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ORIGINAL ARTICLE

Increased level of tumor necrosis factor-α in patients with antiphospholipid syndrome: marker not only of inflammation but also of the prothrombotic state

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Abstract Connections between inflammation and thrombosis are intriguing, especially in a condition such as an antiphospholipid syndrome (APS), a disease characterized by immune-mediated thrombosis. Tumor necrosis factor alpha (TNF-α) is a cytokine which shares proinflammatory and prothrombotic actions, while a soluble form of interlukin-2 receptor (sIL-2R) is considered a typical marker of (auto)immune inflammation with not known direct links to thrombosis. The differences in the pathogenesis of APS as compared to other autoimmune diseases might be connected with different serum levels of both mediators. To answer this question, we studied 147 patients with systemic lupus erythematosus (SLE), 21 with SLE-like syndrome (SLE-LS), 20 with isolated APS (primary antiphospholipid syndrome, PAPS), and 32 healthy controls. Thirty-six patients from the SLE group fulfilled the updated APS criteria (secondary APS, SAPS). In comparison to healthy subjects, TNF-α concentration was increased in all patients, while sIL-2R rose significantly in the SLE group only. APS (both SAPS and PAPS) was characterized by the highest levels of TNF-α. Moreover, patients with lupus anticoagulant or elevated levels of IgG anticardiolipin or IgG anti-β_2-glycoprotein I antibodies had higher TNF-α levels than patients without the presence of any type of antiphospholipid antibodies (aPL). In conclusion, the presence of aPL is associated with higher TNF-α level, whereas increased level of sIL-2R is rather connected with definite SLE where inflammatory processes prevail. It might be hypothesized that TNF-α plays a major role in pathogenesis of APS thrombotic phenomena.

Keywords TNF-α · sIL-2R · Antiphospholipid syndrome · Inflammation · Thrombosis

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Introduction

Antiphospholipid syndrome (APS) is an autoimmune disease which differs from most other systemic autoimmune diseases by its propensity to develop thrombosis. Systemic lupus erythematosus (SLE) is the prototypic example of an immune mediated, generalized inflammatory disease. Both diseases often occur together leading to the interplay between inflammatory and prothrombotic phenomena [1].

In general, systemic inflammation is a potent prothrombotic stimulus. Inflammatory mechanisms upregulate procoagulant factors, downregulate natural anticoagulants and inhibit fibrinolytic activity [2]. Endotoxin, tumor necrosis factor alpha (TNF-α) and interleukin-1α induce tissue factor (TF) expression, primarily on endothelial cells and monocytes/macrophages, promoting blood coagulation [3]. Activation of the complement C5b–C9 complex changes the cell surface to a more procoagulant phenotype by the shift of negatively charged phospholipids from the inner to the outer membrane [4]. Inflammatory reaction is also accompanied by the increase in fibrinogen and C-reactive protein (CRP) blood levels. CRP itself increases TF and decreases TF pathway inhibitor (TFPI) concentrations, what may be important in pathogenesis of arterial thrombosis and myocardial infarction [5]. Of the natural anticoagulants, protein C pathway appears to be the most strongly influenced by inflammation with thrombomodulin

(TM) and the endothelial cell protein C receptor (EPCR) being both downregulated by TNF-α [6].

On the other hand, thrombotic processes enhance inflammatory reactions, mainly through the action of TF and thrombin [7]. Activation of platelets leads to the release of CD40 ligand, which, in turn, induces TF expression and increases IL-6 levels [8]. Thrombin also augments leukocyte adhesion and activation, stimulates endothelial cells to produce platelet activating factor and increases an expression of P-selectin [9].

A common inhibitory pathway for thrombosis and inflammation also exists. Activated protein C (APC) acting directly as an anticoagulant, functions also as an anti-inflammatory and cytoprotective agent through specific receptors: EPCR and protease activated receptor-1 (PAR-1) [10].

It is possible that TNF-α is a proinflammatory cytokine with the strongest prothrombotic action. TNF-α stimulates monocyte and neutrophil adhesion to endothelium, inhibits protein C system, impairs fibrinolysis and increases TF expression on the cell surface [2]. Produced mainly by activated monocytes, macrophages, and T lymphocytes, this cytokine has been found to be elevated in patients suffering from both, SLE [11] and APS [12]. We hypothesized that TNF-α might be more elevated in APS patients, with thrombosis as its prominent feature, than in those with other autoimmune diseases where immune-mediated inflammation prevails.

In contrast to TNF-α, a possible role of soluble interleukin-2 receptor (sIL-2R) in thrombosis and prothrombotic states is unknown. Soluble IL-2R is a key marker of lymphocyte T activation, crucial for the regulation of an autoimmune inflammatory response [13]. Binding of interleukin-2 (IL-2) to its receptor on the surface of T-lymphocyte triggers a series of intracellular signaling events that result in the activation and proliferation of resting T cells. Upon activation of T cells, IL-2 receptor molecules are expressed on the surface and a soluble form is released. It has been found that autoimmune processes are associated with elevated levels of sIL-2R [14, 15].

We speculated that sIL-2R could be less elevated in an isolated form of APS, not associated with other autoimmune diseases, where inflammatory process is less (if at all) pronounced. If both, TNF-α and sIL-2R seem to be markers of autoimmune diseases, we asked whether their serum levels might correlate with the thrombotic state on one hand, and the intensity of autoimmune inflammation from the other. To answer this question, we have measured serum levels of both mediators in subjects with autoimmune diseases with or without presence of antiphospholipid antibodies (aPL) and examined carefully the differences which can be specific for patients fulfilling updated criteria for APS [16].

Patients

We studied 188 patients (165 women and 23 men, aged 18–72; mean age 40.5 years) referred to the Outpatient Clinic for Autoimmune Diseases in Jagiellonian University Medical College. SLE was diagnosed in 147 patients based on the presence of at least four American College of Rheumatology (ACR) criteria [17]. The group of SLE-like syndrome (SLE-LS), defined by the presence of 2 or 3 of these criteria including antinuclear antibodies, consisted of 21 cases. Thirty-six patients in the SLE group fulfilled recently revised APS classification criteria [16] (SAPS subgroup). There was no APS patient in the SLE-LS group. In 20 patients, APS was not accompanied by any features of another autoimmune disease (PAPS). The groups were not statistically different in terms of sex and age. The control group consisted of 32 healthy subjects, matched by sex and age with the group studied.

A detailed history (a uniform set of questions) was taken from all the patients by a trained physician. All available medical records were also carefully analyzed. Objective data confirming typical clinical involvement and laboratory test abnormalities of APS and SLE were required. All thrombotic episodes had to be confirmed by the imaging techniques (USG, CT and/or NMR). Pregnancy complications were defined according to recent updated APS classification criteria [16] and were confirmed by an experienced obstetrician based on available medical records.

From the total group of patients, 66 subjects suffered from thrombosis (45 venous, 17 arterial, and 4 both) and 42 women experienced pregnancy complications. Among 20 patients with PAPS, there were 15 cases of thrombosis (9 venous, 5 arterial, and 1 both) and nine cases of fetal loss (4 women had thrombosis and fetal loss). In the SAPS group 31 patients suffered from thrombosis (22 venous, 7 arterial, and 2 both) and 13 women suffered from pregnancy morbidity. Remaining cases of thrombosis ($n = 20$) and pregnancy complications ($n = 20$) appeared in patients with SLE or SLE-like syndrome without laboratory signs of APS.

The study was approved by the local ethical committee. All of the patients provided informed consent.

Methods

Lupus anticoagulant (LA) was detected in accordance with the three-step procedure recommended by the International Society on Thrombosis and Haemostasis (ISTH) [18]. We used partial thromboplastin time (PTT LA, Diagnostica Stago, France) and diluted Russell's viper venom time (DVV test, American Diagnostica, USA) for screening, and

Staclot LA (Diagnostica Stago, France) and DVV Confirm (American Diagnostica, USA) for confirmation. Tests were run on Behring Coagulation Timer (BCT) and Fibrintimer analyzers (Siemens, Germany).

Serum levels of anticardiolipin (aCL) and anti-β_2-glycoprotein I (aβ_2GPI) antibodies (both of IgG and IgM classes) were measured using an in-house ELISA [19]. Following the recent suggestions [20, 21], monoclonal antibodies HCAL and EY2C9 (Sapporo Standards) against β_2GPI were used as calibrators for the construction of standard curves for IgG and IgM, respectively [22]. All the aPL measured values exceeding 99 percentile of a healthy control group were considered positive, if confirmed by a second determination at least 12 weeks apart.

Serum concentrations of TNF-α and sIL-2R were determined by an immunoenzymatic method using commercially available kits on Immulite 1000 chemiluminescence automatic analyzer (Siemens, Germany). According to the manufacturer, reference ranges for healthy population are lower than 8.1 pg/ml for TNF-α and below 710 U/ml for sIL-2R.

Mann–Whitney test was used for statistical analysis. Correlation of variables was assessed by means of Spearman's rank correlation coefficient.

Results

Among our patients, LA was found in 38 subjects, aCL IgG in 79, aCL IgM in 57, aβ_2GPI IgG in 36, and aβ_2GPI IgM in 40 subjects. Altogether, at least one aPL at a level fulfilling the revised APS classification criteria (at least two measurements exceeding 99 percentile, at least 12 week apart) was found in 93 patients.

Serum concentration of TNF-α was significantly elevated in all groups of patients (SLE, SLE-LS and PAPS) as compared to controls. The highest levels of TNF-α were found in patients with PAPS, followed by these from the SAPS subgroup. In the SLE group, patients fulfilling APS classification criteria had significantly higher TNF-α levels than the remaining patients suffering from SLE (Table 1).

Levels of sIL-2R were also higher in patients than in controls but the difference reached statistical significance for SLE group only. There were no differences in sIL-2R levels between studied groups of patients. Some trend toward higher levels of sIL-2R in SLE as compared to SLE-LS ($P = 0.06$) and PAPS ($P = 0.07$) patients could be seen. Interestingly, there was also a trend to the lower levels of sIL-2R in the PAPS group as compared to those with SAPS ($P = 0.06$) (Table 1).

When aPL-positive patients (regardless of the concomitant presence of clinical APS symptoms) were compared to aPL-negative ones, higher TNF-α values (but not sIL-2R) were seen in the former. Inside the SLE group, these differences were evident for both markers (Table 2).

Table 1 TNF-α and sIL2-R levels in the groups studied

Groups studied	Number of patients (*n*)	TNF-α (pg/ml)	sIL2-R (U/ml)
SLE	147	13.3 ± 7.2[a,c]	789 ± 591[a]
SAPS	36	15.1 ± 7.3[a,c,d]	779 ± 396[a]
SLE-APS-	111	12.7 ± 7.1[a]	791 ± 635[a]
SLE-LS	21	9.8 ± 4.2[a,b]	559 ± 224
PAPS	20	16.6 ± 9.8[a]	569 ± 190
Controls	32	7.7 ± 2.3	427 ± 143

Results are expressed as mean ± standard deviation (SD)

[a] $P < 0.05$ versus controls

[b] $P < 0.05$ versus PAPS

[c] $P < 0.05$ versus SLE-LS

[d] $P < 0.05$ versus SLE-APS

Table 2 TNF-α and sIL2-R levels in patients with and without aPL

Group of patients	Number of patients (*n*)	TNFα (pg/ml)	sIL2-R (U/ml)
All patients	188		
aPL+	93	14.8 ± 11.6[a]	789 ± 660
aPL−	95	11.7 ± 5.8	692 ± 392
SLE group	147		
aPL+	63	14.9 ± 8.3[a]	907 ± 778[a]
aPL−	84	12.0 ± 6.0	709 ± 409

Results are expressed as mean ± SD

[a] $P < 0.05$ aPL+ versus aPL−

Further analysis showed that the levels of TNF-α also depended on the type of aPL. TNF-α was higher in patients showing the presence of LA or ACL IgG or aβ_2-GPI IgG antibodies as compared to those without any antibodies (Fig. 1). A weak, but significant correlation between aCL IgG and TNF-α levels ($r = 0.31$; $P = 0.0001$; by Spearman rank test) was found.

The presence of the APS clinical criteria (thrombosis and pregnancy complications) were associated with a tendency toward the elevation of TNF-α. However, this difference did not reach statistical significance (data not shown).

The levels of cytokines were also analyzed in relation to historical presence of symptoms and signs of SLE (Table 3). Elevated levels of sIL-2R were associated with many SLE clinical symptoms, whereas TNF-α was significantly elevated in SLE patients with only renal involvement. As far as immunologic criteria of SLE is considered, patients with the presence of ds-DNA antibodies as well as those with decreased C4 concentrations showed higher values of both cytokines.

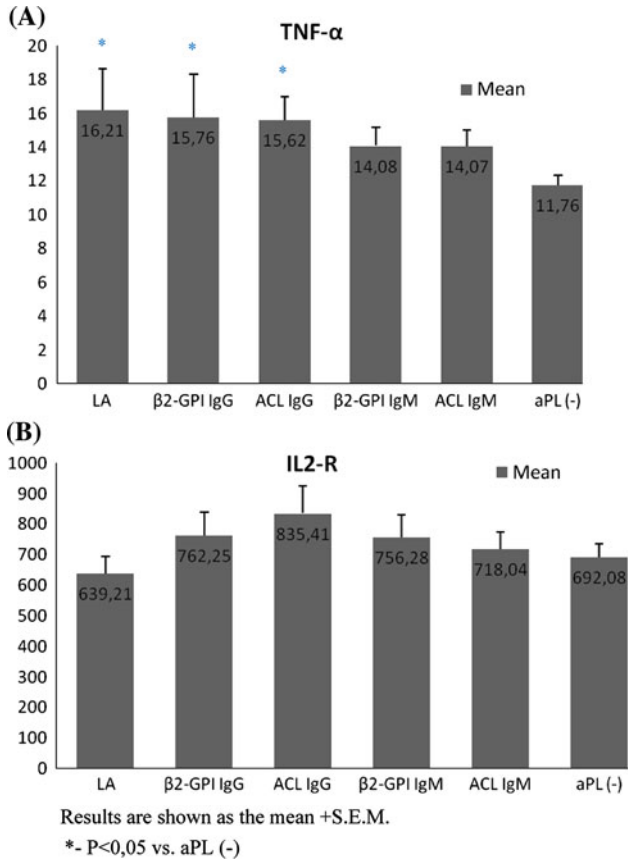

Fig. 1 Levels of TNF-α (**a**) and sIL2-R (**b**) depending on the presence and the type of aPL. Results are shown as the mean + SEM [$*P < 0.05$ versus aPL (−)]

Discussion

In our study, we have shown that: (a) elevated levels of TNF-α are present not only in SLE but also in PAPS patients and (b) whilst the highest levels of TNF-α were detected in patients with APS (PAPS and SAPS), sIL-2R levels were more elevated in SLE patients, especially within those showing symptoms and signs related to the generalized inflammatory reaction.

In SLE group of patients, elevation of both markers and their association with disease activity has already been shown [11, 12, 14, 15, 23]. We support these findings by showing that patients with an active disease (decreased C4 levels, presence of ds-DNA autoantibodies and renal involvement) had significantly higher levels of TNF-α and sIL-2R than the others. Recently, it was published that in PAPS patient higher level of TNF-α is connected with lower level of C3 and C4 and lupus anticoagulant activity [24]. Patients showing other clinical symptoms (arthritis, serositis and skin involvement) show only elevated sIL-2R levels (Table 3). It may be hypothesized that this is due to the fact that sIL-2R is a more sensitive marker of inflammation than TNF-α.

Levels of sIL-2R in SLE-LS and PAPS patients (negligible widespread inflammation) were not different from the controls (Table 1). Possibly in these groups of patients, lymphocyte T activation and inflammatory reactions are much less pronounced than in fully blown SLE. In this respect, the PAPS group seems to be interestingly showing highest levels of TNF-α and a relatively low levels of sIL-2R. Our data may suggest that sIL-2R is a good marker of autoimmune activity but it does not reflect prothrombotic state, typical for APS.

In contrast, serum TNF-α was especially elevated in patients diagnosed with definite APS (Table 1) and those with antiphospholipid antibodies (Table 2). Our results corroborate with those of Bertolaccini et al. [25], who showed elevated TNF-α levels in patients with APS. Also, in accordance with our findings, they were unable to show any differences between its primary and secondary form (Table 1). It may indicate that elevated TNF-α levels are related rather to pathogenesis of thrombosis, common for both forms of APS, than to inflammation—very limited in PAPS. However, such a causal relation between TNF-α and thrombosis is not uniformly accepted. In the experimental setting it has been shown that TNF-α might exert anti-platelet and antithrombotic activity [26].

TNF-α was found to be higher in patients positive for LA or aPL of the IgG class than in those with IgM antibodies. These LA or IgG-positive patients showed significantly higher levels of TNF-α as compared to aPL-negative group (Fig. 1). These findings indirectly strengthen the notion about the important pathogenic role of LA and IgG aPL (as opposed to IgM) in the development of APS-related thrombosis [27]. If eventually proven, it could also serve as yet another argument in the discussion about the negligible clinical significance of IgM aPL in APS [28, 29].

It has been suggested that TNF-α plays an important role in inducing APS-related pregnancy loss [30], and thrombosis [12, 31]. However, in our study, we were unable to find any significant differences in TNF-α levels between patients with and without APS clinical symptoms. This is not surprising as there were usually wide temporal gaps between TNF-α determinations and clinical complications which took place in the distant past.

High level of TNF-α was rather connected with the present level of aPL than with the historical events of thrombosis. In future, hopefully, prospective studies can answer the question which groups of patients have the highest risk of getting thrombosis.

Several studies indicate that aPL [32, 33] and TNF-α [34] can activate endothelium and induce prothrombotic phenotype of endothelial cells, leading to increased thrombin generation. Endothelial cell activation causes in vivo up-regulation of the TF which has been proposed as a main potential mechanism of APS-related thrombosis [35, 36].

Table 3 TNF-α and sIL2-R levels in patients with and without SLE symptoms and signs

SLE symptoms and signs	*n*	TNF-α	*P*	IL-2R	*P*
Skin SLE criteria					
+	107	13.66 ± 7.27	NS	846.01 ± 657	0.0008
–	81	12.74 ± 11.4		598.08 ± 262	
Arthritis					
+	150	13.04 ± 7.28	NS	783.5 ± 590	0.01
–	38	14.15 ± 13.12		583.94 ± 238	
Serositis					
+	29	13.94 ± 8.17	NS	877.2 ± 506	0.01
–	159	13.07 ± 9.56		697.25 ± 540	
Renal disorder					
+	69	14.62 ± 8.05	0.02	864.37 ± 718	0.01
–	119	12.48 ± 9.85		655.35 ± 353	
Neurologic disorder					
–	24	14.07 ± 8.87	NS	762.22 ± 408	NS
–	164	13.15 ± 9.34		733.92 ± 557	
Haematologic disorder					
+	128	13.48 ± 10.03	NS	705.09 ± 372	NS
–	60	12.79 ± 7.43		813.4 ± 793	
Anti-Sm					
+	9	16.17 ± 5.97	NS (0.06)	1,198 ± 756	0.01
–	179	13.12 ± 9.39		716.25 ± 517	
Anti-RNP					
+	24	15.1 ± 7.75	NS (0.07)	1,034.54 ± 1,114	NS
–	164	12.99 ± 9.46		688.75 ± 347	
Anti-Ro					
+	84	12.95 ± 6.54	NS	859.74 ± 716	0.01
–	104	13.52 ± 11.01		634.13 ± 276	
Anti-La					
+	60	12.71 ± 6.6	NS	845.02 ± 770	NS
–	128	13.52 ± 10.29		686.77 ± 371	
Anti-dsDNA					
+	53	15.65 ± 7.8	0.0004	855.11 ± 432	0.001
–	135	12.33 ± 9.64		692.84 ± 567	
C4 decreased level					
+	33	16.37 ± 10.09	0,04	1063.36 ± 1041	0.04
–	155	12.6 ± 8.98		675.12 ± 341	

Once endothelial cells become activated, up-regulation of TF may be further augmented by a synergistic effect of TNF-α and factor Xa [37]. Other changes typical for this prothrombotic phenotype include increased expression of adhesion molecules (ICAM-1, VCAM-1, selectins E and P) [38, 39], and formation of endothelial microparticles [40]. Raschi et al. have shown that anti-beta$_2$GPI antibodies induce translocation of NFκB in a manner similar to that elicited by LPS and TNF-α. Anti-beta$_2$GPI antibodies bind to beta$_2$GPI autoantigen, which acts as a toll-like receptor, and induce activation of endothelium via the MyD88 pathway [41]. The other proposed mechanism for aPL-induced TF expression is the phosphorylation of p38 mitogen-activated protein kinase (MAPK) in endothelial cells and/or platelets [42, 43].

It is not clear whether aPL acts on endothelial cells directly or (as is the case with LPS) through TNF-α. The latter possibility finds support in the article of Dunoyer-Geindre et al. [44]. Independently from the mechanism, prothrombotic state, typical for APS, seems to be associated with both markedly elevated levels of aPL and increased concentrations of TNF-α.

Our results suggest that while sIL-2R seems to be a good marker of a generalized (auto)immune inflammation, TNF-α might be crucial to the prothrombotic action of pathogenic aPL antibodies. If confirmed, it would offer an

intriguing possibility of using TNF-alpha blockade as a therapeutic option in APS.

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