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Increased activity of interleukin-23/interleukin-17 cytokine axis in primary antiphospholipid syndrome

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ABSTRACT

The aim of the study was to investigate serum concentrations of interleukin (IL)-17 and IL-17-inducing cytokines IL-23 and transforming growth factor (TGF)-β, as well as IL-17 single nucleotide polymorphism (SNP) rs2275913 in patients with primary antiphospholipid syndrome (PAPS). We studied fifty patients with PAPS and fifty age- and sex-matched healthy controls. The cytokine levels were measured by ELISA, while the rs2275913 SNP located in promoter region of IL-17 gene was genotyped using real-time PCR. The significantly higher levels of IL-17 ($p = 0.002$), IL-23 ($p < 0.001$) and TGF-β ($p = 0.042$) were found in PAPS patients (median 13.1, 9.4, and 125.6 pg/ml, respectively) compared to the control group (6.8, 4.9 and 44.4 pg/ml). There was a significant positive correlation between concentrations of IL-17 and IL-23 ($r = 0.540$, $p < 0.001$), but not between those of IL-17 and TGF-β. No statistically significant differences were observed in the distribution of genotypes and alleles of the IL-17 rs2275913 variants in patients with PAPS compared to healthy subjects. The blood concentrations of IL-17 did not differ in subjects with different rs2275913 genotypes or patients with or without antiphospholipid antibodies. Finally, a trend toward higher IL-17 levels ($p = 0.063$) and the significantly higher IL-17 concentrations ($p = 0.012$) were observed in PAPS patients with deep vein thrombosis and thrombocytopenia, respectively. These data demonstrate that IL-23/IL-17 axis, stimulated independently of TGF-β increase IL-17A gene polymorphism and antiphospholipid antibody production, might contribute to vascular manifestations of PAPS.

Introduction

Antiphospholipid syndrome (APS) is a systemic autoimmune disease in which the excessive blood clotting and/or certain complications of pregnancy (fetal loss or premature birth) are associated with the presence of antibodies that recognize anionic phospholipid–protein complexes (Ruiz-Irastorza et al. 2010). Although antiphospholipid antibodies such as those against cardiolipin, β2 glycoprotein 1 or lupus anticoagulant are both diagnostic markers for, and pathogenic drivers of APS (Sherer et al. 2007), the exact pathophysiological mechanisms of this disorder have not been established. The primary antiphospholipid syndrome (PAPS) occurs in the absence of any other related disease, while in secondary APS the presence of antiphospholipid antibodies is associated with the systemic lupus erythematosus (Shoenfeld et al. 2009). The patients with APS may also have cardiac, neurological, renal, cutaneous or hematologic symptoms, the latter including mild to moderate thrombocytopenia (Gigante et al. 2009; Rodrigues et al. 2010; Soltesz et al. 2007; Uthman et al. 2008; Weinstein and Piette 2008).

Interleukin (IL)-17A (referred to hereafter as IL-17) is the prototype member of the IL-17 cytokine family, comprising of several structurally similar proteins involved in the immune response, as well as in the homeostasis of tissues in health and disease beyond the immune system (Gaffen 2011; Moseley et al. 2003). IL-17-producing T helper (Th)17 cells have recently been identified as a unique subset of helper T cells responsible for induction and propagation of autoimmune responses in animal models and possibly in humans (Fouser et al. 2008). The lineage commitment and/or

Abbreviations: β2GPIβ, 2 glycoprotein-I; CL, cardiolipin; LA, lupus anticoagulant; PAPS, primary antiphospholipid syndrome; SNP, single nucleotide polymorphism.

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expansion/maintenance of Th17 is controlled in an autocrine or paracrine manner by several cytokines, including IL-23 and TGF-β (Gutcher et al. 2011; Li et al. 2007; McGeachy et al. 2009; Stritesky et al. 2008). The proposed pivotal role of IL-23/IL-17 axis in pathogenesis of autoimmunity has been supported by the increased levels of these cytokines in various organ-specific and systemic autoimmune/inflammatory diseases, such as rheumatoid arthritis (Melis et al. 2010; Ziolkowska et al. 2000), multiple sclerosis (Matusevicius et al. 1999; Vaknin-Dembinsky et al. 2006), systemic lupus erythematosus (Wong et al. 2008), Sjögren's syndrome (Mieliauskaite et al. 2012), ankylosing spondylitis (Wang et al. 2009), inflammatory bowel disease (Fujino et al. 2003; Liu et al. 2011), psoriasis (Coimbra et al. 2010) and systemic sclerosis (Komura et al. 2008; Kurasawa et al. 2000). Moreover, genome-wide association studies have demonstrated that single-nucleotide-polymorphisms (SNPs) in the IL-17 and IL-23 receptor gene confer a significant risk for development of multiple autoimmune diseases (Arisawa et al. 2008; Duerr et al. 2006; Huber et al. 2008; Illes et al. 2008; Kim et al. 2011; Nordang et al. 2009). Several gene polymorphisms have been reported as risk factors for APS development (Castro-Marrero et al. 2009) and IL-17-inducible proinflammatory cytokines tumor necrosis factor (TNF) and IL-6 have been found elevated in APS patients (Ahmed et al. 1992; Bertolaccini et al. 2001; Forastiero et al. 2005; Soltesz et al. 2008). However, no study thus far investigated the association of Th17 cytokine levels or their gene polymorphisms with APS.

In the present study we demonstrate for the first time that blood levels of IL-17, as well as those of Th17-promoting cytokines IL-23 and TGF-β, are increased in patients with PAPS. The increase in IL-17 levels was independent of rs2275913 SNP located in promoter region of IL-17 gene, and was apparently associated with vascular manifestations of PAPS.

Materials and methods

Subjects

We studied a group of 50 patients with PAPS, who all satisfied the Sydney classification criteria for APS (Miyakis et al. 2006), including the presence of lupus anticoagulant (LA), anticardiolipin (aCL) IgG and IgM, and anti-β2 glycoprotein-I (aβ2GPI) IgG and IgM antibodies. The patients were classified as PAPS according to the proposed exclusion criteria (Piette et al. 1993). None of the patients were on immunosuppressive treatment. All thrombotic episodes were confirmed by standard imaging techniques. Thrombocytopenia was defined as platelet counts equal or less than 120×10^9 platelets/l on at least two separate occasions. The control group consisted of 50 healthy age- and sex-matched volunteers of the same ethnic group, with no previous history of any significant clinical manifestation. At the time of investigation, none of the study individuals suffered from an infectious disease. The study conformed to the ethical guidelines of the declaration of Helsinki and all subjects signed a consent form approved by the human studies committees of the participant institutions.

Antibody and cytokine measurement

All patients and controls were tested for aCL and aβ2GPI antibodies, while LA was tested only in patients with PAPS. Antiphospholipid antibodies (aCL IgG and IgM, aβ2GPI IgG and IgM) were measured using commercial enzyme linked immunosorbent assay (ELISA) kits (Bindazyme, Birmingham, UK) and LA was tested by diluted Russel viper venom test, phospholipid sensitive PTT reagent and platelet neutralization procedure. Serum concentrations of IL-17, IL-23 and TGF-β1 were measured by commercial ELISA kits according to the manufacturer's instructions (eBioscience, San Diego, CA). The lower limits of detection were 4 pg/ml for IL-17 and 8 pg/ml for IL-23 and TGF-β, as reported by manufacturer (IL-17, TGF-β) or determined in our laboratory (IL-23).

Table 1
Demographic, laboratory/clinical parameters and Th17 cytokine levels (mean ± SEM) in PAPS and control group (n.a. – not assessed).

	PAPS ($n = 50$)	Control ($n = 50$)
Age (mean ± SEM)	45.3 ± 1.8	44.8 ± 1.8
Sex (F/M)	37/13	34/16
aCL (IgG)	25 (50%)	2 (4%)
aCL (IgM)	33 (66%)	3 (6%)
aβ2GPI (IgG)	22 (44%)	1 (2%)
aβ2GPI (IgM)	27 (54%)	0
LA	36 (72%)	n.a.
Venous thrombosis	16 (32%)	n.a.
Thrombocytopenia	12 (24%)	n.a.
IL-17 (pg/ml)	16.2 ± 2.2	8.5 ± 1.1
IL-23 (pg/ml)	14.3 ± 2.3	7.8 ± 2.2
TGF-β (pg/ml)	411.3 ± 79.6	158.7 ± 39.8

DNA extraction and genotyping

Peripheral venous blood from patients with PAPS and healthy controls was collected in EDTA tubes. Genomic DNA was purified from frozen whole blood using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The SNP rs2275913 located in the promoter region of IL-17 gene was genotyped using commercial pre-synthesized TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA) on the ABI Prism 7500 Real-Time PCR platform (Applied Biosystems). PCR reaction containing 2.25 μl genomic DNA, 7.5 μl universal PCR Master Mix (2×), 0.75 μl of probe mix (20×) and 4.5 μl of purified water, was performed in 96-well plates by using the standard protocol in a total volume of 15 μl.

Statistical analysis

Statistical analysis was carried out using the SPSS software package (version 15; IBM Corporation, Armonk, NY). Since Kolmogorov–Smirnov test confirmed that the cytokine concentrations were not normally distributed, the differences in cytokine levels were analyzed using Mann–Whitney rank sum test or Kruskal–Wallis *H* test for comparing two or more groups, respectively. The differences in frequency of cytokine detection were evaluated by Chi-square test, while the Spearman correlation test was employed to assess the correlation between different parameters. The *p* values of less than 0.05 were considered significant.

Results

Th17 cytokine levels are increased in PAPS patients

The main demographic, laboratory and clinical parameters of patients with PAPS and controls, as well as their blood concentrations of IL-17, IL-23 and TGF-β are presented in Table 1. No significant difference was observed in age or sex distribution between PAPS patients and healthy controls ($p = 0.981$ and $p = 0.509$, respectively). Both IL-17 and IL-23 were more frequently detected in PAPS patients (92% and 62%, respectively) than in control subjects (78% and 28%, respectively), but only the difference in IL-23 detectability was significant ($p = 0.093$ and $p = 0.001$, respectively). TGF-β was detected in 94% of samples in both PAPS and control group. The serum levels of all three cytokines were significantly higher in PAPS patients compared to healthy individuals (Table 1 and