Etanercept Increases Tumor Necrosis Factor-Alpha Level but not sFas Level in Patients with Rheumatoid Arthritis

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ABSTRACT: **Background:** Targeted anti-tumor necrosis factor-alpha (TNFα) therapy in patients with rheumatoid arthritis (RA) has resulted in dramatic improvement in the disease course and prognosis. One of the features of RA is hyperplasia of synovial cells, particularly RA synovial fibroblasts (RA-SF), caused partially by impaired apoptosis of RA-SF cells. It has been shown that TNFα may inhibit apoptosis in RA-SF cells and this process may be reversed by the use of TNFα antagonists.

Objectives: To determine the influence of etanercept, an anti-TNFα agent, on sFas (CD 95) receptor.

Methods: We analyzed serum levels of sFaS and TNFα in a group of 26 patients with high RA disease activity who were selected to start treatment with etanercept. Assessment of sFas receptor and TNFα levels was performed before and 6 months after treatment with etanercept.

Results: Treatment with etanercept resulted in increased TNFα levels (log TNFα 0.602 vs. 1.17, $P < 0.05$) but no change in sFas levels (log sFas 3.17 vs. 3.11, $P = 0.37$). As expected, treatment resulted in significant reduction in both disease activity and levels of inflammatory markers.

Conclusions: Etanercept may increase TNFα levels in patients with RA. We also speculate that the Fas pathway is not the main apoptotic pathway in patients with RA treated with etenercept, since sFas, a marker of apoptotic activity, remained unchanged and was not influenced by disease activity and concomitant treatment.

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KEY WORDS: apoptosis, etanercept, rheumatoid arthritis (RA), tumor necrosis factor-alpha (TNFα), biological treatment

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation and destruction of cartilage and bone in the joints. In RA the synovium is infiltrated by inflammatory cells, and these cells produce inflammatory cytokines and growth factors leading to the progressive joint destruction.

Fibroblast-like cells (RA synovial fibroblasts, RA-SF) have been assigned a key role in the pathogenesis of RA [1]. It is commonly accepted that the inflammatory environment stimulates RA-SF. Specifically, tumor necrosis factor-alpha (TNFα), one of the key cytokines that drives inflammation and triggers the activation of other immunocompetent cells, has been shown to stimulate proliferation of RA-SF [2]. Additionally, some data suggest that TNFα may modulate RA-SF cell apoptosis mainly via transcription factor NF-κB pathways, and TNFα is believed to be an important link between inflammation and synovial hyperplasia [3,4]. It has also been shown that TNFα inhibits apoptosis in RA-SF; however, the precise mechanism of this phenomenon is only partially understood. One of the possible mechanisms responsible for reduction in apoptosis in RA-SF is inhibition of Fas/CD95-induced apoptosis due to upregulation of surface-bound and soluble Fas/CD95 receptors [5]. Elevated levels of soluble Fas have been found in synovial fluid of patients with RA. Based on the ability of sFas to antagonize the Fas signaling pathway, sFas receptor could be the main negative regulator of apoptosis in synovial cells [6].

Apoptosis can be induced by internal mitochondria-dependent and external death receptor-dependent pathways. The latter comprises two main pathways that utilize Fas (CD95/Apo-1) and p55 TNF receptor (TNFRI). The activation of apoptosis through CD95 molecules is caused by the specific ligand for CD95 (CD95L/FasL). Interaction of Fas-FasL is recognized as an important modulation of apoptosis in RA-SF [7]. This second pathway also utilizes TNFα and its receptor may be involved. However, it has been shown that the p55 receptor of TNFα transmits signals that may result in either apoptosis or proliferation.

Treatment of RA with anti-TNFα agents has resulted in dramatic improvement in both disease control and prognosis [8]. Among agents used in this indication, etanercept, a recombinant human TNFα dimeric receptor fusion protein, has proven safe and efficacious in patients with RA. Etanercept consists of the extracellular portion of two p75 receptors fused to the Fc portion of immunoglobulin G-1 (IgG1). It may be hypothesized that inhibition of TNFα may result in increased apoptosis, which could explain in part the therapeutic mechanism

of etanercept in RA. In this context we wished to evaluate the interaction between the two main external apoptosis pathways dependent on Fas and TNFα receptor in a group of patients with RA, and assess whether inhibition of TNFα modulates serum levels of both ligands.

PATIENTS AND METHODS

We prospectively recruited RA patients resistant to treatment with conventional disease-modifying anti-rheumatic drugs (DMARDs) who had been selected to start treatment with the anti-TNFα antagonist etanercept. All patients met the 1987 American College of Rheumatology criteria for RA and were characterized by high disease activity despite methotrexate treatment (mean DAS 5.8 ± 1.2). We excluded patients with uncontrolled hypertension, overt or latent heart failure (defined as left ventricular ejection fraction < 40%, as assessed by conventional echocardiography), history of malignancy, renal impairment and liver disease. Eleven women served as healthy age-matched controls. The study protocol was approved by the Ethics Committee at the Medical University of Silesia, Katowice, Poland, and informed consent was obtained from each patient prior to participation in the study.

The patients received etanercept 25 mg twice a week subcutaneously for 6 months. Age, gender, RA activity (DAS-28), TNFα, sFas levels, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF) and anti-citrullinated antibodies (ACPA) were measured and routine laboratory tests were performed.

Blood samples were collected from a peripheral vein after the patient had rested in the supine position for at least 10 minutes. Samples were stored at -80°C until analysis using appropriate techniques. Blood samples were obtained from the patients before the study and repeated 6 months later. TNFα and sFas were assessed in the serum by enzyme-linked immunosorbent assay (ELISA) kits (Biomedica, Poland) in accordance with the manufacturer's instructions.

Two subgroups of patients were analyzed: one group received methotrexate once weekly at an average dose of 9.3 ± 0.53 mg/week (7.5–25 mg/week) and folic acid 15 mg once a week; the other group was not on treatment with methotrexate. All patients received steroids as required.

STATISTICAL ANALYSIS

Statistical analyses were performed using STATISTICA 9.0 PL (StatSoft Polska, Kraków, Poland). The results are presented as median values/IQR (interquartile range). Figures are presented as box-plots, where whiskers denote mean ± 95% confidence interval, hinges denote standard errors, and points refer to mean value. Distribution of variables was evaluated by the Shapiro-Wilk test. Homogeneity of variances was assessed by the Levene test. To compare two dependent groups (before and after treatment) as well as cases and control groups before treatment, the non-parametric Mann-Whitney U test was used. In case of heavy skewed distribution, normalization with the logarithmic function was performed. Pearson linear correlation was used to assess dependency between variables. All results were considered statistically significant if the *P* value was < 0.05. All tests were two-tailed.

RESULTS

We recruited 26 patients (age 48.3 ± 11 years). Duration of disease before initiation of anti-TNFα was 7.1 ± 1.0 years. Two patients did not complete the study and their results were excluded from the analysis. All patients received prednisone at a mean dose of 5.3 mg daily. Eighteen patients were RF/ACPA positive. Fourteen patients received methotrexate at a mean dose of 10.5 mg (15–25 mg) once a week together with folic acid 15 mg once a week.

Treatment with etanercept resulted in reduced disease activity in the whole RA group (median DAS-28 was 6.06 vs. 4.90, $P < 0.01$). There was also a reduction in ESR and CRP levels over the course of the study [Table 1]. Clinical response was good in 12 (50%) of the treated patients and moderate in 6; the remaining 6 patients were treatment resistant. However, when we analyzed seropositive and seronegative subgroups separately, we observed a statistically significant reduction in ESR, CRP, DAS and VAS only in the seropositive subgroup, and a significant elevation of TNFα in both groups [Table 2].

Plasma sFas levels in patients at baseline did not differ significantly in comparison with healthy subjects (median 1321 vs. 1533 pg/ml). Treatment with etanercept did not influence sFas levels in the treatment group as a whole, or when responders, non-responders, seropositive and seronegative subgroups were analyzed separately [Table 2 and Figure 1]. There were no significant differences in baseline (median 1369 vs. 1809 pg/ml, $P = 0.1$) or post-treatment serum sFas levels between patients who were taking methotrexate and those who were not

Table 1. Influence of etanercept on TNFα levels, sFAS and disease activity parameters in patients with rheumatoid arthritis (seropositive and seronegative groups together)

Parameter	Before	After	*P*
TNFα (pg/ml)	5.27 / 9.94	16.07 / 47.97	< 0.05
sFas (pg/ml)	1533 / 1063	1281 / 1258	0.373
ESR (mm/hr)	41 / 47	21 / 22	< 0.01
DAS-28 (points)	6.06 / 1.32	4.90 / 2.07	< 0.01
CRP (ng/ml)	18.6 / 32.5	7.2 / 5.9	< 0.05
VAS (mm)	60 / 30	50 / 50	< 0.05

Data presented as median/IQR (interquartile range)

Table 2. Changes in sFas, TNFα levels and disease activity parameters in patients with RA with regard to RF factor positivity

Parameter	Control	Seropositive			Seronegative		
		Before*	After**	*P*	Before*	After**	*P*
sFas (pg/ml)	1321/1170	1385/832	1290/ 199	0.904	1954/1790	1211/1769	0.160
TNFα (pg/ml)	2.14/7.33	5.62/20.32	13.75/37.26	‹ 0.05	3.05/9.31	18.39/60.87	‹ 0.001
DAS-28 (points)		6.10/0.87	5.01/0.97	‹ 0.01	5.00 1.80	3.15/2.43	0.074
CRP (ng/ml)	1.05/1.32	23.35/31.95	7.20/2.80	‹ 0.05	14.10 55.50	10.00/12.30	0.455
ESR (mm/hr)	1 /7	52/39	18/23	‹ 0.05	26 58	24/18	0.306
VAS (mm)		70/20	50/35	‹ 0.05	50/30	40/60	0.620

*Before treatment
**After treatment
Data presented as median/IQR (interquartile range)

(median 1361 vs. 1174 pg/ml, $P = 0.28$). To examine the effect of disease activity parameters on sFas changes we correlated sFas levels with DAS-28, CRP, ESR, and tender and swollen joint count. We did not observe any correlation between sFas and disease activity parameters. The same was true when changes of sFas (ΔsFas) were correlated with ΔDAS28, ΔCRP and ΔESR, respectively. Interestingly, we showed that post-treatment sFas levels were unrelated to basal sFas values, indicating that changes in sFas are highly unpredictable [Figure 2].

Surprisingly, we observed a significant increase in TNFα level in patients at the end of the study [Table 1 and Figure 1]. When analyzing groups according to RF status, statistically significant increases in TNFα levels were observed in both groups [Table 2]. There were no differences in TNFα levels in subgroups with regard to clinical response (good, moderate, none). TNFα levels were statistically higher in the seropositive group ($P < 0.05$) compared to seronegative patients. This difference in TNFα levels remained significant at the end of the study. We observed that TNFα levels were numerically lower in patients receiving methotrexate (MTX) than in those on monotherapy; however, the difference did not reach statistical significance ($P = 0.1$). Treatment with etanercept resulted in a reduction of CRP in the seropositive group, and this reduction was more pronounced in the MTX group than in patients not receiving MTX.

DISCUSSION

Evidence is accumulating that apoptosis is implicated in the pathogenesis of rheumatoid arthritis. One of the main features of RA is synovial hyperplasia. The mechanisms leading to uncontrolled synovial proliferation are not completely understood. One of the possible mechanisms involved in this process is an imbalance between cell hyperplasia and cell apoptosis. In RA, apoptosis may be regulated via two main pathways: Fas ligand (FasL) and TNFα. Introduction of anti-TNFα agents to the treatment repertoire in RA may thus modulate the course of apoptosis in RA patients. The role of TNFα in apoptosis in RA is, however, controversial. While TNFα may stimulate apoptosis via interaction with p55 TNF receptor, it can also express a variety of molecules which act as anti-apoptotic molecules resulting in inhibition of apoptosis [9]. This is true for RA-FS where TNFα does not induce apoptosis but rather promotes proliferation of synovial cells. Indeed, Drynda et al. [5] demonstrated the inhibitory effect of TNFα on Fas-mediated apoptosis. They demonstrated that TNFα protected RA-SF from Fas/CD95-induced apoptosis in a dose-dependent manner that was paralleled by upregulation of cell surface-bound and soluble Fas receptor. Similar results have been seen in in vitro studies with RA synovial

Figure 1. Changes in TNFα and sFas in patients treated with etanercept. The decimal logarithmic transformation of TNFα and sFas values. Statistical significance $P < 0.05$

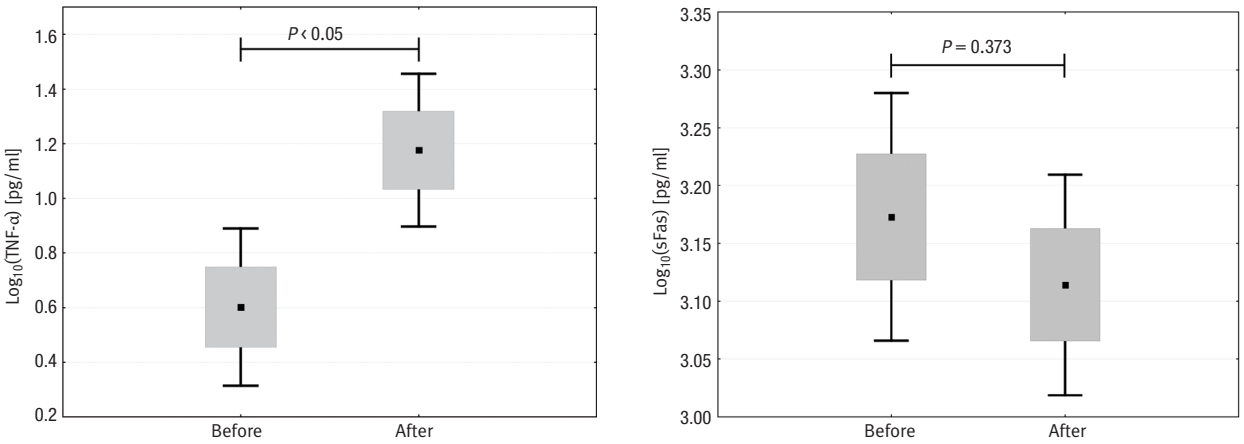
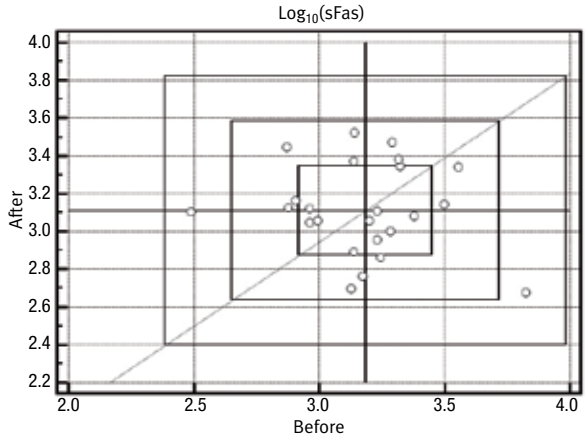


Figure 2. Relationship between sFAS measurements before and after treatment in patients with rheumatoid arthritis (seropositive and seronegative groups together)



The Youden plot which shows the relationship between sFAS measurements before and after treatment in the whole group of patients

Rectangular boxes show 90%, 95% and 99% confidence intervals respectively

cells where TNFα inhibited Fas-mediated apoptosis, and this process was completely reversed by TNFα antagonist [10]. Less is known about sFas in this regard. However, given that sFas is one of the main negative regulators of apoptosis, Fas may precisely reflect anti-apoptotic activity of TNFα.

In the present study we explored the balance between sFas and TNFα pathways during the treatment of RA with the TNFα antagonist etanercept. Previous publications have shown conflicting results, with some showing different sFas levels in RA patients compared to healthy controls [6,11] while others confirmed our results of similar Fas levels [12,13]. We did not identify any relationship between levels of sFas and applied treatment with MTX, disease activity, response to treatment, or RF/ACPA positivity. The latter is surprising as the presence of ACPA antibodies may herald a more aggressive course of RA [14].

To our knowledge, this study is the first to address the changes in soluble sFas in patients receiving the anti-TNFα agent etanercept. Treatment with etanercept had no influence on sFas regardless of clinical response, previous treatment, or concomitant use of MTX. It supports the view that sFas levels remain stable and are not influenced by disease activity or treatment. Lack of correlation between sFas and disease activity was previously reported by Goel et al. [13], despite evidence that TNFα increases levels of sFas and enhanced sFas level is believed to be one of the anti-apoptotic mechanisms of TNFα [5]. This is consistent with data showing that TNFα-dependent activity is exerted only when survival signals are blocked.

We found that TNFα levels were significantly lower in control subjects than in RA patients before treatment; however, no correlation was observed between sFas and disease activity and levels of inflammatory markers. These results should be interpreted cautiously, since we did not examine apoptosis at the cellular level. It may also be speculated that changes in sFas levels in synovial fluid may better reflect anti-apoptotic activity, and increased level of sFas locally may prevent apoptosis in inflamed joints. Dubikov and Kalinichenko [15] previously reported higher levels of sFas in the synovial fluid of patients with both early and longstanding RA in line with this theory. On the other hand, sFas reflects only one apoptotic pathway and it may be that the TNFR1-dependent pathway is also used for transmission of apoptotic signals.

Interestingly, we observed a significant increase in TNFα level in the serum measured by ELISA following treatment with etanercept. Although the background of this phenomenon is unclear, it may be speculated that the observed phenomenon is related to a specific structure of etanercept. Etanercept has a unique structure, contrary to the other TNF antagonist, namely a fusion protein consisting of p75 receptor and Fc portion of IgG. The molecular structure of this agent may explain, at least partially, why its administration resulted in higher TNFα levels. Such elevation of the cytokine has been reported previously [16-19].

It was recently shown that etanercept increases immunoreactivity and bioactivity of TNFα and can shift the balance between monomers, dimers and homotrimers of the cytokine in favor of biologically active TNFα homotrimers, which may explain our findings [18]. However, changes in TNFα had no effect on sFas levels. This raises the possibility that apoptosis in patients treated with anti-TNFα is mediated mainly through abrogation of the inhibitory effect of TNFα at the level of regulatory molecules in the Fas death receptor pathway, but not at the level of Fas receptor. Another theory is that this is due to cross-talk between TNFα receptors and Fas, as proposed by Rothe and co-authors [20]. Therefore, it may be speculated that raised TNFα levels in patients treated with etanercept may cause an imbalance between TNFR1 and Fas-mediated death pathways and favor TNFR1 receptor signaling. The weak point of this speculation is that sFas levels did not differ between patients and controls, so in this model we cannot exclude lack of influence of the rheumatoid process on serum sFas levels. Further limitations of our study are the lack of assessment of apoptosis at the synovial level, utilization of only one anti-TNFα agent, and assessment of TNFα levels by ELISA which measures "total" (i.e., free and receptor bound) levels of the cytokine. Mann et al. [18] have shown that etanercept does increase bioactivity of TNFα when the concentration of anti-TNFa is much higher than that of TNFα. This may mean that the lack of influence of etanercept on sFas level is caused at least partially by the increase in TNFα bioactivity.

To summarize, despite the significant therapeutic effect of etanercept, we failed to show any influence of the drug

on sFas levels but observed a significant increase in TNFα levels. This finding may support the hypothesis that during anti-TNFα treatment apoptosis is mediated via blocking survival signals, but the treatment does not interact with Fas and TNFR1 receptor activity [4,21].

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