the spin column with DEPC-treated water.

cDNA. cDNA was synthesized on the basis of manufacturer's instructions (PrimeScript II strand cDNA Synthesis Kit, Takara, Japan). RNA (3 μL) was added to master mix containing RT enzyme and was incubated at 37 °C for 900 s. Then, it was inactivated at 80 °C for 5 s and was kept at –80 °C.

SREBP-2 expression. The buffy coat SREBP-2 expression value was measured by QuantiFast *SYBR Green PCR Kit* (Qiagen, Germany) (temperature cycles, n=30; denaturation, 95 °C for 20 s; annealing and extension, 63 °C for 25 s). The primers for SREBP-2 gene were designed with Genomics Expression Software (ver. 1.1) (5'-CTACGGTGCAGACAGTTGCT-3', 5'-CCAGGGTTGGTACTTGAAGGG-3') and normalized with beta-actin gene (5'-TCCCTGGAGAAGAGCTACG-3', 5'-GTAGTTTCGTGGATGCCACA-3').

Statistical analysis

Data was analyzed with a statistical software package (Version 16.0, Chicago, SPSS Inc.). The Kolmogorov Smirnov test was applied to evaluate the normal distribution. The partial covariates for binary regressions were controlled by age, gender and BMI parameters. Differences between gender groups were statistically evaluated with Student-t test. The $2^{(-deltaCT)}$ value was calculated to determine the relative expression of SREBP-2 gene.

Results

Population characteristics

Table I shows some biochemical parameters in the study population. The serum sdLDL-C value (0.91± 0.05 mmol/L) was nearly ¼ of serum LDL-C value (3.12±0.7 mmol/L). The LDL-C value (0.08±0.03 mol/g) was nearly ½ of total cholesterol value (0.17± 0.027 mol/g) in cellular fraction. Moreover, the results did not show significant associations between gender and serum LDL-C value (p=0.15), and also between gender and serum sdLDL-C value (p=0.35).

Serum LDL-C

There were no significant linear correlations between age and serum LDL-C value (r=0.039, p=0.69) and also between cellular LDL-C/protein and serum LDL-C values (r=-0.12, p=0.39). Similar data were obtained for cellular total cholesterol/protein and serum LDL-C values (r=0.14, p=0.32). However, a significant correlation was observed between serum sdLDL-C and LDL-C values (r=0.34, p=0.001) (*Table II*).

Serum sdLDL-C

The serum sdLDL-C values did not correlate to age (r=0.127, p=0.18), total cholesterol (r=0.053, p=0.57), cellular LDL-C/protein value (r=0.04, p=0.79) and cellular total cholesterol/protein value (r=0.19, p=0.18). However, a significant correlation was observed between serum sdLDL-C and total triglyceride values (r=0.2, p=0.04). In contrast with serum LDL-C value, we did not consider as significant the correlation between serum sdLDL-C and PCSK9 values (r=-0.08, p=0.57) (*Table III*).

Cellular LDL-C and total cholesterol

Results showed a significant correlation between cellular LDL-C/protein and total cholesterol/protein values (r=0.55, p=0.009). Also, we observed inverse correlations between cellular LDL-C/protein and estimated *de novo* cholesterol/protein values (r=–0.5, p=0.001) and between the SREBP-2 expression level and cellular LDL-C/protein value (r=–0.52, p=0.004) (*Table IV*).

Table I Characteristics of the study population.

Parameter	Mean±SD (n%)
Age (years)	44.32±11.56
Sex (male/female)	63 (50.8%)/61 (49.2%)
Serum LDL-C (mmol/L)	3.12±0.7
Serum HDL-C (mmol/L)	1.42±0.28
Serum total cholesterol (TC, mmol/L)	4.74±36.31
Serum total triglyceride (mmol/L)	2.39±1.41
Serum LDL-C/HDL-C	2.26±0.66
Serum LDL-C/TC	0.68±0.15
Serum PCSK9 (μg/L)	3.62±1.5
Serum small dense-LDL-C (sdLDL-C, mmol/L)	0.91±0.05
Total cholesterol (serum sdLDL-C fraction, mmol/L)	1.93±0.94
Cellular LDL-C/protein (mol/g)	0.08±0.03
Cellular total cholesterol/protein (mol/g)	0.17±0.027

Table II Correlations between serum LDL-C value and study parameters.

Parameter	Correlation coefficient (r)	P value
Age (years)	0.039	0.69
sdLDL (mmol/L)	0.34	0.001
Cellular LDL-C/protein	–0.12	0.39
Cellular cholesterol/protein	0.14	0.32
PCSK9 (ng/mL)	0.29	0.03

Controlled by age, gender and BMI parameters

Table III Correlations between serum sdLDL-C value and study parameters.

Parameter	Correlation coefficient (r)	P value
Age (years)	0.127	0.18
Total triglyceride (mmol/L)	0.2	0.04
Total cholesterol (mmol/L)	0.053	0.57
Cellular LDL-C/protein (mol/g)	0.04	0.79
Cellular cholesterol/protein (mol/g)	0.19	0.18
PCSK9 (μg/L)	–0.08	0.57

Controlled by age, gender and BMI parameters

Table IV Correlations between cellular LDL-C/protein value and study cellular parameters.

Parameter	Correlation coefficient (r)	P value
Cellular cholesterol/protein (mol/g)	0.55	0.009
De novo cholesterol/protein (mol/g)	–0.5	0.001
Relative expression level, SREBP-2 ($2^{-\Delta CT}$)	–0.52*	0.004

*; Spearman
Controlled by age, gender and BMI parameters

Discussion

The serum LDL-C value is known as one of the most important classic risk factors in the pathogenesis of cardiovascular diseases (CVD). In recent decades, the studies have been directed at introducing statins as drugs lowering the serum LDL-C level. Moreover, a new approach following in clinical trials is suggested to target the circulating PCSK9 in order to diminish the LDLR internalization and serum LDL-C level (13, 14). In this study, we investigated the correlations between LDL-C and sdLDL-C values and some lipid parameters. Also, we evaluated the effect of circulating PCSK9 on the serum LDL-C and sdLDL-C values and the association of SREBP-2 expression level with the cellular LDL-C value so that it may help to explain the results of clinical trials.

Although LDL-C value is known as a strong risk factor for CVD, it is not always elevated in CVD patients. Numerous studies suggested that serum sdLDL value is thought to be a more atherogenic factor so that its measurement may be useful for evaluating CVD risk (15). Furthermore, a number of studies reported that serum sdLDL is a primary factor for the development of atherogenic events (16). In agreement with these reports, we showed a significant correlation between serum LDL-C and sdLDL-C values. Moreover, we found a correlation between sdLDL and total triglyceride values as reported by others (17). However, there were no similar results for serum sdLDL-C and total cholesterol values. This may be due to the sdLDL cholesterol content that is reported to be less than that of large LDL particles (18) and the role of total triglycerides as an important risk factor in the study population.

On the other hand, it is well known that the serum LDL-C value is associated to circulating PCSK9, as we showed in this study. Furthermore, the approaches targeting PCSK9 have tried to explain the effects of complementary therapies with statins to reduce serum LDL levels (19). We did not observe significant correlations between serum LDL-C and sdLDL-C levels and their values in the cellular fraction. The results also suggested that circulating PCSK9 is able to regulate the cellular LDL influx while it was not correlated to serum PCSK9 value. Previous

studies reported that sdLDL particles have lower affinity for LDL receptors (20) thus, we proposed that the PCSK9 regulatory mechanism may not be involved in sdLDL influx. Furthermore, we suggested that it may be still a strong risk factor for CVD even after targeting PCSK9.

The results also showed that the estimated *de novo* cholesterol value is inversely correlated to cellular LDL-C value. Moreover, we observed similar results between the cellular LDL-C value and SREBP-2 expression level. It was enough to propose that the reduced LDL influx causes elevation of the cellular cholesterol level through the SREBP-2 regulatory system (21).

In conclusion, the results showed that serum sdLDL-C value correlating with the serum LDL-C value is not related to circulating PCSK9. Furthermore, we considered the role of the SREBP-2 regulatory system to elevate the cellular cholesterol level after reducing LDL influx. We suggested investigating the cellular sdLDL fate and the involvement of lipid synthesis pathways in PCSK9-targeting studies.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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