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Gene Expression of Tumor Necrosis Factor-Alpha in Etanercept-Treated Rheumatoid Arthritis Patients

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Abstract

Fifty-one rheumatoid arthritis (RA) patients were enrolled to assess the gene expression of tumor necrosis factor-alpha (TNF-α) by reverse transcription quantitative polymerase chain reaction (qRT-PCR) in etanercept-treated RA patients, with some emphasis on clinical and biological markers of disease. The results revealed that the ΔCt mean range in total, male and female RA patients and controls was 1.286 ± 1.226 - 4.023 ± 0.856 and the differences were not. Laboratory and clinical findings in subgroups of patients also showed no significant variations in the distribution of $2^{-\Delta\Delta Ct}$ means, with the exception of anti-cyclic citrullinated peptide (ACCP) antibodies. The lowest expression was observed in moderate positive patients (1.566 ± 1.104) compared to low and high positive patients (4.061 ± 1.366 and 9.668 ± 3.518, respectively) for ACCP antibodies, and the difference was significant (p = 0.043). Inspecting the $2^{-\Delta\Delta Ct}$ means in duration of disease and gender revealed that male patients recorded a lower mean than female patients (0.827 ± 0.550 *vs.* 4.143 ± 1.317) at <5 years disease duration, while at >10 years duration of disease, female patients showed a lower mean than male patients (1.242 ± 0.372 *vs.* 5.607 ± 3.334). However, both differences were not significant. It is concluded that etanercept was effective in normalizing the *TNF* gene expression, but variations that were related to gender, duration of disease and some biological markers of disease, were observed.

Keywords

Rheumatoid Arthritis, Tumor Necrosis Factor Gene Expression, Etanercept, (qRT-PCR)

1. Introduction

Rheumatoid Arthritis (RA) is a chronic inflammatory autoimmune disease of the connective tissues. Genetic and environmental factors are involved in RA initiation, as well as the severity of disease course [1]. It is characterized by a synovial inflammation of small joints (hands and feet) and large joints (shoulder and knees). The joint synovitis leads to progressive destructions in bones and cartilages, and causes significant disabilities and even permanent functional loss due to the erosion of bones surfaces if it is untreated [2]. The global prevalence of RA in adult populations is approximately 0.5% - 1.0%, and it is more common in women than in men (two- to four-folds), with a most common age at onset between the fourth and sixth decade of life [3]. Pathologically, RA is mainly three steps process that begins with autoimmunity development, followed by a local inflammation and in the final step, bone destruction is induced [4]. Localized inflammation of joints is progressed (*i.e.* synovitis) and results in cartilage and bone destruction. Both innate and adaptive immune responses are involved in the inflammatory process, in which cytokines play important role in the inflammation and advancing the disease [5].

Tumor necrosis factor-alpha (TNF-α) is one of these cytokines. It plays recognized roles in regulating innate immunity, adaptive immunity, inflammation, and autoimmunity. A deregulation of *TNF* gene expression and signaling can cause chronic inflammation, which may result in a development of autoimmune diseases and tissue damage [6]. It is mainly produced by activated macrophages, T lymphocytes, and natural killer cells, but lower levels of expression have been reported in various other cells, for instance, fibroblasts, mast cells and tumor cells [7]. TNF-α has been implicated in the stimulation of T cell immune response, upregulation of proteolytic enzymes, prostaglandins and chemokines, and over-expression of adhesion molecules [8].

Elevated serum levels of TNF-α have been reported in RA patients and such increase has been linked with inflammation and joint destruction that are observed in RA patients, and it is considered as one of the main mediators of these pathologies [6]. Therefore, TNF-α has been a target for immunotherapy, and etanercept is one of these therapies.

Etanercept (Enbrel® Amgen-Pfizer) is a fusion protein consisting of a recombinant human TNF receptor (rTNF-p 75), bound to the Fc portion of an immunoglobulin, which binds strongly to soluble TNF-α [9]. It is a biological response modifier that binds and inactivates TNF-α in RA patients. Etanercept-treated RA patients have shown a more rapid improvement in disease activity and a slower rate of radiographic progression than methotrexate-treated patients [10]. However, the response to anti-TNF-α is heterogeneous with success in only 65% of patients and a consideration of some clinical and biological aspects are useful in the attempt to define and predict the response of RA patients to inhibitors of TNF-α [11].

Accordingly, the present investigation was designed to assess the gene expression of TNF-α in RA patients treated with etanercept, with some emphasis on

clinical and biological markers of disease.

2. Materials and Methods

2.1. Patients

The ethical committee of the Iraqi Ministry of Health approved the study, in which 51 RA patients were enrolled, and their age range was 20 - 63 years. The diagnosis was made by the consultant medical staff at the Rheumatology Unit (Baghdad Teaching Hospital), and it was based on a clinical examination, X-ray findings, and laboratory tests. The diagnosed was according to the revised diagnostic criteria established by the American College of Rheumatology, 2010 [12]. The patients were under therapy, which was a single weekly subcutaneous dose of 25 mg of etanercept (Enbrel) for a period of 3 - 5 years.

The patients were assessed for erythrocyte sedimentation rate (ESR), rheumatoid factors (RFs), C-reactive protein (CRP) and anti-cyclic citrullinated peptide (ACCP) antibodies. The patients were sub-grouped according to these principles; positive and negative for RFs and CRP, and weak (20.0 - 39.9 U/ml), moderate (40.0 - 59.9 U/ml) and strong (≥60.0 U/ml) positive for ACCP antibodies. A further sub-grouping of patients was based on Disease Activity Score (DAS)-28. The DAS-28 is a system developed and validated by the EULAR (European League Against Rheumatism) to measure the progress and improvement of RA, and based on four assessments, which are TEN 28 (number of joints with tenderness upon touching), SW 28 (number of swollen joints), ESR and SA (subjective assessment of disease activity by the patient during the preceding seven days on a scale between 0 and 100). A DAS-28 value of >5.1 corresponds to a high disease activity, 3.2 - 5.1 corresponds to a moderate disease activity and <3.2 corresponds to a low disease activity [13]. The calculations of DAS-28 were carried out using the DAS-28 calculator, which is available free online (http://www.das-score.nl). The duration of disease (<5, 5 - 10 and >10 years) was also considered in subgrouping of patients.

In addition to patients, 45 apparently healthy individuals (control group) were also enrolled in the study, and their age range was 25 - 52 years. Details of patients and controls are given in **Table 1**.

2.2. Isolation of Total RNA

The blood was collected from participating RA patients in EDTA tubes during their visit to the Rheumatology Unit to take their subcutaneous dose of etanercept (*i.e.* after seven days of the previous dose and before the latter dose). A ready-to-use reagent (TRIzolTM LS Reagent; Thermo Fischer Scientific; USA) was used to isolate total RNA from blood samples, and instructions of the manufacturer were followed.

2.3. Gene Expression of TNF-α

The *TNF* gene expression was determined by the reverse transcription-quantitative polymerase chain reaction (qRT-PCR) method using SaCycler (Sacace,

Table 1. Characteristics of rheumatoid arthritis patients and controls.

Parameter	Rheumatoid Arthritis Patients (No. = 51)			Controls (No. = 45)
Gender				
Males (%)		22 (43.1)		15 (33.3)
Females (%)		29 (56.9)		30 (66.7)
Age (Years; Mean ± SEM)				
Total		44.9 ± 1.5		41.3 ± 1.3
Males		44.4 ± 2.6		45.6 ± 2.1
Females		45.3 ± 1.8		39.2 ± 1.5
Duration of Disease (%)	Total	Males	Females	
<5 years	14 (27.5)	6 (27.3)	8 (27.6)	-
5 - 10 years	26 (51.0)	9 (40.9)	17 (58.6)	
>10 years	11 (21.5)	7 (31.8)	4 (13.8)	
DAS-28 (%)	Low 2 (3.9)	Medium 29 (56.9)	High 20 (39.2)	-
ACCP Antibody Positivity (%)	Weak 24 (47.1)	Moderate 8 (15.7)	Strong 19 (37.2)	Not tested
Rheumatoid Factors (%)	Positive 27 (52.9)		Negative 24 (47.1)	Not tested
C-reactive Protein (%)	Positive 33 (64.7)		Negative 18 (35.3)	Not tested

ACCP: Anti-Cyclic Citrullinated Peptide.

Italy). The GoTaq® One-Step qRT-PCR System kit (Promega, USA) was used to assess the expression. It is a reagent system for quantitative analysis of RNA by using a one-step qRT-PCR protocol.

5'-TCTTCTCGAACCCCGAGTGA-3' and 5'-CCTCTGATGGCACCACCAG-3' were the forward and reverse primers, respectively. They were adopted from previously published sequences [14], in which the forward (5'-AGCCGAGCC-ACATCGCT-3') and reverse (5'-CAGCCCTGGTGACCAGGC-3') primers of the housekeeping gene *GAPDH* (reference gene: glyceraldehyde-3-phosphate dehydrogenase) were also given.

The procedure was carried out in a reaction volume of 20 µl according to the manufacturer's instructions (Promega, USA): 10 µl master mix, 0.5 µl RT mix, 2 µl of each primer, 5 µl RNA and 0.5 µl nuclease-free water. The following PCR cycling conditions were employed: cDNA synthesis at 37˚C for 15 minutes (1 cycle), initial denaturation at 95˚C for 5 minutes (1 cycle), followed by 40 cycles of denaturation at 95˚C (30 seconds), annealing at 60˚C (30 seconds) and extension at 72˚C (30 seconds).

2.4. Gene Expression Calculations

The double delta Ct analysis was used to assess the expression of *TNF* gene, in which *GAPDH* was the housekeeping reference gene. First, the mean of Ct values for the housekeeping gene and the gene being tested in patients and controls was calculated. Such calculation returned with four Ct values; The Ct value for *TNF* gene tested in patients (TP), Ct value for *TNF* gene tested in controls (TC), Ct value for housekeeping gene tested in parallel with patients (HP) and Ct value

for housekeeping gene tested in parallel with controls (HC). Second, the difference between TP and HP (TP-HP) and TC and HC (TC-HC) was calculated. Such calculation returned with ΔCt values for patients (ΔCtP) and controls (ΔCtC). Third, the difference between ΔCtP and ΔCtC (ΔCtP-ΔCtC) was obtained to get the Double Delta Ct Value (ΔΔCt). To get the expression fold change for *TNF* gene, the $2^{-\Delta\Delta Ct}$ was calculated, which represents the Relative Fold Change. Therefore, the results were expressed as a fold change in the expression level of the target gene that was normalized to endogenous control (housekeeping gene) and relative to calibrator, which is the target gene in control subjects [15].

2.5. Statistical Analysis

Data were given as mean ± standard error of mean (SEM), and a significant difference between means was assessed by analysis of variance (ANOVA) followed the least significant difference (LSD). The statistical package SPSS version 13.0 was used to carry out these analyses. A *p*-value ≤0.05 was considered significant.

3. Results

The range of ΔCt mean in the investigated groups (total, male and female RA patients and controls) was 1.286 ± 1.226 - 4.023 ± 0.856, and there was no significant difference between these means. The $2^{-\Delta\Delta Ct}$ mean in patients was 5.759 ± 1.834, and again there was no significant variation between male and female patients (4.957 ± 1.650 *vs.* 6.320 ± 2.919; $p > 0.05$), as shown in Table 2.

Laboratory and clinical findings in subgroups of patients also showed no significant variations between them in the distribution of $2^{-\Delta\Delta Ct}$ means, with exception of ACCP antibodies. The lowest expression fold was observed in moderate positive patients (1.566 ± 1.104) compared to low and high positive patients (4.061 ± 1.366 and 9.668 ± 3.518, respectively), and the difference was significant ($p = 0.043$) (Table 3).

Inspecting the $2^{-\Delta\Delta Ct}$ means in duration of disease and gender revealed that male patients recorded a lower mean than female patients (0.827 ± 0.550 *vs.* 4.143 ± 1.317) at <5 years of disease duration, while at >10 years duration of disease, an opposite picture was drawn, in which female patients showed a lower

Table 2. Expression of TNF mRNA in rheumatoid arthritis patients and controls.

Groups		Number	ΔCt (Mean ± SEM)	LSD *p*-value	$2^{-\Delta\Delta Ct}$ (Mean ± SEM)
Total	Patients	51	2.462 ± 0.448	NS	5.759 ± 1.834
	Controls	45	2.253 ± 0.790		
Males	Patients	22	2.947 ± 0.798	NS	4.957 ± 1.650
	Controls	15	1.286 ± 1.226		
Females	Patients	29	2.123 ± 0.522	NS	6.320 ± 2.919
	Controls	30	4.023 ± 0.856		

NS: Not significant ($p > 0.05$).

Table 3. Expression fold ($2^{-\Delta\Delta Ct}$) of TNF mRNA in rheumatoid arthritis patients distributed by laboratory and clinical findings.

Groups		Number	$2^{-\Delta\Delta Ct}$ (Mean ± SEM)	ANOVA *p*-value
Duration of Disease	<5 years	14	2.722 ± 0.889	NS
	5 - 10 years	26	8.129 ± 3.421	
	>10 years	11	4.020 ± 2.168	
Disease Activity Score (DAS-28)	Low	2	1.927 ± 0.920	NS
	Medium	29	7.541 ± 3.076	
	High	20	3.557 ± 1.335	
Rheumatoid Factors	Positive	27	3.851 ± 1.085	NS
	Negative	24	7.905 ± 3.697	
C-reactive Protein	Positive	33	6.401 ± 2.735	NS
	Negative	18	4.581 ± 1.449	
ACCP antibodies	Weak positive	24	4.061 ± 1.366	**0.043**
	Moderate positive	8	1.566 ± 1.104	
	Strong positive	19	9.668 ± 3.518	

ACCP: Anti-Cyclic CitrullinatedPeptide; NS: Not significant ($p > 0.05$).

mean than male patients (1.242 ± 0.372 *vs.* 5.607 ± 3.334). However, the difference in both cases was not significant (Table 4).

4. Discussion

In 2004, Schwartzman and colleagues addressed the following question: do anti-TNF agents have equal efficacy in patients with RA? [16] It is possible to address a further related question: do RA patients respond similarly to etanercept irrespective of their age, gender, duration of disease or their clinical biomarkers. The presented results may answer such question, and the effects of the anti-TNF agent etanercept on *TNF* gene expression showed variations that were related to some parameters in patients, although most of them were not significant.

Gender and duration of disease were among these parameters, and although total male and female patients responded similarly to etanercept, the profile was different at earlier (<5 years) and later (>10 years) duration of disease. The $2^{-\Delta\Delta Ct}$ at <5 years was increased four-folds in female patients than in male patients, while an opposite observation was made at >10 years of disease duration. This may suggest a different manner of TNF mRNA expression in response to etanercept therapy in male and female patients when duration of disease is considered. There is no direct evidence that can support such findings, but it has been observed that formation of neutralizing antibodies against anti-TNF agents (infliximab and adalimumab but not etanercept) is associated with lower serum levels of these agents in patients with rheumatic diseases, and this leads to lower efficacy and higher withdrawal rate of the agent [17]. The formation of these antibodies has been confirmed in an animal model of arthritis (mouse with collagen-induced arthritis; CIA) treated with etanercept. The therapeutic efficacy of etanercept was reduced in CIA mice that developed anti-etanercept antibodies, and the authors concluded that these antibodies can considerably reduce the

Table 4. Expression fold ($2^{-\Delta\Delta Ct}$) of TNF mRNA in rheumatoid arthritis patients distributed by duration of disease and gender.

Duration of Disease		Number	$2^{-\Delta\Delta Ct}$ (Mean ± SEM)	LSD *p*-value
<5 years	Males	6	0.827 ± 0.550	NS
	Females	8	4.143 ± 1.317	
5 - 10 years	Males	9	7.043 ± 2.700	NS
	Females	17	8.704 ± 5.098	
>10 years	Males	7	5.607 ± 3.334	NS
	Females	4	1.242 ± 0.372	

NS: Not significant ($p > 0.05$).

effects of etanercept (anti-arthritic and anti-osteoporotic effects) [18]. Therefore, the formation of anti-etanercept antibodies may interfere with the expression of TNF mRNA, but such subject is a matter of controversy, and more recently, anti-etanercept antibodies have not been detected in psoriatic patients treated with etanercept [19].

A further different manner of TNF mRNA expression was observed when the status of RFs, CRP and ACCP antibodies was considered. Patients seronegative for RFs showed increased expression compared to seropositive patients, and this may suggest that seronegative patients were less responder to etanercept therapy. In contrast, patients seropositive for CRP were less responder compared to seronegative patients. In the case of ACCP antibodies, strong positive patients (≥60.0 U/ml) were the less responder to etanercept therapy compared to low or moderate positive patients, as the TNF mRNA showed approximately 9 fold expression. In this context, it has been reported that presence of RFs and ACCP antibodies are associated with a poorer response to anti-TNF agents. A poorer response has also been associated with CRP levels at initiation of therapy [20].

Distributing RA patients by their DAS-28 revealed that patients with a moderate activity (3.2 - 5.1) showed an increased expression compared to patients with low (<3.2) or high (>5.1) disease activity. No direct evidence that can support such finding, but etanercept has been shown to be efficacious for treating RA patients that have moderate and severe disease in early and established RA [21]. Moreover, a further analysis revealed that etanercept treatment was associated with a better disease status than severe disease [22].

5. Conclusion

The effectiveness of etanercept as anti-TNF therapy in RA patients might be subjected to gender, duration of disease, disease activity and some biological markers of disease. However, the findings are limited by the investigated sample of patients, and the results can be more fruitful if firstly diagnosed RA patients, who have not received etanercept therapy, are included in the assessment of *TNF* gene expression.

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