Possible implications of Serum levels of Tumor necrosis factor-α (TNF- α) Among Rheumatoid Arthritis Patients In Kerbala Province

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Abstract

Background: Anti- Tumor necrosis factor -α (anti-TNF) therapies have shown to slow and even halt structural joint damage in Rheumatoid Arthritis (RA). However, therapeutic inhibition of TNF-α has approved to lead to an increase in infections and lymphoma, hence it is mandatory to identify which patients with rheumatoid arthritis may benefit from anti-TNF therapies.

Aim of the study: in this study we attempt to find out if any link could be drawn between serum levels of TNF-α and some disease processes and manifestations in RA. Such presumed link may help providing bases for patients' selection to anti-TNF therapies.

Methods and Material: thirty two RA patients were enrolled in this study. The patients were diagnosed based on the American College of Rhuematology (ACR) criteria and non of them had received anti-TNF therapy. Blood sample was taken from each patient at time of attending; sera were separated immediately and kept frozen at –20^{o}C until used. Disease Activity Score (DAS) was calculated using DAS28-3 formula. Radiographs were read by expert radiologists. TNF-α was measured using solid phase Enzyme Amplified Immunoassay (EASIA), while Sandwich Enzyme-linked Immunosorbent Assay (ELISA) was used for the separate quantitative detection of RF of the IgG, IgM and IgA classes in serum.

Results: Among the 32 Rheumatoid Arthritis (RA) patients who were enrolled in this study, there were statistically significant positive correlations between the serum TNF-α levels with radiological joint damage and with serum Rheumatoid Factor (RF-Latex), (p=0.015, and p=0.011 respectively). However, no correlation could be found between serum TNF-α level with any of other disease processes and outcomes.

Conclusions: These results may further support the importance of serum TNF-α level of erosive RA and may reflect a causal relationship between TNF- α and joint damage. In addition, radiological changes and Latex-RF may be promising inclusion criteria in patients' selection for anti-TNF therapies. However, a further detailed study using a larger sample of patients is recommended to support our results.

تمهيد: وجد إن العلاجات المثبطة لعامل التنخر نو ع الفا تستطيع أن تبطئ أو حتى توقف التلف المفصلي في مرض التهاب المفاصل الرثوي. ألا أن هذه العلاجات تؤدي إلى زيادة نسب الإصابة بالاخماج والأورام اللمفاوية. لذلك فأن من المهم تحديد المرضى الذين يحتاجون هذه العلاجات بصورة دقيقة.

الهدف من الدراسة: لقد حاولنا في هذه الدراسة إيجاد رابط بين المستويات المصلية لعامل التنخر نوع الفا مع بعض مظاهر مرض التهاب المفاصل الرثوي, علما بأن وجود مثل هذا الرابط ممكن إن يساعد

في إيجاد الأسس الملائمة في اختيار المرضي الذين يتم اخضاعهم للعلاج بمضادات عامل التنخر نوع الفا.

المواد وطرق العمل: اشتملت الدراسة على اثنين وثلاثون مريضا مصابا بالتهاب المفاصل الرثوي والذين تم تشخيص المرض لديهم من قبل طبيب مختص وبالاعتماد على دلائل الكلية الأمريكية لعلم المفاصل (ACR criteria) وتم اخذ عينات دم من جميع المرضى وتم فصل الأمصال وحفظها مجمدة لحين الاستخدام. تم تحديد درجة فعالية المرض DAS باستخدام الصيغة الحسابية المسماة DAS28-3 كما تم فحص الصور الإشعاعية من قبل أطباء اختصاص في مجال الاشعة التشخيصية. كما تم قياس مستوى عامل التنخر نوع الفا بواسطة فحص (EASIA) في حين تم قياس العامل الرثوي ضمن أصناف الأجسام المضادة IgG, IgM و IgA وذلك بأستخدام تقنية (ELISA).

النتائج: أظهرت الدراسة ان من بين اثنان و ثلاثون مريضا مصابا بالتهاب المفاصل الرثوي فان هناك ارتباط معنوي مفيد إحصائيا بين المستويات المصلية لعامل التنخر نوع الفا مع تلف المفصل الظاهر من قبل الصور الشعاعية و كذلك مع المستوى المصلي للعامل الرثوي المقاس بطريقة تلازن اللاتكس (p=0.015 و p=0.011 بالتتابع) . الا انه لم تظهر الدراسة وجود أي ارتباط بين المستويات المصلية لعامل التنخر نوع الفا مع بقية المتغيرات.

الاستنتاجات: تؤكد نتائج هذه الدراسة على اهمية المستوى المصلي لعامل التنخر نوع الفا في تلف المفصل في مرض التهاب المفاصل الرثوي مما قد يعكس علاقة سببية بينهما. بالإضافة إلى ما تقدم فان هذه الدراسة قد تؤشر أهمية كل من التغيرات الاشعاعية و تلازن العامل الرثوي كمؤشرات لاختيار المرضى للخضوع لعلاجات المثبطة لعامل التنخر نوع الفا. مع ذلك فإننا نقترح إجراء دراسة مفصلة تشمل عينة اكبر من المرضى وذلك لتأييد نتائج هذه الدراسة.

Introduction

Tumor necrosis factor-α (TNF-α) was originally named for its ability to trigger necrosis of transplanted tumor cells in mice [1]. However, its major role appears to be in early inflammatory responses and the host response to intracellular organisms. TNF-α, along with interleukin- 6 (IL-6), primarily mediates the acute-phase response. It is capable of inducing the expression of adhesion molecules that regulate migration of neutrophils in an early inflammatory response, and it induces the expression of nitric oxide (NO) in mice and an unknown mediator in humans that is required for intracellular killing of mycobacteria [2-5]. Given these important roles, it is no surprise that therapeutic inhibition of TNF-α leads to an increase in infections and lymphoma [6, 7].

TNF- α is produced primarily by activated macrophages (monocyte) and, to a lesser extent, by lymphocytes. It is initially synthesized and expressed as a transmembrane molecule, the extracellular portion of which is subsequently cleaved by TNF- α converting enzyme (TACE) to release the soluble 17 kDa molecule. Soluble TNF-α circulates as a homotrimer and engages its cognate receptors on cell surfaces [8].

TNF receptors (TNF-R) are expressed by nearly every mammalian cell. This ubiquitous expression, in conjunction with cell-specific effecter molecules that are triggered by the TNF-R, may explain the variety of effects of TNF which include apoptosis, the synthesis of protein and lipid inflammatory molecules, and transcription factors. TNF-α is capable of binding to each of the two TNF-R designated as TNF-RI (or p55) and TNF-RII (or p75). Interaction of TNF with its receptor triggers a conformational change and dimerization or clustering of receptors which, in turns, triggers the cellular response [9].

In vitro studies suggested that TNF is a critical and proximal mediator of the inflammatory pathway in the rheumatoid joint. Proof-of-concept for this hypothesis has now been provided by animal studies and clinical trials. Not only does TNF inhibition dramatically reduce markers of inflammation but it also slows or halts structural damage, and these

effects appear to be as potent in early disease as they are in late disease. In human terms, these efficacies should translate to less functional disability and higher quality of life [10]. Taking in consideration the potential side-effects from anti TNF-α therapies and precautions that need to be taken in their use, it is mandatory to indentify which adult patients with rheumatoid arthritis (RA) may benefit from anti-TNF therapies (the potential risks versus the benefits need to be considered for each individual case).

In this study, we tried to find out if any link could be drawn between serum TNF-α level with disease manifestations and processes. Such presumed link may help giving bases for patients' selection to anti-TNF therapies.

Patients and Methods

The present work represents a cross sectional study conducted on 32 RA patients who attend private clinic and rheumatology consultation clinic in Al-Hussein Teaching Hospital (Holy Kerbala Province) during the period from June 2008 to January 2009. Non of the patients had received anti-TNF therapy The patients were diagnosed based on American College of Rheumatology (ACR)[11] classification criteria in addition to simple laboratory assays (Patients were diagnosed by Specialist Rheumatologist). ACR diagnostic criteria include: morning stiffness for more than one hour, arthritis of more than 3 joint groups, arthritis of hand, symmetrical joint involvement, rheumatoid nodules, positive rheumatoid factor (RF), radiographic changes (erosions and local decalcification). Serum RF and ESR were evaluated in routine laboratory techniques. Blood sample was taken from each patient at time of attendant; serum was separated within 1 h of blood collection after spinning for 15 min at 1500 g. The serum was stored without preservative at -20 oC and then thawed just prior to testing. Baseline data about patients obtained from clinical and laboratory examinations were arranged in a questionnaire for each patient and entered in SPSS software.

Disease Activity Score (DAS)

Disease activity of individual patients was assessed by Disease Activity Score (DAS). DAS including a 28-joint count (DAS28)-erythrocyte sedimentation rate (ESR) was calculated from the tender joint count (TJC), swollen joint count (SJC) and ESR according to the authorised formula (http://www.das-score.nl/, accessed 18 December 2009)[17] and as follows:

$$DAS28\text{-}3 = (0.56 \times \sqrt{(TJC28)} + 0.28 \times \sqrt{(SJC28)} + 0.70 \times \ln(ESR)) + 1.08$$

Where TJC28= number of tender joints from 28joints; SJC28= number of swollen joints from 28joints; and ESR= erythrocyte sedimentation rate in mm/ 1st hour.

Twenty-eight tender and swollen joint scores include shoulders, elbows, wrists, metacarpophalangeal joints, proximal interphalangeal joints and knees. The DAS has a continuous scale ranging from 0-10, and according to the EULAR criteria,) the level of disease activity can be interpreted (> 5.1= High disease activity, <5.1-> 3(.)2= Moderate disease activity, <3.2= Low disease activity, and <2.6= Remission. Radiographs of hands and feet are used to asses the structural joint damage (erosions) in RA. Those radiographs were read by at least two expert radiologists.

Enzyme-Amplified Immunoassay (EASIA) to Measure TNF-α serum level

The TNF-α EASIA kit was purchased from BIOSOURCE (BioSource Europe S.A. Belgium). The instructions of the manufacturer were followed in performing the assay and blow is a brief description of the procedure. Standards or serum samples containing TNF-α react with capture monoclonal antibodies (MAbs1) coated on the microtiter well. After incubation, the occasional excess of antigen is removed by washing. Mab 2, the horseradish peroxidase (HRP)-labelled-antibody, is then added. After an incubation period allowing the formation of a sandwich: coated MAbs 1- TNF-α – Mab 2- HRP, the microtiter plate is washed to remove unbound enzyme labeled antibodies. Bound enzyme-labelled antibodies are measured through a chromogenic reaction. Chromogen Solution (TMB+ H2O2) is added and incubated. The reaction is stopped with the addition of Stop Solution (H2SO4) and the microtiter plate is then read at the appropriate wavelength. The amount of substrate turnover is determined colourimetrically by measuring the absorbance which is proportional to the TNF-α concentration. A standard curve is plotted and TNF-α concentrations in serum samples is determined by interplotation from the standard curve.

ELISA for IgG-RF, IgM-RF and IgA-RF

Sandwich Enzyme-linked Immunosorbent Assay (ELISA), was used for the separate quantitative detection of RF of the IgG, IgM and IgA classes in serum. The assay is based on the use of ELISA plate coated with highly purified Fc fragment of human IgG, which is then used to 'capture' the relevant autoantibody in the test serum. The antibody-autoantibody complex is then reacted with third antibody specific to certain autoantibody isotype. This complex is then detected by measuring the activity of an appropriate enzyme that had previously been covalently attached to the third* antibody. ELISA kits were purchased from Aida Gmbh, Germany. Due to the lack of international reference calibration this assay is calibrated in arbitrary units (U/ml) for IgG and IgA RFs. For IgM RFs, the assay is calibrated against the international WHO standard and results are given in IU/ml. According the manufacturer instructions the following cutoff values were used: 18 U/ml for IgG-RF, 18 IU/ml for IgM-RF and 15 U/ml for IgA-RF.

Statistical analysis was performed using SPSS version 15.

Results

This study included 32 rhumatoid arthritis patients. Table (1) shows the patient's characteristics that include some demographic, physical and laboratory findings. Among those patients, 28 (87.5%) were females and 4 (12.5%) were males. Age mean was 49.5 (± 13.9) years old. Extra-articular manifestations were recorded in 9 (28.1%) cases. Radiological changes (joints erosions) were shown in 22 (68.8%) of the cases. Rheumatoid nodules were found in 4 cases (13.3%) out of 30 RA patients (data about presence of rheumatoid nodules were missing for 2 cases).

Regarding the Disease Activity Score (DAS); 8 (25.8%) were found to have high disease activity, 16 (51.6%) have moderate disease activity, 4 (12.9%) have low disease activity and 3 (9.7%) have remission (Disease Activity Score was not available for one of the cases).

Laboratory findings showed that 24 (75%) were positive for serum rheumatoid factor as measured by slide

latex agglutination test. Concerning the RF isotypes measured by ELISA, the highest positivity was found in the IgG isotype (IgG-RF) {25 (78.1%)} , followed by the IgM-RF {19 (59.4%)}, the least positivity was found with the IgA-RF {16 (50%)}. Furthermore, serum TNF-α was positive in 17 (53.1%) cases.

Table 1. Characteristics of patients with rheumatoid arthritis (n = 32)

Characteristic	Number
Age (year) mean (±SD)	49.5 (±13.9)
Female gender (%)	28(87.5%)
Disease duration, mean (SD)/month	40.4 (±37.04)
Disease Activity Score (DAS28-3) mean (±SD) High Disease Activity Moderate Disease Activity Low Disease Activity Remission Total	 4.6 (±1.7) 8 (25.8) 16 (51.6) 4 (12.9) 3 (9.7) 31(100)
Radiological changes (erosions) (%)	22 (68.8%)
Extra-articular manifestations (%)	9 (28.1%)
Rheumatoid nodules (%)	4 out of 30 (13.3%)
Serum RF (Latex) (%)	24 (75%)
Tumor Necrosis Factor-α (TNF-α) (%)	17 (53.1%)
Rheumatoid Facor IgG (ELISA) (%)	25 (78.1%)
Rheumatoid Facor IgM (ELISA) (%)	19 (59.4%)
Rheumatoid Facor IgA (ELISA) (%)	16 (50%)
Serum TNF-α (%)	17 (53.1%)

Table (2) shows the distribution of TNF-α Positivity according to some patients' characteristics, in addition to some physical and laboratory findings. Out of 9 RA cases who have extra-articular manifestations, only 4 cases demonstrated positive serum TNF-α levels. While among 23 cases without extra-articular manifestations, 13 cases showed positive serum TNF-α levels, however, this difference was statistically insignificant (*p=0.411*).

Regarding joint erosions that are indicated by the finding of radiological changes; 15 out of 22 cases with radiological changes were shown to have positive serum TNF-α levels, this in contrast to cases without radiological changes where only 2 out of 10 were shown to be positive to serum TNF-α levels. This difference was statistically significant (*p=0.015*).

Three out of 4 RA cases associated with rheumatoid nodules were shown to be positive for serum TNF-α levels. In contrast, among 26 RA cases that are not associated with rheumatoid nodules, 13 cases were positive for serum TNF-α levels. Nevertheless, this apparent difference was found to be statistically insignificant (*p=0.352*). It is worthy to mention here that there is a clear difference in positivity for serum TNF-α levels among cases associated with rheumatoid nodules in comparison to cases not associated with rheumatoid nodules. However, this difference was not significant which might be due to the limited number of cases associated with rheumatoid nodules in this study (only 4 cases). Therefore, it is strongly advisable to further studying this difference in another study that apply a larger patients sample size.

Patients with positive serum RF latex test had higher serum levels of TNF-α (14 out of 25) in comparison with negative serum RF latex test (1

out of 8). This difference was statistically significant (*p=0.011*).

Regarding DAS, serum levels of TNF in High Disease Activity Scores case was positive in (4 out of 8) in High Disease Activity cases, (8 out of 16) in Moderate Disease Activity cases, (2 out of 4), in Low Disease Activity cases, (2 out of 3) in Remission cases. Those results were not statistically significant (p=0.697).

Table 2. Distribution of TNF-α Positivity according to different attributes of Rheumatoid Arthritis among the study subjects.

Disease Attribute		TNF-α		Total
		Negative	Positive	
Extra-articular Manifestations	Absent	10	13	23
	Present	5	4	9
Total		15	17	32
Chi-square		0.411		
Radiological changes (Erosions)	Absent	8	2	10
	Present	7	15	22
Total		15	17	32
Chi-Square		0.015*		
Rhuematoid nodules	Absent	13	13	26
	Present	1	3	4
Total		14	16	30
Chi-Square		0.352		
Serum RF (Latex)	Negative	7	1	8
	Positive	8	16	24
Total		14	17	32
Chi-Square		0.011*		
Disease Acitivity Score (DAS28)	High Disease Activity	4	4	8
	Moderate Disease Activity	8	8	16
	Low Disease Activity	2	2	4
	Remission	1	2	3
Total		15	16	31
Chi-Square		0.697		
Rheumatoid Facor IgG (ELISA)	Negative	4	3	7
	Positive	11	14	25
Total		15	17	32
Chi-Square		0.424		
Rheumatoid Factor IgM (ELISA)	Negative	8	5	13
	Positive	7	12	19
Total		15	17	32
Chi-Square		0.155		
Rheumatoid Factor IgA (ELISA)	Negative	9	7	16
	Positive	6	10	16
Total		15	17	32
Chi-Square		0.240		

* Statistically significant (*P*< 0.05)

Concerning RF isotypes measured using ELISA, positive IgG-RF, IgM-RF and IgA-RF had higher serum TNF in comparison with cases with negative cases. However, this difference was not statistically significant (p=0.424, p=0.155, p=0.240, respectively)

Discussion

In this study, TNF-α was measured using a solid phase Enzyme Amplified Immunoassay (EASIA) performed on microtiter plate. This assay is based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs) directed against distinct epitopes of TNF- α are used. The use of a number of distinct MAbs avoids hyperspecificity and allows high sensitive assays with extended standard range and short incubation time. This make EASIA is superior to conventional ELISA procedures[13].

RA is an autoimmune inflammatory disease characterized by cartilage destruction and extracellular matrix degradation. Studies have shown that TNF-α is among the cytokines that play a major role in the pathogenesis of the disease, being able to induce bone resorption and cartilage destruction[14]. Treatment with TNF blocking agents, alone or in combination with methotrexate, has high clinical efficacy and delays joint destruction in RA[15].

Extra-articular manifestations (vasculitis, rheumatoid nodules) occur, especially in patients with long-standing RA [16]. This study has failed to find any statistically significant association between presence of the extra-articular manifestations and serum levels of TNF-α. This result might indicate that TNF-α does not play a role in the development of extra-articular manifestations.

One of the characteristics of RA is the presence of certain autoantibodies. Rheumatoid factor (RF) is a family of autoantibodies that recognizes the 'fraction crystallizable' (Fc) part of IgG molecules and exists as IgA-, IgG- and IgM-isotypes. Rheumatoid factor is detected in majority of patients with established disease, and constitutes one of the American College of Rheumatology (ACR) classification criteria [17]. Nevertheless, in this study no association was found between serum levels of TNF-α and any of the above rheumatoid factor isotypes. Surprisingly, we found a statistically significant association between the serum levels of TNF-α and RF measures by latex agglutination ($p= 0.011$). However, to the best of our knowledge there is no similar study to compare our results with its results.

Current disease activity can be assessed, both for clinical and research purposes, by a joint disease activity score (DAS28-3) comprising swollen joint count (SJC), tender joints count (TJC) and ESR[18]. In the current study, DAS28-3 was assessed only in 31 patients and the majority of cases were found to have a moderate DAS (51.6%). However, no statistically significant association could be found between DAS and the serum TNF-α. This result clearly indicate that the increase in serum TNF-α did not associated with raising DAS, nevertheless, this result does not exclude the possibility to use DAS to monitor the patients response to anti-TNF-α therapies.

Furthermore, in the current study; there were statistically significant associations between the presence of radiological joint changes with serum TNF-α as measured by chi –square ($p=0.015$). These results came in line with the results of other studies [18]. These results may indicate that TNF-α could be used as prognostic marker for joint damage. In addition, according to this results, TNF-α appear to be a promising criteria for selecting patients who may get benefit from anti-TNF-α therapy. Furthermore, these results confirm other prospective studies that showed a clear association

of TNF-α levels with the development of erosions.

In conclusion our results may further support the importance of serum TNF-α level of erosive RA and may reflect a causal relationship between TNF- α and joint damage. In addition, radiological changes and Latex-RF may be promising inclusion criteria in patients' selection for anti-TNF therapies. However, a further detailed study using a larger sample of patients is recommended to support our results.

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