Serum Levels of TNF-α, TNF-αRI, TNF-αRII and IL-12 in Treated Rheumatoid Arthritis Patients

Ali Asghar Ebrahimi1*, Hamid Noshad2, Shahram Sadreddini1, Mohammad Saeid Hejazi3, Yashar Mohammadzadeh Sadigh4, Yashar Eshraghi4, Morteza Ghojazadeh5

1 Departments of Rheumatology, 2 Nephrology, 5 Physiology, 3 Immunology, 4 Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

ABSTRACT

Background: Rheumatoid arthritis (RA) is a chronic multisystem autoimmune disease common in all races and ethnicities. Cytokines and cytokines receptors play an important role in RA pathogenesis and clinical presentation. Objective: To investigate the serum levels of TNF-α, TNF-α RI, TNF-α RII and IL-12 in RA patients and healthy control group. Methods: In this study 43 patients fulfilling the revised criteria of American College of Rheumatology (ACR) for RA and 13 healthy cases as a control group were selected for TNF-α, TNF-αRI, TNF-αRII and IL-12 serum level analysis. The patients' age was 42.2 ± 22 and the age of healthy group was 40.1 ± 19.2 years (p=0.1). The patients had an active disease with at least six swollen and ten tender joints. Minimum ESR was 28 mm at first hours of the morning. Early morning stiffness in patients lasted longer than 45 minutes. Results: Our study showed that IL-12 serum level of the patients (91.69 ± 43.07 pg/ml) and control (61.79 ± 40.08 pg/ml) group was significantly different (p<0.001). The serum level of TNF-αRI was 2.36 ± 0.77 ng/ml in the patient and 1.73 ± 0.37 ng/ml in the control group (p<0.01). TNF-αRII serum concentration in patients was 8.89 ± 2.3 ng/ml, while that of control group was 7.06±1.30 ng/ml (p=0.03). The serum level of TNF-α in patients was 32.90 ± 19.27 pg/ml and that of the control group was 24.27± 8.28 pg/ml (p=0.08) with no significant difference between the two. Conclusions: It is concluded that IL-12, TNF-αRI and TNF- αRII serum concentrations are more important and better predictive factors than TNF-α in RA course and in the active forms of the disease.

Keywords: TNF-α, Interleukin-12, Rheumatoid Arthritis
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic multisystem autoimmune disease that can cause irreversible joint deformities and functional organ impairment (1,2). It is characterized by the synovial proliferation and inflammatory cell infiltration (3). The skeletal complications of RA consist of focal erosions in marginal and subchondral bone, juxtaarticular osteoporosis and generalized bone loss with reduced bone mass (4,5). Although the pathogenesis of RA is not clearly known, cytokines are supposed to play an important causative role (6,7,8). TNF-α, sTNF-R and Interleukin-12 (IL-12) are responsible for several clinical manifestations of RA (3,4,9-11). Early detection of proinflammatory factors in the biopsy specimens of involved joints and cell culture established their importance in joint inflammation. Among them, the significance of TNF-α in mediating the arthritogenic response has been demonstrated in anti-TNF-α treated animal models of arthritis (12-14) and in human disease (12,15). Although the results of surveys in genetically modified mice showed that TNF-α and sTNF-R are not necessary for the development of collagen induced arthritis (CIA) (4,16,17), but the severity of CIA is attenuated by anti-TNF-α even in the established disease (4,18). Moreover, blockade of TNF-α in RA patients using either chimeric anti-TNF-α antibodies or soluble p75 TNF receptor-Fc fusion proteins substantially decreases inflammation, improves their function and vitality and attenuates cartilage and bone erosion (4,19). TNF-α not only makes adverse effects on synovial tissue but also stimulates other proinflammatory cytokines including interleukin 1, GM-CSF, PG-E and matrix metaloproteinases involved in the degradation of synovial tissue. TNF-α also activates the release of soluble TNF receptors which inhibit its function and result in a negative signalling message (9,20). TNF-αRII is involved in the stimulation of immune system in the affected joints and in the regulation of the cascade of events mediated by TNF-α R1 by controlling the binding of TNF-α to the receptor (21-24). Synovial tissue and cartilage pannus junction of RA patients express TNF-αRI and TNF-αRII. Comparing with control groups, synovial levels of TNF-αRII were emphasized to increase in RA patients (21, 25, 9, 26).

In addition elevated production of IL-12 was seen in RA patients (3, 11). In patients with rheumatoid arthritis in vitro IL12 p70 induces IFN-γ dominant cytokine production by infiltrating T-cells in chronically affected joints, suggesting that IL-12 may play an important role in rheumatoid T-cells with Th1 cytokine profiles production. Some previous studies showed that IL-12 levels reflect RA disease activity and that IL-12 is involved in the production of proinflammatory cytokines (3).

Based on above observations, this study was designed to evaluate the possible relationship between serum levels of TNF-α, TNF-α RI, TNF-α RII and IL-12 in treated RA patients.

PATIENTS AND METHODS

Patients. Forty three patients who fulfilled the revised criteria of the American College of Rheumatology (ACR) for RA were enrolled in our study. They were referred to the outpatient rheumatology clinic of Sheykh-ol Rais affiliated to Tabriz University of Medical Sciences and Sina Teaching Hospitals. The RA patients' age was 42.2±22 years. Disease duration ranged from 1 to 15 years. The patients had the radiological damage index of I to IV (2). All enrolled patients had active disease, defined as at least
six swollen joints and ten tender joints. The disease activity was evaluated by clinical manifestation and laboratory findings (e.g. ESR or CRP) (2). Our exclusion criteria were primary autoimmune diseases other than RA, congestive heart failure (CHF), any active infections and impaired renal function (Cr>1mg/dl). Control group consisted of 13 healthy individuals without any rheumatic disease. Peripheral blood was collected from median cephalic vein using a 21 gauge syringe. Blood was transferred to sterile tubes and the serum was separated by centrifugation at 4000 rpm, and stored at -70°C until analysed.

**CRP, ESR and Hb Assay.** Hb concentration (gr/dL) and ESR (mm/h) at the first and the second hours were estimated according to Westergren Method; CRP and IgM-RF were measured using commercial agglutination tests.

**TNF-α, TNF-αRI, TNF-αRII and IL-12 Assay.** The serum levels of TNF-α, TNF-αRI, TNF-αRII and IL-12 were measured using commercially available ELISA kits (Biosource, Belgium). All samples and reagents were prepared according to the manufacturer’s instruction. Briefly, for measuring the level of TNF-α and IL-12, chromogenic (TMB) solution was freshly prepared upon diluting 0.2ml of concentrated chromogenic solution with the substrate buffer (H2O2 in acetate/citrate buffer). The chromogenic solution was used within 15 minutes and was kept in the dark. The wash solution was also freshly prepared, and all of the reagents were equilibrated to room temperature before use. Samples containing TNF-α or IL-12 reactive with capture monoclonal antibodies coated on the microtiter wells. Following two hours incubation, excess antigen and serum were washed out completely. The second monoclonal antibody, conjugated with horse radish peroxidase (HRP), was added to the wells, incubated and chromogenic solution was added to produce a blue colored product. Finally to stop the reaction, phosphoric acid was added which changed the color from blue to yellow. Then the absorbances were measured at 600nm using ELISA reader Awareness Statfax-2100, USA. The concentration of cytokines in the samples was determined using a standard curve. The concentration of TNF-αRI and TNF-αRII were measured using appropriate kits and reagents. After pipeting the samples into the wells, labeled sTNF-αRI and sTNF-αRII solutions were added. Following the incubation period, wells were washed thoroughly. Chromogenic solution plus substrate (TMB + H2O2) was then added to produce a blue colored product. Adding phosphoric acid as the stop solution changed the color from blue to yellow and the reaction was terminated and the absorbances were read in an ELISA reader as mentioned above. The expected normal ranges for various cytokinase were as followed. For TNF-α, 0-29 (pg/ml), IL-12, 10-98 (pg/ml), TNF-αRI, 0.3-2.1(ng/ml) and TNF-αRII, 1.91-6.51 (ng/ml).

**Statistical Analysis.** Data were analyzed using SPSS version 13.0 (SPSS, Chicago. IL, US). Data were expressed as mean ± SD. For comparing means between patient and control groups, an independent sample t-test was used. A p<0.05 was considered statistically significant.

**RESULTS**

**Patients.** Disease duration was 1 to 15 years, with a mean of 5.8 years (Table 1). All Patients who enrolled in the study received anti-inflammatory drugs (combination therapy...
with NSAIDs, steroids, DMARDs, Hydroxychloroquin and sulfasalazine, MTX). None received anti-TNF-α agents.

The ESR level of patients ranged from 4 to 90 mm in the first hour, and the CRP of the patients was between 1+ and 3+.

### Table1. Patients’ characteristics.

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Mean</th>
<th>Max</th>
<th>Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women/men</td>
<td>33/10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>-</td>
<td>42.15</td>
<td>65</td>
<td>11</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>-</td>
<td>5.8</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>ESR(mm/1st hr)*</td>
<td>-</td>
<td>32.3</td>
<td>90</td>
<td>4</td>
</tr>
<tr>
<td>CRP**</td>
<td>-</td>
<td>3+</td>
<td>1+</td>
<td></td>
</tr>
<tr>
<td>Hb</td>
<td>-</td>
<td>12</td>
<td>14.3</td>
<td>11.1</td>
</tr>
</tbody>
</table>

*Erythrocyte sedimentation rate; ** C-reactive protein

### Serum Levels of TNF-α and TNF-αRs.

The patients’ serum level of TNF-α was 32.9 ± 19.27 pg/ml in RA patients (ranging from 10.7 to 87 pg/ml), compared with 24.27 ± 8.28 pg/ml in the control group (ranging from 8.6 to 35.1 pg/ml). No significant differences in serum level of TNF-α were seen between patients and the control group (p=0.08) (Table2).

### Table 2. Serum levels of TNFα, TNFαRs and IL-12 in patients and control groups.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Patient</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα (pg/ml)</td>
<td>32.9±19.27</td>
<td>24.27±8.28</td>
<td>0.08</td>
</tr>
<tr>
<td>TNFαRI(ng/ml)</td>
<td>2.4±0.77</td>
<td>1.7±0.37</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TNFαRII(ng/ml)</td>
<td>8.9±2.3</td>
<td>7.06±1.3</td>
<td>0.03</td>
</tr>
<tr>
<td>IL-12(pg/ml)</td>
<td>91.61±43.07</td>
<td>61.79±40.08</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

1TNF-α= tumor necrosis factor α, TNF- α RI= tumor necrosis factor receptor I, TNF- α RII= tumor necrosis factor receptor II and IL-12= interleukin 12.

ELISA results showed that the serum level of TNF-αRI in patients varied between 0.98 and 4.6 ng/ml and that of controls varied between 1.22 and 2.51 ng/ml. The mean of TNF-αRI concentration in patients was 2.36 ± 0.77 ng/ml, while its mean concentration in the control group was 1.73 ± 0.37 ng/ml (p<0.01) (Table 2).

The serum level of TNF-αRII in patients ranged from 5.93 to 13.9 and that of the controls varied between 5.3 and 8.69 ng/ml. The mean of TNF-αRII concentration in patients and healthy control were 8.89 ± 2.3 and 7.056 ± 1.3 (p=0.0.3) respectively. Data is summarized in Table 2.

### IL-12 Serum Level.

The patients’ serum level of IL-12 was 91.61 ± 43.07 pg/ml (ranging from 22.2 to 231.8 pg/ml), while its mean concentration in the control group was 61.79 ± 40.08 pg/ml (ranging from 18.09 and 125.28 pg/ml). A significant difference in serum level of IL-12 was observed between the patients and the control group (p<0.01).

### DISCUSSION

Both TNF-α and IL-12 have been considered important cytokines in the pathogenesis of RA. They are involved in almost all aspects of articular inflammation and destruction
TNF-α, particularly, plays a pivotal role in the pathogenesis of RA (8,18). It is present at biologically significant levels in RA synovial tissue and fluid, but not in osteoarthritis synovium or systemic lupus erythematosus kidney tissue. Moreover, the TNF-α levels seem to parallel the severity of both inflammation and bone erosion (28-30). Some previous studies demonstrated high levels of TNF-α and its receptors in patients with RA (8,27,31). They showed that serum concentration of TNF-α in RA patients was significantly higher than the levels obtained from healthy controls. Moreover, a strong correlation was found between serum TNF-α and CRP, ESR and the number of swollen joints. However other surveys did not demonstrate any relationship between TNF-α levels and the inflammatory parameters including ESR or CRP. Our results show no significant difference in the serum levels of TNF-α between the patient and the control groups. Furthermore, clinical markers of disease activity did not correlate with the serum level of TNF-α. There are two surface receptors of molecular weight 55kDa (TNF-αRI) and 75kDa (TNF-αRII) that TNF-α binds to. These receptors are thought to be produced by synovial cells and the circulating peripheral cells. TNF-α upregulates the generation of TNF-αRs (8,32).

Some investigators reported elevated serum concentrations of TNF-αRs in RA cases (2, 4). TNF-αRs were not associated with ESR and CRP, but they correlated with the number of swollen joints, Ritchie index and DAS (8,32,33). However, some studies failed to find similar relations (8,22). In this study serum concentrations of TNF-αRs were not significantly higher in RA patients in comparison with the healthy controls (p=0.08). IL-12 is able to prime the selective expansion of T helper cells with a Th1-type pattern of cytokine production which has an important role in host protection against intracellular pathogens (34-36). In RA, T cell cloning experiments, staining of freshly isolated cells for intracellular cytokines, and RT-PCR analyses of biopsy samples have all demonstrated that CD4+ lymphocytes present in the synovial fluid or membranes produce IL-12 (34,37,38). Studies using the animal models of RA showed that administration of IL-12 enhanced disease expression and severity (3,40). Furthermore, it has been documented that synovial lining cells and macrophages express IL-12 in RA patients (3,40). Some previous studies showed higher levels of IL-12 p70, a biologically active form of IL-12, in the sera and synovial fluid, compared with osteoarthritis (OA) patients and the control group (3,27,41,42). However, this was not confirmed by other surveys (39,43). It was demonstrated that the patients with elevated serum levels of IL-12 had higher tender joint scores, more swollen joint scores and higher CRP (3). In the present study, circulating levels of IL-12 were significantly higher than the control group, confirming previous reports (41, 42).

It was shown that circulating TNF-α and its soluble receptor TNF-αRI increase significantly in patients with RA complicated by systemic amyloidosis, as compared with carefully matched RA control patients without amyloidosis, and that the increased TNF-α levels may be implicated in the pathogenesis of the renal manifestations and anemia of chronic disease (ACD) in reactive amyloidosis. The findings may also suggest that anti-TNF-α directed therapeutic strategies may be beneficial in RA patients with amyloid nephropathy (44). However it is possible that another gene or genes, in vicinity of TNFα/B microsatellites and distinct from TNF-α itself, would be the primary causative agent. For instance, lymphotoxin-α (LTA ) or lymphotoxin-β (LTB), which lye very close in the class III region of the MHC and encode for lymphotoxins α and β, share many biologic functions with TNF-α (45).
ACKNOWLEDGMENTS

This work was supported by a grant from Tabriz University of Medical Sciences.

REFERENCES

Ebrahimi AA, et al