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The significance of diminished sTWEAK and P-selectin content in platelets of patients with pulmonary arterial hypertension

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ABSTRACT

Pulmonary arterial hypertension (PAH) is a progressive disease characterized by proliferative changes in pulmonary arteries. There is growing evidence suggesting that soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) and P-selectin could be involved in PAH development and progression. Here we investigate whether circulating platelets may be a source of sTWEAK and contribute to diminished availability of sTWEAK and P-selectin in PAH patients.

We have prospectively enrolled two independent study groups of stable patients with confirmed PAH and age matched controls: derivation (10 PAH; 15 controls) and validation (20 PAH; 12 controls). P-selectin and sTWEAK concentrations were measured in platelet-poor plasma and platelet lysate. To avoid procedural bias, in each group we employed different protocols for platelet isolation.

Consistently, both in derivation and validation groups PAH patients presented significantly lower sTWEAK content in platelets than control group with no significant differences in plasma levels. Similarly, patients presented comparable to controls plasma P-selectin concentrations and lower concentration in platelet lysate. Kaplan-Meier analysis revealed that patients with low platelet sTWEAK/total protein concentration ratio had more frequently detoriation of PAH in the follow-up (16.51 ± 3.32 months), log-rank test, p = .03.

Patients diagnosed with pulmonary arterial hypertension present diminished sTWEAK and P-selectin storage capacity in platelets. Thrombocytes appear to be a major source of sTWEAK that could be released upon local injury and its decreased availability could have an impact on pathophysiology and prognosis in PAH.

1. Introduction

Pulmonary arterial hypertension (PAH) is a destructive disease characterized by proliferative changes in pulmonary arteries that produce a gradual increase in pulmonary vascular resistance and lead to deterioration of cardiopulmonary function. Despite the availability of new drugs for specific PAH therapy the prognosis is dependent on fast diagnosis to prevent advanced right ventricular dysfunction [1]. Various echocardiographic and clinical parameters are used nowadays to determine severity level of disease or predict the survival. However, there is still a need for new biological biomarkers, which could be useful in diagnosis as well as in prediction of PAH deterioration, since no single hemodynamic parameter or exercise test adequately reflects the prognosis for an individual patient.

An increasing role in pathogenesis of PAH is attributed to inflammation, especially occurring locally in the lung tissue and pulmonary vasculature. Recent studies have confirmed the role of inflammatory modulators e.g. kynurenine metabolites, interleukin-6, IL-1beta in development of PAH [2–4]. One of them is soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK), which belongs to the tumor necrosis factor-alpha (TNF-alpha) superfamily [5,6]. This cytokine is involved in numerous biological responses associated with tissue damage and repair like apoptosis, cell growth or angiogenesis [7]. One study (using a pool of 20 healthy subjects) confirmed that upon activation, human platelets expose TWEAK antigen and release it as a soluble form (sTWEAK) [6]. It has been proven before that sTWEAK has a potential role in pathogenesis of various cardiovascular diseases like chronic heart failure or acute coronary syndromes [8,9]. sTWEAK

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via binding to the fibroblast growth factor-inducible 14 receptor (Fn14) is able to regulate right ventricular hypertrophy/fibrosis processes [10,11]. sTWEAK–Fn14 pathway has also been found to play a role in the development of atherosclerosis [12,13]. A prognostic value of this cytokine was observed in patients with ST-elevation myocardial infarction where increased sTWEAK levels predicted worse prognosis [14]. On the other side low sTWEAK concentration correlated with higher mortality in advanced non-ischemic heart failure, while such effect was not observed in ischemic HF patients [8]. Our previous research revealed that PAH patients present lower serum sTWEAK concentrations, however exact mechanism by which sTWEAK is reduced in these patients and how it could be involved in the pathogenesis of the disease is still unclear [4]. We hypothesized that platelets may be an important source of sTWEAK in PAH and thus may have an impact on PAH development and progression.

P-selectin is a thrombo-inflammatory molecule stored in the Weibel-Palade bodies of endothelial cells and the α-granules of platelets. This cytokine is transferred to the outer membrane surface upon platelet or endothelial activation, thus participating in hemostasis and in initial recruitment of leukocytes at endothelial cell injury. Increased plasma levels of soluble form of P-selectin were found in inflammatory disorders like atherosclerosis or connective tissue diseases [15].

In this pilot study, we assessed the mechanisms of altered serum concentration of sTWEAK and P-selectin by evaluating their concentrations in platelet poor plasma and platelet lysates of patients with pulmonary hypertension and healthy controls and attempted to estimate the contribution of platelets as main source of circulating sTWEAK.

2. Material and methods

2.1. Population

We have prospectively enrolled PAH patients and age matched healthy controls in two independent study groups: derivation group consisted of 10 stable PAH patients, 48.34 ± 20.23 years old (7 females) and 15 healthy controls 46.32 ± 15.18 years old (8 females) and validation group of 20 stable PAH patients 46.81 ± 17.34 years (16 females) and 12 healthy controls 45.22 ± 11.15 years (6 females).

Altogether 30 stable patients (from two PAH expert centers in Poland - Bialystok and Lublin) enrolled into this study had diagnosis of PAH confirmed by right heart catheterization [mean pulmonary artery pressure (mPAP) ≥25 mmHg, pulmonary artery wedge pressure (PAWP) ≤15 mmHg] and the use of an algorithm that included respiratory function tests, perfusion lung scan, echocardiography, computed tomography to rule out secondary pulmonary hypertension causes according to the European guidelines [1]. The exclusion criteria were: patients in IV WHO class, Eisenmenger physiology, PAH associated with prevalent systemic-to-pulmonary shunts due to moderate to large defects (according to the European guidelines) [1], group II, III, IV, V of pulmonary hypertension. During the baseline evaluation we also performed physical examination, six-minute walk test, laboratory tests e.g. serum B-type natriuretic peptide (BNP), complete blood count, renal function parameters. An echocardiography was performed to assess morphology and function of right ventricle. Right heart catheterization was carried out with a standard technique using a balloon-tipped 7F Swan-Ganz catheter; cardiac output was measured by thermodilution method; pulmonary vascular resistance (PVR) was calculated with the formula PVR = (mPAP-PAWP)/cardiac output (CO), expressed in Wood's units (WU).

Fasting peripheral venous blood samples were obtained from patients with PAH as well as controls. Plasma and platelet concentrate aliquots were stored at −80 °C for future analysis.

2.2. Cytokines' measurements

In both derivation and validation groups concentration of sTWEAK and P-selectin was determined (according to the manufacturer instructions) using commercially available ELISA kits (sTWEAK: eBioscience, Austria; P-selectin: R&D Systems, USA). The detection limits were 9.7 pg/ml for sTWEAK and 0.5 ng/ml for P-selectin. All measurements were performed in duplicate. To avoid procedural bias, in each group (derivation and validation) we employed different protocols for platelet isolation. In derivation group platelets were isolated from citrate plasma; after sampling, 4 ml of citrate-anticoagulated blood was centrifuged at 300*g* for 10 min then platelet-rich plasma was centrifuged at 1000*g* for 10 min to isolate platelets and they were homogenized in phosphate-buffered saline solution (PBS) for analysis of cytokines content [16]. In validation group platelets were isolated from EDTA plasma by repeated centrifugation and also homogenized in PBS [17]. The amount of protein contained in the platelet lysate in both groups was determined by a Bradford method, with a use of dye reagent kit (BIO-RAD, UK). To better present platelet storage of sTWEAK and P-selectin, independently of the platelet count, the amounts of these cytokines were normalized to the concentration of protein in the platelet lysates.

The clinical follow-up lasted 19.54 ± 4.56 months in derivation group and 14.59 ± 3.11 months in validation group. Clinical deteriorations were used as combined clinical endpoint (CEP) for Kaplan-Meier analysis and defined as patients' death, hospitalization due to PAH/heart failure progression or need for escalation of PAH-targeted therapy.

2.3. Statistical analysis

The distribution of all variables was verified with Kolmogorov-Smirnov test. Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using t-student test for continuous data and χ^2 test for categorical variables. Pearson's correlation coefficient was used to examine the relationship between 2 continuous variables. To investigate the occurrence of clinical endpoints Kaplan Meier method with log-rank test was implemented. A statistical software package STATA13 (USA) was used for analysis.

The study was approved by Bioethics Commission at Medical University of Bialystok.

3. Results

3.1. Clinical characteristics

Most PAH patients were in WHO class III (66.8%, n = 20). Idiopathic etiology constituted 60% (n = 18); PAH associated with connective tissue diseases (systemic scleroderma and mixed connective tissue disease) 13% (n = 4) and congenital heart diseases with small left-right defects 27% (n = 8). 5 patients (16%) were incident cases while the rest of the study group was receiving PAH specific treatment at the time of survey. Seven patients were on prostacyclin therapy during the study (2 patients – inhaled iloprost; 5 patients - subcutaneous treprostinil). The clinical and hemodynamic characteristics as well as laboratory data and echo results of validation group subjects are indicated in Table 1.

There was significant difference observed in mean 6MWD between PAH and healthy subjects (353.45 ± 76.56 m vs 554.45 ± 80.54 m, $p < .001$). In laboratory test, BNP and uric acid concentrations in blood were significantly higher in PAH group (Table 1). In echocardiographic examination, PAH patients were characterized by indices of impaired RV morphology and global function with higher sPAP and lower AcT, TAPSE comparing to controls. They also manifested significantly lower ventilatory efficiency during exercise (cardiopulmonary exercise test), characterized by elevated VE/VCO2 slope,