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Baseline level of platelet-leukocyte aggregates, platelet CD63 expression and soluble P-selectin concentration in patients with posttraumatic stress disorder: a pilot study

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Abstract

Platelets may have an important role in development of cardiovascular disease (CVD) as a result of chronic stress. We conducted a pilot study to evaluate the effect of posttraumatic stress disorder (PTSD) on baseline platelet activation. Platelet-leukocyte aggregates (PLA) and CD63 expression were measured by flow cytometry and soluble (s)P-selectin concentration was determined in sera of 20 Croatian male combat veterans with PTSD and 20 healthy civilians. Groups were matched in sex, age, body mass index (BMI) and traditional CVD risk factors. Our data showed no differences in measured parameters. Other platelet activation markers should be determined and bigger sample size used in our future study.

Keywords: war veterans, cardiovascular disease, flow cytometry

1. Introduction

Recent studies provide convincing evidence that psychosocial factors contribute to pathogenesis and expression of coronary artery disease (Rozanski et al., 2005). Similarly, the evidence linking exposure to traumatic stress and cardiovascular disease (CVD) is compelling and supported by different epidemiologic studies (Sibai et al., 1989; Falger et al., 1992; Boscarino and Chang, 1999). Platelets play an important role in pathogenesis of atherosclerosis and acute coronary syndromes through their thrombotic and proinflammatory properties (Furman et al., 1998; Nijm et al., 2005). Posttraumatic stress disorder (PTSD) and increased CVD risk may be connected through mechanisms of platelet activation with substantial impact of acute or chronic stress (Brydon et al., 2005), particularly depression (Bruce and Musselman, 2005). This association might be formed through increased sympathetic output associated with PTSD (Yehuda et al., 1992, 1998). Catecholamines bind to α2-adrenergic receptors on platelet surface with consequent platelet activation (von Kanel and Dimsdale, 2000). Platelet activation results in surface expression of the adhesion molecule P-selectin, which binds to the leukocyte receptor, P-selectin glycoprotein ligand (PSGL-1) leading to the formation of platelet-leukocyte aggregates (PLA) (Rinder et al., 1991). If platelet activation occurs repeatedly, for example during hyperarousal episodes in PTSD patients, proinflammatory factors released from platelets bound to leukocytes could promote atherosclerosis (Nijm et al., 2005). Furthermore, importance of platelet physiology in PTSD patients is emphasized by studies of platelet α-2 adrenergic receptors number and reactivity (Perry et al., 1987), platelet peripheral-type benzodiazepine receptor density (Gavish et al., 1996), cAMP activity (Lerer et al., 1987), serotonin measures (Cicin-Sain et al., 2000; Pivac et al., 2002) and monoamine oxidase activity (Kozaric-Kovacic et al., 2000).

We assumed that platelet activity might be elevated in PTSD patients and conducted a pilot study to determine baseline platelet function in war veterans diagnosed with combat-related chronic PTSD. Circulating PLA and platelet activation dependent marker CD63 (lysosomal glycoprotein GP53) expression were determined by flow cytometry, and soluble (s)P-selectin (platelet α-granule glycoprotein GP140) concentrations were measured in sera.

2. Methods

A total of 40 men, i.e. 20 Croatian combat veterans with PTSD and 20 age-comparable healthy civilians with no combat experience participated in this study. Veterans were PTSD outpatients from Vrapče Psychiatric Hospital. The study was approved by the local ethics committee, and written informed consent was obtained from all subjects after the study design had been fully explained. All patients met ICD-10 PTSD criteria (World Health Organization, 1992), official classification in Croatian psychiatric practice. For purposes of this study, the diagnosis of PTSD was made by DSM-IV (American Psychiatric Association, 1994) based Clinician-Administered PTSD Scale (CAPS-DX) (Blake et al., 1996). All 20 patients met DSM-IV criteria for chronic PTSD. The PTSD-group was relatively homogenous related to the severity of the illness. The average severity of PTSD was qualified as moderate (mean=4), according to CGI-S scale (Guy 1976). The specificities of clinical picture (frequency and intensity of all and selected PTSD symptoms during the period of one month and one week prior to rating) were determined by CAPS scale (reexperiencing (mean±SD, 18.50±2.21), avoidance/numbing (26.35±2.32), hyperarousal (15.85±3.65)). No psychiatric premorbidity or comorbidity, including depression, was found in PTSD group when entering this study. The study was carried out 9 to 15 (median=13) years after veterans' traumatic experience.

Prior to blood drawing, healthy civilians were examined by experienced physician and relevant data for purposes of this study were recorded on basis of patients' histories. They had negative history of any psychiatric disorder and no symptoms or signs of acute or chronic physical illness other than conditions that were controlled for (Table 1).

All participants were free of any psychotropic medication, drug or alcohol abuse for at least one month, and did not suffer from any infectious or allergic disorder. They had negative history of myocardial infarction, stable, non-stable angina pectoris or stroke. During the seven days prior to study, they haven't used any non-steroid anti-inflammatory drugs.

Peripheral blood was collected by venipuncture using 4.5 mL sodium citrate, theophyilline, adenosine and dipyridamole (CTAD) Vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA) for CD63 and PLA determination. To minimize platelet activation during blood drawing, only a light tourniquet and 20-gauge needles were used and the first 10 mL of blood were discarded. Samples were processed within 30 minutes of collection. Sera for (s)P-selectin determination were stored at -80°C until assayed.

Flow cytometry was done with LSR II flow cytometer (BD Biosciences, Mountain View, USA) and data were analyzed with FACSDiva software (BD Biosciences). Standard filters and mirrors for fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin chlorophyll protein (PerCP) fluorescence analysis were used.

For PLA determination we used previously described assay (Li et al., 1999) with modifications. Briefly, 10 μL of whole blood were added to 90 μL of phosphate-buffered saline (PBS) containing 2 μL of BD Simultest™ LeucoGATE™ (BD Biosciences, Heidelberg, Germany) (FITC-conjugated anti-CD45 and PE-conjugated anti-CD14) and 10 μL of PerCP-conjugated anti-CD42a (BD Biosciences) (glycoprotein IX, platelet specific marker). Saturating amounts of antibodies were determined by titration using sample stimulated by 20 μM adenosine diphosphate (ADP). After incubation in the dark at room temperature (RT) for 20 minutes, samples were fixed using 900 μL of 0.5% formaldehyde saline. By adjusting threshold to fluorescence channel 1 (FITC emission signal), cytometer was set to acquire only CD45 positive events (leukocytes) at flow rate of 30 events/second until at least 2000 CD14 events (monocytes) were acquired. Lymphocytes, monocytes, and neutrophils were discriminated according to their side scatter properties and CD45 expression. Proportions of CD42a positive events in monocyte, neutrophil and lymphocyte gates represented percentages of platelet-lymphocyte (P-Lym), platelet-monocyte (P-Mon) and platelet-neutrophil (P-Neu) aggregates.

For CD63 determination, whole blood samples were diluted 10 fold with PBS and 10 µL were added to 40 µL of PBS containing 5 µL of FITC-conjugated anti-CD63 (BD Biosciences) and 5 µL of PerCP-conjugated anti-CD42a (BD Biosciences). Appropriate isotype controls (BD Biosciences) were used for discrimination of nonspecific binding. The samples were gently mixed and incubated in the dark at RT for 20 minutes and fixed with 450 µL of 0.5% formaldehyde saline. Cytometer threshold was set in fluorescence channel 3 (PerCP emission signal) in order to exclude all CD42a-negative events from analysis. A total of 20 000 CD42a-positive events were acquired at flow rate set on 300 events/second. Proportions of CD63 positive events were determined with 0.5% of nonspecific binding allowed.

(s)P-selectin concentrations were measured using a commercially available immunoenzymatic method with the human (s)P-selectin kit (R&D Systems Inc., Minneapolis, USA). All samples were analyzed following the manufacturer's protocol.

Statistical analyses were done using Fisher's exact chi-square tests for categorical data. Non Gaussian numerical data were normalized, tested for multivariate normality using Mardia test and analyzed with MANCOVA controlling for age, BMI and smoking. Partial correlations were performed controlling for the same factors. Power analysis was conducted for two-sample Hotelling's T-squared test (variant of MANOVA used for comparison of two groups). All tests were considered significant if $P<0.05$.

3. Results

Results are summarized in Table 1. Groups were similar in all assessed characteristics except work status with more PTSD patients being retired and majority of healthy controls being employed. Traditional CVD risk factors (hypertension, hyperlipidaemia, diabetes, smoking) were equally distributed among groups.

No significant differences in P-Mon, P-Neu, P-Lym, CD63 and (s)P-selectin were found between groups. Our sample sizes with 20 participants per group achieved only 18% power to detect an effect size of 0.26 which represents the differences between the group means of the five response variables, adjusted by the variance-covariance matrix. To achieve 80% power for given effect size, at least 98 patients per group are needed. Effect sizes for single parameters yielded 0.40 (95% confidence interval, -0.23 to 1.03) for P-Mon, 0.06 (-0.56 to 0.68) for P-Gran, -0.32 (-0.95 to 0.30) for P-Lym, 0.04 (-0.58 to 0.66) for CD63 and -0.06 (-0.68 to 0.56) for (s)P-selectin.

No one of response variables correlated significantly with scores for avoidance/numbing cluster of symptoms (P-Mon, r=0.14, P=0.585; P-Gran, r=0.04, P=0.865; P-Lym, r=-0.19, P=0.465; CD63, r=-0.29, P=0.243; (s)P-selectin, r=-0.14, P=0.600). P-Lym were in negative correlation with reexperiencing scores (r=-0.55, P=0.022) and CD63 expression negatively correlated with hyperarousal scores (r=-0.51, P=0.036). Any other variable didn't correlate significantly either with reexperiencing scores (P-Mon, r=0.27, P=0.292; P-Gran, r=-0.24, P=0.346; CD63, r=0.02, P=0.943; (s)P-selectin, r=-0.10, P=0.694) or hyperarousal scores (P-Mon, r=0.18, P=0.466; P-Gran, r=-0.09, P=0.717; P-Lym, r=-0.04, P=0.878; (s)P-selectin, r=0.39, P=0.124).

4. Discussion

In this pilot study we have evaluated the effect of chronic combat-related PTSD on baseline platelet activity. Comparison with healthy civilians showed no group differences in measured parameters. Platelets in PTSD patients served mainly as peripheral model for studying neurotransmitters and their receptors but, to our knowledge, a question of platelet activity hasn't been addressed yet. Designed as a pilot, this study has low sample sizes resulting in low power to detect group differences. Although not statistically significant, these differences are possibly clinically important so we decided to report calculated effect sizes as guidance for further research of the subject.

While earlier studies mainly measured quantity of soluble circulating factors (e.g. platelet factor 4, beta-thromboglobulin), flow cytometry is nowadays widely used to assess platelet function in psychiatric disorders. Whole blood flow cytometry enables direct analysis of platelet function in their physiological milieu with minimal manipulation, preventing *in vitro* platelet activation (Michelson et al., 2000). Thus, it can detect very small changes of platelet function *in vivo*. Right choice of surface or soluble marker of platelet activation is very important and depends on condition being studied. Although platelet surface P-selectin (CD62P) is very sensitive measure of platelet function, we preferred to measure its soluble fraction because it is known that platelets rapidly loose CD62P and maintain their function (Michelson et al., 1996). Although (s)P-selectin, because of its endothelial portion (Semenov et al., 1999), is not exclusive measure of platelet activity, there is a strong evidence proving that changes in (s)P-selectin levels directly reflect platelet disturbances (Blann et al., 2003). We should note that serum values differ from plasma values of (s)P-selectin (Caine and Blann, 2003; Thom et al., 2004) and future studies should address citrated plasma concentrations to confirm our results.

Upon activation, a portion of surface CD62P is involved in formation of PLA. Beside serving as indirect measure of CD62P, PLA provide more accurate measure of platelet function (Michelson et al., 2001). CD63 is another platelet surface activation dependent molecule possibly involved in interaction with leukocytes (Israels et al., 2001) and platelet spreading (Israels and McMillan-Ward, 2005).

Flow cytometry studies of baseline platelet activity in other psychiatric disorders, particularly depression, demonstrated controversial results (von Kanel, 2004). Among various activation dependent markers studied, no elevation in baseline expression of platelet CD63 was found in depression (Walsh et al., 2002a; Lederbogen et al., 2004) or schizophrenia (Walsh et al., 2002b). Unexpectedly, CD63 negatively correlated with hyperarousal scores in our study. Although not in line with hypothesis of platelet hyperactivity in PTSD patients, this finding, along with negative correlation of P-Lym with reexperiencing scores, suggests involvement of sympathetic nervous system in regulation of platelet function. This is further supported by absence of any correlation with avoidance/numbing scores. PLA have not been studied before in the context of psychiatric disease while (s)P-selectin has been measured only in depressed with cardiovascular disease (Serebruany et al., 2003; Pasic et al., 2004). Nevertheless, Steptoe et al. (2003) reported higher baseline PLA in men with lower socioeconomic status and attributed it to cumulative effect of frequent daily stress. Although possible influence of prolonged stress and frequent hyperarousal episodes in PTSD patients on baseline PLA wasn't confirmed in this study, we have noticed that P-Mon tend to be higher in PTSD group with higher effect size (0.40) than other parameters.

It is proposed that mechanisms linking mental stress and platelet activity might be mediated by activating properties of epinephrine and norepinephrine through α-2 receptors on platelets (Camacho and Dimsdale, 2000). This hypothesis is supported by findings of reduced number and increased reactivity of α-2 adrenergic receptors on platelets (Perry et al., 1987) and

elevated 24-hour urine epinephrine and norepinephrine values in PTSD patients (Yehuda et al., 1992). Moreover, numerous studies showed elevated basal cardiovascular activity in PTSD (Buckley and Kaloupek, 2001).

Studies of baseline plasma catecholamine levels generally found no changes in PTSD (Southwick et al., 1999). Our results are in line with these findings suggesting that platelet activity is not substantially altered by catecholamine disturbances at baseline level. On the other hand, increased reactivity to internal or external trauma associated cues, both in terms of elevated catecholamine levels and physiological arousal, are common findings in PTSD (Blanchard et al., 1991). Since platelet activation changes are relatively short-lived with platelet life-span of approximately seven days, similar studies are needed to assess *in vivo* platelet reactivity to psychological stimuli or *in vitro* reactivity to various platelet agonists, especially catecholamines.

Although this pilot study indicates that baseline platelet activity is not altered in PTSD, we are far from reaching final conclusion. Other platelet activation markers should also be investigated and, considering the magnitude of changes, bigger sample sizes used. If platelet function in PTSD patients reflects findings related to sympathetic nervous system activation, we can expect them to have exaggerated platelet reactivity to psychological stimuli or physiological agonists and this could contribute to enhanced CVD risk.

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Table 1

Participant characteristics and variables

Variable	PTSD (*n*=20)	Healthy controls (*n*=20)	*P*-value[a]
Age (years)	45.3±8.2	42.8±10.4	0.396
BMI	25.04±2.57	26.78±3.76	0.123
Documented Hypertension[b]	3 (15)	6 (30)	0.451
Hyperlipidaemia (total cholesterol >5 mmol/L)[b]	2 (10)	3 (15)	1.000
Tobacco use	13 (65)	9 (45)	0.341
Diabetes type II[b]	1 (5)	1 (5)	1.000
Marital status			0.191
Married	10 (50)	15 (75)	
Unmarried/divorced/widower	10 (50)	5 (25)	
Education			0.135
Elementary school	2 (10)	1 (5)	
High school	16 (80)	12 (60)	
University education	2 (10)	7 (35)	
Work status			0.008*
Employed	11 (55)	19 (95)	
Retired	8 (40)	1 (5)	
Unemployed	1 (5)	0 (0)	
P-Mon (%)[c]	5.23 (3.97-8.10)	4.60 (2.83-6.95)	
P-Neu (%)[c]	1.32 (0.99-1.55)	1.22 (1.03-1.61)	
P-Lym (%)[c]	0.85 (0.66-0.97)	0.88 (0.76-1.09)	
CD63 (%)[c]	1.06 (0.65-1.36)	0.91 (0.62-1.48)	
(s)P selectin (ng/mL)[c]	172.8 (114.3-200.0)	152.3 (128.2-184.9)	

Values are means ± SD, *n*(%) or medians (interquartile range). BMI = body mass index; P-Mon = platelet-monocyte aggregates; P-Neu = platelet-neutrophil aggregates; P-Lym – platelet-lymphocyte aggregates; (s) = soluble

[a] Two-sided values obtained using Fisher's exact test or t-test.

[b] Participants were using appropriate therapy.

[c] Initial values before transformation. Hotelling's Trace= 0.034, $F(5,31)=0.21$, $P=0.954$ (performed on transformed values). Since MANCOVA wasn't significant, no additional ANCOVAs were performed.

[*] Statistically significant.