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Significant limitations associated with the analysis of human plasma soluble CD36 performed by ELISA

Analiza osoczowych stężeń CD36 metodą ELISA oraz jej ograniczenia

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ABSTRACT

Introduction: Plasma sCD36 is generally considered to be a marker of macrophage activation and inflammation, accelerated fat accumulation in the vascular wall, and atherosclerosis. The objective of the study is to develop a methodology for sCD36 determination.

Materials and methods: The material for sCD36 determination by ELISA assays was collected from fasting blood samples from healthy cases.

Results: The study identified possible problems in sCD36 plasma concentration measurement. The presented data also suggested that the interrelationship of sCD36 and various metabolic stimuli can significantly affect sCD36 plasma concentration.

Conclusion: The ELISA assay can be used to determine sCD36 concentration in human plasma.

Keywords: sCD36 determination; plasma; ELISA; methodology.

ABSTRAKT

Wstęp: Badania wskazują, że stężenie białka sCD36 w osoczu mogłoby być markerem aktywacji makrofagów i stanu zapalnego oraz przyspieszonej akumulacji lipidów w ścianie naczyniowej, które w rezultacie prowadzą do miażdżycy tętnic.

Celem badania było opracowanie wiarygodnej metodyki oznaczania sCD36.

Materiały i metody: Jako materiał do opracowania metodyki oznaczania stężenia sCD36 w osoczu testem ELISA posłużyły pobrane na czczo próbki krwi od losowo wybranych zdrowych osób, które zgłosiły się na obowiązkowe badania kontrolne w Przychodni Medycyny Pracy.

Wyniki: Wykazano możliwe problemy w pomiarze stężenia sCD36 w osoczu metodą ELISA. Wskazano również, że interakcje sCD36 z innymi czynnikami mogą wpływać w szerokim zakresie na stężenie sCD36 w osoczu.

Wnioski: Uwzględniając pewne ograniczenia, test ELISA można wykorzystać do oznaczania stężenia sCD36 w osoczu ludzkim.

Słowa kluczowe: oznaczanie stężenia sCD36; osocze; ELISA; metodyka.

INTRODUCTION

CD36 is a major receptor for the uptake of oxidized LDL [1]. It is a glycoprotein situated in plasma membranes of platelets, monocytes, macrophages, adipocytes and muscles [2, 3]. The available data suggest [4, 5, 6, 7, 8, 9] that the monocyte-to-macrophage differentiation by CD36 expression may play a critical role in the initiation and progression of atherosclerosis by facilitating the formation of lipid-engorged macrophage foam cells. The *CD36* gene is located on chromosome 7q11.2 [10]. The plasma circulating product of the *CD36* gene, soluble CD36 (sCD36), is not derived from proteolytic cleavage of the extracellular part of the CD36 protein [11], but may be present as the unbound protein in a peptide fraction or in microparticles shed from cells after being triggered by various stimuli or during cell apoptosis [12, 13]. It has been proposed in some studies [10, 14] that sCD36 could be a marker of macrophage activation and inflammation, accelerated fat accumulation in the vascular wall, and atherosclerosis. The analysis of sCD36 is currently hampered by the lack of widely accepted standardized methods of plasma sCD36 determination. Therefore, the study aims to develop a methodology for sCD36 determination by ELISA assays.

MATERIALS AND METHODS

The material for sCD36 determination comprised 35 healthy subjects, 8 men and 27 women, older than 70 years, without accompanying diseases. The study complies with the principles

of the Declaration of Helsinki and was approved by the local Institutional Ethics Committee (BN-001/162/04). Informed consent was obtained from participating subjects. Fasting blood samples were taken for sCD36 measurements.

EDTA-anticoagulated blood samples were centrifuged for 10 min at 4000 g and then stored at –30°C. Plasma concentrations of human antigen CD36 (also called platelet membrane glycoprotein IV) were measured, according to the manufacturer's instructions, with the use of ELISA kits, which is a commercially available enzyme-linked immunosorbent assay (EIAab, Wuhan EIAab Science Co., Ltd., China). All the samples were diluted 5000× with sample diluent. It was impossible to determine the concentration of sCD36 on the curve in this assay with lower dilution of the sample, thus only extrapolation was possible rather than accurate determination. The standard was reconstituted with 1.0 mL of sample diluent and set aside for 15 min with gentle agitation prior to making serial dilutions. This reconstitution produced a stock solution of 10.0 ng/mL. The undiluted standard served as the high standard (10.0 ng/mL). The sample diluent served as the zero standard (0 ng/mL). A monoclonal antibody specific for sCD36 was pre-coated onto a microplate. Standards and samples were pipetted into the wells and any sCD36 present was bound by the immobilized antibody. This was followed by a 2-hour incubation at 37°C. Detection reagents were diluted to the working concentration using assay diluent 1:100. After removing liquid from any unbound substances, a biotin-conjugated polyclonal antibody specific for sCD36 was added to the wells and again incubated for another 60 min at 37°C. Following a wash to remove any unbound antibody-enzyme reagent, an avidin conjugated with Horseradish Peroxidase (HRP) solution was added to the wells. Wash buffer was warmed to room temperature and mixed gently. 30 mL of wash buffer concentrate was diluted into distilled water to prepare 750 mL of wash buffer. The wash process was repeated 3 times to remove any remaining wash buffer by decanting. After the last wash the plate was inverted and blotted with clean paper towels. In the next step, after washing away any unbound substances, a 90 µL of substrate solution was added to the wells for 30 min at 37°C. The substrate solution was TMB-tetramethylbenzidine. The colour development was stopped with 50 µL of sulfuric acid. In each step of the procedure the total dispensing time for the addition of reagents to the assay plate did not exceed 10 min. Absorbance was read at 450 nm with correction at $\lambda = 630$ nm. We used an automated Microplate Reader ELX 808IU (Bio-Tek Instruments Inc). The results were analyzed using a quadratic curve fit. The calibration was performed with recombinant human CD36 in concentration range 0.15–10 ng/mL.

RESULTS AND DISCUSSION

In our study the median plasma sCD36 concentrations with lower quartile (Q_1) and upper quartile (Q_3) was 19.0 µg/mL (Q_1: 13.50 µg/mL; Q_3: 23.3 µg/mL). Due to limited funding, validation of the test used in the study was not performed in order to compare it to other manufacturers' tests. For the same reasons, a duplicate determination of sCD36 concentration was performed only for some blood samples. The precision of intra-assay and inter-assay in used ELISA was not determined by the manufacturer either. The range of plasma sCD36 concentrations (7.14–78.9 µg/mL) measured with the use of the kit was much higher than described in 2 previous studies. One of them [15] reported plasma sCD36 in healthy Caucasian subjects in the range of 20.0–45.0 ng/mL, while the other [16] reported 0.05–250 ng/mL plasma sCD36 in 10 undefined subjects. Significant differences in plasma sCD36 concentrations measured by 2 commercial ELISA: Adipobioscience and Cusabio Biotech sCD36 assays were also reported recently [17]. The first assay measured 10 samples in the range 0–250 ng/mL, while the other measured the same 10 samples in the range 0.05–1.0 ng/mL. Only one study [18] – with obese diabetic Australian individuals – determined the absolute levels of sCD36 protein in the plasma samples to range from undetectable to as high as 22.9 µg/mL. Unlike all other authors [6, 12, 14, 19, 20] only relative or arbitrary units of sCD36 concentration were reported in the study. This limitation may be a major cause of inconsistent results of different studies concerning sCD36 and prevents the comparison of the data reported by the various authors. Moreover, there are several other possible explanations for significant differences between results obtained during measurements of concentration of sCD36 in plasma reported by several authors with range from ng/mL to µg/mL. The differences may have originated from differences in patient population, different sources of tissue, differences in antibody specificity, or differences between the structure of the protein used as a standard and native sCD36 molecule found in the plasma.

One of the possible causes for the high concentration of sCD36 in the plasma samples may be the presence of platelets, but it seems that in our study residual cells and platelets were effectively removed from plasma at the centrifugation step (4000 g for 10 min). Other authors [21] prepared platelet-free plasma by serial double centrifugation of fresh blood samples at 3000 g for 15 min at room temperature and isolated circulating sCD36 from platelet-free plasma by immunoprecipitation. The cited study established that more than 90% of circulating microparticles are thought to be platelet derived present in the plasma of normal healthy individuals. The second possible cause for the high concentration of sCD36 in plasma samples may be the presence or absence of various stimuli of CD36 expression. sCD36 is not subject to post-translational modification. The small size of our study group does not allow us to draw advanced conclusions, but it is necessary to identify possible problems in sCD36 plasma concentration measurement.

CONCLUSIONS

To conclude, it is crucial to standardize the methods of plasma sCD36 determination in further research before using sCD36 concentration as a potential risk factor of metabolic syndrome.

ELISA can be one of the methods of plasma sCD36 determination. However, presented data suggest that sCD36 as a marker is difficult to evaluate, because the inter-relationship of sCD36 and various stimuli can significantly affect sCD36 plasma concentration.

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