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Original Article

DIAGNOSTIC AND PROGNOSTIC POTENTIAL OF OXIDATIVE STRESS MARKERS IN ADULTS WITH IMMUNE THROMBOCYTOPENIA

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Summary

Immune thrombocytopenia (ITP) is one of the most common causes of clinically overt hemorrhage. Despite the progress made in recent years in clarifying the pathogenesis of the disease, the exact unlock mechanisms still remain unclear. The aim of the study was to correlate the oxidative stress markers and the severity of immune thrombocytopenia in adults and to investigate their predictive value of transforming the acute form of ITP into chronic ITP. We studied a total of 58 subjects (14 patients with newly diagnosed ITP, 13 patients with chronic form of ITR, and 31 controls). The plasma levels of human pantetheinase (vanin-1) and lipid hydroperoxides were measured using commercial assay kits. We found that the form of the disease was not significantly related to the plasma vanin-1 levels (p=0.120). A significant difference in the vanin-1concentrations was observed between newly diagnosed IPT and the controls (p=0.046). Further studies with larger and more homogenous groups of patients and including more indicators of oxidative stress are needed to be able to draw statistically valid conclusions about the role of oxidative biomarkers in diagnosing and treatment of ITP.

Key words: immune thrombocytopenia, oxidative stress, vanin-1, lipid hydroperoxides

Introduction

Immune thrombocytopenia (ITP) is among the most common causes of hemorrhagic manifestations in clinical practice and could serve as a model of autoimmune disease. The primary ITP is an immune-conditioned disorder characterized by isolated thrombocytopenia with a platelet count below 100 x 10^9/L, and the absence of other causes or disorders, which can be associated with thrombocytopenia [1]. Despite the progress made in recent years in clarifying the pathogenesis of the disease, the exact unlock mechanisms and the mechanism of onset of certain immunity disorders still remain unclear. Moreover, the clinical manifestations of the disease are highly variable, and today there is no specific diagnostic marker or etiology-specific therapy.

According to clinical and experimental data, oxidative stress is involved in various stages of the pathogenesis of autoimmune diseases, and particularly in the etiological, regulatory and

effector mechanisms of the autoimmune processes [2-6]. The data on the role of oxidative stress in the pathogenesis and progression of ITP is sparse and, in some cases, contradictory. Some studies have reported changes in levels of oxidative biomarkers in the blood, especially the changes in levels of serum malondialdehyde, reduced/oxidized glutathione ratio, total antioxidant status and the activity of certain antioxidant enzymes [7-9]. There is also evidence for available overexpression of the vanin-1 (VNN1) in peripheral leukocytes, a sensitive sensor to oxidative stress [10]. Furthermore, the results published suggest the use of gene overexpression of VNN1 as a sensitive and specific marker for differentiating a newly diagnosed chronic form of the disease [11].

The primary objectives of this study was to establish whether VNN1 alone or in a complex with ROOH can be regarded as a predictor of the process of transformation of the acute form of ITP to chronic ITP and whether there is a correlation between markers of oxidative stress [plasma VNN1 and lipid hydroperoxide (ROOH)] and the severity of immune thrombocytopenia in adults.

Patients and Methods

Subjects Tested

The present study was conducted from October 2015 to May 2016 and comprised 27 patients, of whom 14 were subjects with newly diagnosed form of ITP and 13 – with chronic form of ITR. Thirty one healthy male and female subjects of a similar age range were included in the control group. The patients had been treated and registered for follow-up at the Clinic of Hematology of the University Hospital in Pleven. The diagnosis was made based on the International Working Group (IWG) – 2009 criteria, in accordance with the methodological instructions that are part of the standards in clinical hematology. The study did not include individuals with diseases possibly affecting the levels of VNN1, i.e. inflammatory bowel disease, colorectal cancer and coronary heart disease (CHD). Informed consent was obtained from each subject. The study was approved by the Ethics Committee at Medical University – Pleven.

Biochemical Tests

Peripheral venous fasting blood samples, anticoagulated with EDTA, were collected from the tested subjects upon admission to hospital. The blood count test was carried out by standard methods. Plasma was separated within 30 min after blood withdrawal. For this purpose, the blood samples were centrifuged at 1000 g at 2-8°C for 15 min, after which the separated plasma was stored at -20°C before testing.

Determination of Human Pantetheinase (VNN1) in Plasma

VNN1 levels in plasma were measured by ELISA kit (Cusabio Biotech Co., Ltd).The samples were placed with a standard pipette onto microplates previously coated with a pantetheinase-specific antibody. The pantetheinase available bound to the immobilized antibody. After removing the unbound substances, pantetheinase-specific biotin-conjugated antibody was added to the wells. After washing, avidin conjugated to horseradish peroxidase (HRP) was also added to the wells. After washing and removal of the unbound avidin, a substrate solution reagent was added. The coloration obtained was proportional to the bound pantetheinase, whose concentration was measured by colorimetry.

Determination of Lipid Hydroperoxide in Plasma

The concentration of lipid hydroperoxides in the plasma was determined spectrophotometrically with Assay Kit Cayman 705002. The method is based on direct measurement of hydroperoxides through redox reaction with iron ions. Initially, ROOH is extracted into chloroform, and thus eliminates their ability to react with endogenous iron ions. Samples were assessed by spectrophotometry at λ=490 nm, using a 96-well plate reader.

Statistical Analysis

Statistical analysis was performed with the Statistical Program for Social Sciences 17.0 (SPSS Inc.,Chicago, IL). Comparison of proportions between patient groups was made using the χ^2-test. Interval variables are presented as means (standard deviation) or median (25^{th} - 75^{th} percentile), depending on the type of data distribution. Normality of distribution was checked with the Shapiro-Wilk test. The significance of differences between the groups in normally distributed data was established using one-way ANOVA, and in those deviating from the normal distribution – with the Mann-Whitney

or Kruskal-Wallis tests. Two outliers in the group of patients and the two control groups, identified by SPSS through the stem-and-leaf plots and the box plots, were removed. A value $p<0.05$ was considered as a threshold of statistical significance.

Results

Some basic and biochemical characteristics of the subjects studied are given in Table 1.

No difference was found between the age groups studied (one-way ANOVA; F=0.632; p=0.535). The groups were also indistinguishable in terms of plasma ROOH concentration (F=0.332; p=0.719). The form of the disease was not significantly related to the levels of plasma VNN1 (Kruskal-Wallis test; χ^2=4.245; p=0.120). A significant difference in the concentrations of VNN1 (Figure 1) was found between newly diagnosed IPT and the controls, after excluding four outliers, as stated in the Statistical Methods section (Mann-Whitney test; Z=-1.993, p=0.046).

Table 1. Some basic and biochemical characteristics of patients and controls

Parameter	Patients		Controls
	Newly diagnosed	Chronic ITP	
Age [SD] (yrs)	50.0±15.4	43.5±13.8	47.5±15.3
Gender (Male/Female)	5/7	6/7	15/16
PLT (x10^9/L)	31 (15-39)	41 (30-68)	286 (235-387)
Splenectomy (%)	0	62	
ROOH (ng/mL)	4.8±1.6	5.1±1.9	5.4±2.4
VNN1 (ng/mL)	0.81 (0.64-0.89)	0.68 (0.60-0.82)	0.65 (0.57-0.75)

* Interval variables are expressed as mean ± standard deviation or median (25^{th} - 75^{th} percentile)

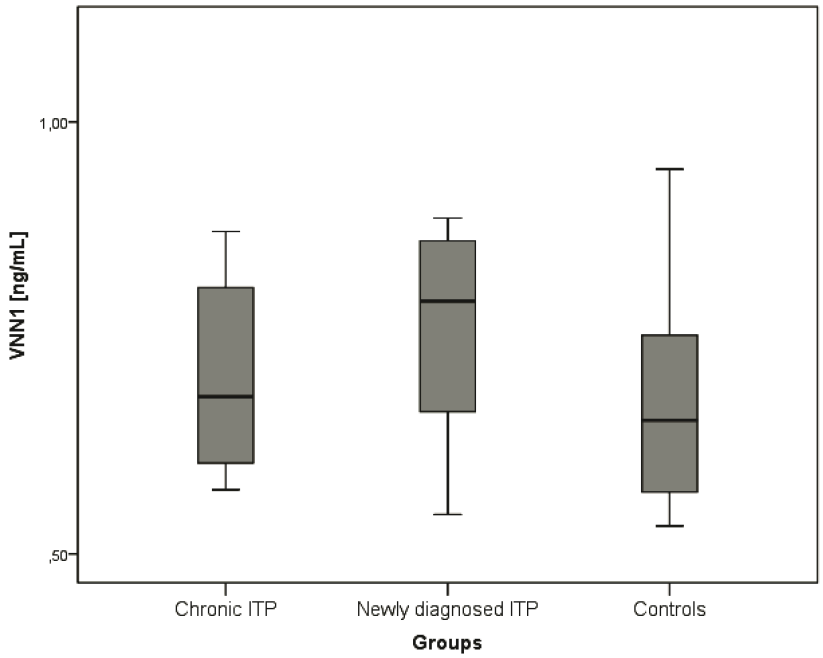

Figure 1. Plasma vanin-1 concentrations depending on the form of immune thrombocytopenia

Discussion

Despite the progress in studies on the etiology and pathogenesis of ITP, the immediate cause of the disease (formation of autoantibodies) is still not fully understood [12, 13]. The purpose of this study was to find out if there exists a connection between available to easily determinable markers

of oxidative stress and the clinical form of ITP in adults as defined according to modern classification of IWG – newly diagnosed, persistent and chronic ITP.

We found increased levels of plasma pantetheinase (VNN1) in newly diagnosed ITP patients, as compared with the healthy controls. However, no changes were established between the groups of patients and healthy individuals as regards the plasma levels of lipid hydroperoxides, a marker of oxidative damage in circulation.

There is multiple evidence, indicating that oxidative stress is a potential initiation mechanism of autoimmune diseases [2, 3, 5, 7, 8]. However, results from few studies on the role of oxidative stress in ITP have been reported and some of these results are contradictory. Elevated levels of oxidative stress in patients with ITP based on elevated levels of malondialdehyde, increased total oxidant status and reduced/oxidized glutathione ratio have been reported [14]. The published data on total antioxidant capacity of blood are controversial – it was found to be low or unchanged in patients when compared to controls [15, 16]. Changes in serum levels of NO, superoxide dismutase, catalase, glutathione peroxidase, and ascorbic acid have also been reported [17]. Recently, Zhang et al. [11] have found increased gene expression of VNN1, the latter being the most significant one in the chronic form of the disease. On the other hand, Elsalakawy et al. [10] used VNN1 expression in peripheral leukocytes as a criterion to differentiate the forms of the disease. Other authors did establish gender-specific differences such as in the pathophysiology of ITP, such as higher levels of oxidative stress in women with ITP, as compared with healthy controls and women and men with ITP [18].

Modern advances in medical science place higher demands on clinicians against the background of increased patient expectations. Despite their high scientific value, many high-tech methods (eg. molecular genetic study of the expression of antioxidant genes) remain too expensive to be used in everyday practice. In this study, plasma concentrations of VNN1, and the plasma concentration of ROOH were determined. These investigations are more affordable and with reliable results. This makes them more applicable and helpful in making timely decisions in routine practice.

At this stage, our results do not allow to consider the studied markers of oxidative stress as indicators of severity or as predictors of disease.

This study has some limitations. ITP is a rare disease and the groups studied were relatively small. In the current classification of IWG, newly diagnosed ITP cases are presented as a relatively heterogeneous group, and only part of them can be classified as acute ITP. The study group included adult patients, and comorbidity might be expected to have an influence on the results, when comparing the study group and the control group.

Further studies with larger and more homogenous groups of patients and including more indicators of oxidative stress are needed to be able to draw statistically valid conclusions about the role of and the possibility of using oxidative biomarkers in diagnosing and treatment of ITP.

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