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TNF-A, IL-1β and IL-6 Cytokine Gene Expression in Synovial Fluid of Rheumatoid Arthritis and Osteoarthritis Patients and Their Relationship with Gene Polymorphisms

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Abstract

Objective: To analyse and compare TNF-α, IL-1β and IL-6 mRNA expression of rheumatoid arthritis (RA) and osteoarthritis patients (OA) and to study the relationship between mRNA expression and promoter polymorphisms of these genes in RA patients.

Method: A total of 20 RA, 10 OA and 10 healthy controls were taken in this study. The mRNA expression was quantified by qRT-PCR method and polymorphic analysis done by PCR-RFLP method.

Result: TNF-α, IL-1β and IL-6 mRNA expression was significantly increased in the synovial fluid of both RA and OA patients as compared to healthy controls but with a higher fold change in RA than OA with TNF-α: 10.70 fold in RA vs 3.58 fold in OA; IL-1β: 5.61 fold in RA vs 2.42 fold in OA and IL-6: 2.58 fold in RA vs 1.70 fold in OA. The TNF-α and IL-1β mRNA expression was higher in RA patients with higher DAS28 Score ($p = 0.02$ and $p = 0.009$ respectively). TNF-α and IL-1β mRNA expression was significantly higher in the carriers of TNF-α -238 GG ($p = 0.04$) and IL-1β-511 TT ($p = 0.03$) genotypes respectively.

Conclusion: We conclude that TNF-α, IL-1β and IL-6 cytokines play an important role in the pathogenesis and disease severity of RA. Also, the difference in cytokine gene expression between RA and OA is more quantitative than qualitative. Therefore, suggesting a more intense inflammatory nature of RA than OA. Moreover, the TNF-α-238 GG and IL-1β-511 TT genotypes are associated with higher mRNA expression, therefore are susceptibility markers to RA.

Keywords: Rheumatoid arthritis; Osteoarthritis; Cytokines; mRNA; Polymorphism

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by chronic inflammation of the synovial joints, hyperplasia and overgrowth of synoviocytes, with ensuing destruction of articular cartilage [1,2]. Although the precise etiology of the disease is unknown, genetic and environmental factors seem to be involved in its pathogenesis [3]. A broad array of macrophage and fibroblast cytokines, most importantly IL-1β, IL-6 and TNF-α have been suggested to play a key role in the pathogenesis and disease manifestations of RA and biological agents against these have proven to be the efficient therapy for RA thus far [4-6].

The pathogenic basis of osteoarthritis (OA) compared to RA is less clear cut. Variable degrees of inflammation are common in OA, both clinically and pathologically. Inflammation at the onset of OA in the synovium may be secondary to cartilage destruction or may be the primary cause. The histological features, which include the presence of mononuclear cells, may be indistinguishable from RA [7,8] Although OA is a synovial disease, experimental models and clinical observations in humans suggest that mechanical factors may be important. There is synovitis in the mechanical models of OA; hence the role of cytokines within the synovium cannot be excluded [9].

Cytokine expression has been shown to be modulated by polymorphisms within the promoter regions of the genes. Studies have indicated that the promoter polymorphisms may modulate the magnitude of the secretory response of these cytokines and thereby affect disease susceptibility and severity [10,11]. Several polymorphisms have been found in the cytokine gene promoters, including TNF-α -308G/A and -238G/A, IL-1β-511T/C and IL-6 -174G/C polymorphisms. All these polymorphisms have been reported as a risk factor for increased susceptibility to RA. These polymorphisms mediate their influence on transcription by modifying the binding site of specific transcription factors [12,13].

With this in mind we aimed our study to examine and compare TNF-α, IL-1β and IL-6 mRNA expression of RA and OA patients and to correlate the mRNA expression and promoter polymorphisms of these genes in RA patients.

Materials and Methods

Study population

Our study group consisted of 20 RA patients, 10 OA and 10 healthy controls (Table 1).

Variables	RA	OA Cases	Controls
Age ≤ 45 years > 45 years	4 16	2 8	3 7
Gender Females Males	17 3	7 3	6 4
RF Positive Negative	17 3	---	---
ACCP Positive Negative	18 2	---	---
SJC ≤ 5(96) > 5(54)	5 15	---	---
ESR ≤ 20mm/hr > 20mm/hr	1 19	---	---
DAS 28 Score ≤ 5 > 5	6 14	----	---

Table 1: Clinical and laboratory features of controls and patients.

All the patients were recruited from the Division of Rheumatology, Department of Internal Medicine, Sher-i-Kashmir Institute of Medical Sciences hospital. Data from all the patients was obtained from personal interviews with patients and/or guardians, and from clinical examination. Both the patients and controls gave informed consent to participate in the study. The study was approved by ethics committee of the institute.

Calculation of DAS28

Disease activity in RA patients was accessed using the DAS28 scoring. DAS28 with three variables was used, based on the counts of tender joints and swollen joints (28 joints were assessed which included 10 PIP's, 10 MCP's, 2 wrists, 2 elbows, 2 shoulders and 2 knees) and ESR. The value was calculated by using the formula DAS28 = 0.56 * √((tender joints))+0.28 * √((swollen joints))+0.70 * ln (ESR) +0.014 * Patient Global Health. Disease activity was determined as follows: DAS28 < 2.6 = remission, DAS 28 < 3.2= low disease activity, DAS 28 < 5.1 = moderate disease activity, DAS 28 > 5.1 = high disease activity.

RNA extraction and cDNA synthesis

About 6 ml of synovial fluid (SF) from the knee joints of RA and OA patients was aspirated by heparinised syringes. SF was incubated with 10 µl/mL hyaluronidase (1000 U/mL, endotoxin tested, formulated for injection) at 37°C for 15 minutes to reduce viscosity. 3.5 ml of peripheral blood was collected in heparinised tubes. Blood was diluted with an equal volume of RPMI. Both SF and peripheral blood were layered onto Ficoll-Paque to separate the mononuclear cell population.

Total RNA was isolated from mononuclear cell population by Trizol reagent (Invitrogen) according to the Chomiczyki and Sacchi method [14]. The RNA concentration was determined by spectrophotometry and the RNA integrity was corroborated on 1% agarose gel. The complementary DNA (cDNA) synthesis was performed using oligo (dT) primer. Briefly, 2.2 g of total RNA was used in a reaction containing RNase-free water to 9 µl and 1 µl oligo (dT) 12-18 Primer. The reaction was incubated at 75°C for 15 minutes followed by 5 minutes on ice. After that, were added 4 µl of 5x first strand buffer, 4 µl 2.5 mM of each dNTPs (Invitrogen), 0.25 2l of 10 U/2l RNase inhibitor, 1 µl of 200 U/2l Moloney murine leukemia virus reverse transcriptase, and 0.75 2l RNase-free water. The reactions were incubated at 75°C for

1 hour followed by 5 minutes of incubation at 95°C to denature reverse transcriptase. The cDNA samples were stored at 80°C until the real-time PCR assays.

Quantitative Real-time PCR

The reactions were performed in MicroAmp 96-well plate capped with MicroAmp optical caps. Five microlitres of cDNA were amplified in 50 µl reaction containing PCR buffer [50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$] with 1.25 U of Taq polymerase (Biotools) and 0.2 mM of the appropriate sense and antisense primers. Samples were denatured at 94°C (1 min), annealed at 60°C (1 min) and extended at 72°C (2 min). Primer pairs were synthesized for TNF-α, IL-1β, IL-6 and GAPDH (Table 2).

Gene	Primer Sequence	Tm (°C)	Product size (bp)
GAPDH	F: 5'-AGAAGGCTGGGGCTCATTTTG-3' R: 5'-AGGGGCCATCCACAGTCTTC-3'	*60*	258
TNF-α	*F*: 5'-CCCAGGGACCTCTCTCTAATC -3' *R*: 5'-ATGGGCTACAGGCTTGTCACT -3'	*60*	*110*
IL-6	*F-5'-ACAACCACGGCCTTCCCTAC-3'* *R-5'-CATTTCCACGATTTCCCAGA-3'*	*60*	*89*
IL-1β	F-5'ACAGATGAAGTGCTCCTTCCA -3' F-5'GTCGGAGATTCGTAGCTGGAT -3'	*60*	*75*
F = forward primer; R = reverse primer; Tm = annealing temperature; bp = base pair			

Table 2: Primer sequences and annealing temperatures of IL-1β, IL-6, TNF-α and GAPDH qRT-PCR.

GAPDH was used as a reference gene. Negative control reactions for every PCR experiment were tubes without Taq polymerase, cDNA or primers. The mRNA expression levels were quantified using the critical threshold value (Ct). Relative gene expression levels were obtained using the 2-ΔΔCt method (expressed as relative expression units). Each sample was tested by triplicate in both groups studied.

DNA extraction and Genotyping

For polymorphic analysis, 3 ml of peripheral blood was collected from RA patients and healthy controls in EDTA containing tubes and DNA was isolated using Zymogen DNA extraction kit. The quality of the extracted DNA was accessed by agarose gel electrophoresis.

The genotyping was done by PCR-RFLP method. PCR was carried out in a final volume of 25 mL containing 50 ng genomic DNA template, 1X PCR buffer (Biotools) with 2 mM MgCl, 50 mM dNTPs (Biotools), and 0.5 U DNA polymerase (Biotools) 0.4 mM of each primer using the standard program described previous [15,16].

For RFLP, the PCR products of TNFα-308G/A, TNFα-238 G/A, IL-1β-511C/T, and IL-6-174G/C polymorphisms were digested with Nco1 (1 U at 37°C for 16 h), Msp1(1 U at 37°C for 16 h), AvaI (1 U at 37°C for 16 h) and) and NlaIII (1 U at 37°C for 16 h) (Fermentas) enzymes, respectively. The digested products were visualized on 3.5% agarose gel and/or 20% PAGE.

Statistical Analysis

The statistical analysis was performed using Student t test. Probability values less than 0.05 were considered statistically significant. The analysis was performed using SPSS version 10.0 (SPSS, Inc, Chicago, IL), Epi Info version 2002 (Centers for Disease Control and Prevention, Atlanta, GA), and GraphPad Prism 5 software (GraphPad Software, La Jolla, CA).

Results

mRNA expression

TNF-α mRNA expression in the SF of both RA and OA was significantly higher than in the normal PBMCs with a 10.70 and 3.58 fold changes respectively. IL-1β mRNA expression was also increased significantly in SF of both RA and OA than in the normal PBMCs with a 5.61 and 2.42 fold increase respectively. The expression of IL-6 mRNA was increased in RA to 2.58 fold and 1.70 fold in OA patients (Table 3).

	Controls	RA			OA		
Gene	**ΔCt (n=10)**	**ΔCt (n=20)**	**P value**	**Fold Change (2-ΔΔCt)**	**ΔCt (n=10)**	**P value**	**Fold Change (2-ΔΔCt)**
TNF-α	3.74 ± 0.88	0.32 ± 01.07	< 0.0001	10.70	1.9 ± 0.79	< 0.0001	3.58
IL-1β	5.96 ± 0.33	3.47 ± 0.65	< 0.0001	5.61	4.68 ± 0.61	< 0.0001	2.42
IL-6	4.23 ± 0.18	2.86 ± 0.70	< 0.0001	2.58	3.46 ± 0.74	0.005	1.70

Table 3: TNF-α, IL-1β and IL-6 mRNA expression in synovial fluid samples of RA and OA patients.

The relationship between mRNA expressions with the disease severity in RA patients as measured by DAS28 score was also carefully analysed (Table 4). We found TNF-α mRNA and IL-1β mRNA expression was higher in RA patients with high DAS28 Score as compared to RA patients with low/moderate DAS28 score (p = 0.02

and p = 0.009 respectively). However, no significant correlation was observed with IL-6 mRNA expression and DAS28 scoring in RA patients (p = 0.84).

Gene	DAS28 Score	mRNA Expression	Number	p-value
		(mean ± SD)		
TNF-α	5	1.14 ± 1.06	6	0.02
	5	-0.02 ± 0.89	14	
IL-1β	5	4.14 ± 0.73	6	0.009
	5	3.19 ± 0.36	14	
IL-6	5	2.92 ± 0.60	6	0.84
	5	2.85 ± 0.79	14	

Table 4: Association of TNF-α, IL-1β and IL-6 mRNA expression with DAS28 score in RA patients

On comparing the mRNA expression result with different polymorphisms in RA patients (Table 5), we found a significant association of TNF-α -238 GG and IL-1β-511 TT genotypes with increased TNF-α (p = 0.04) and IL-1β mRNA expression (p = 0.03) respectively. However no association was found between TNF-α -308G/A and IL-6-174G/C polymorphisms with the mRNA expression.

Genotypes	mRNA Expression	Number	p-value
	(mean ± SD)		
TNF-α -308			
GG	0.378 ± 1.15	15	0.69
GA	0.164 ± 0.33	5	
TNF-α -238			
GG	0.02 ± 0.98	6	0.04
GA	1.03 ± 0.93	14	
IL-1β -511			
TT	3.08 ± 0.42	7	0.03
CT+CC	3.69 ± 0.69	13	
IL-6 -174			
GG	2.37 ± 1.00	4	0.43
GC	2.92 ± 0.74	16	

Table 5: Association of TNF-α, IL-1β and IL-6 mRNA expression in SF of RA patients with polymorphism.

Discussion

Biomarker-based clinical trials are becoming increasingly important as a means to evaluate the efficacy of novel therapeutic agents for RA. While peripheral blood is readily available for analysis, the target organ (synovium), likely provides the most accurate assessment of disease activity as the synovitis is the hallmark of RA. We have focused our study on gene expression in the synovium, which is a primary source of cytokines.

In our study we observed TNF-α, IL-1β and IL-6 mRNA expression in the SF of RA patients was highly increased with a 10.70 fold, 5.61 fold and 2.58 fold respectively as compared to normal PBMCs. Also, a higher fold increase in the TNF-α and IL-1β mRNA expression was observed in patients with high DAS28 score. Our results revealed the expression of TNF-α mRNA was highest followed by IL-1β mRNA followed by IL-6 mRNA.

Increased hyperplasia of the synovial membrane imposed by TNF-α, IL-1β and IL-6 proinflammatory cytokines has been suggested to play a crucial role in the pathogenesis and progression of RA. In rheumatoid arthritis, joint pathology has been shown to be associated with high IL1-β and TNF-α production for RA so far [5,17,18] thereby supporting our finding of higher fold expression of these two cytokines in patients with higher DAS28 Score. Studies have shown that IL-1β in SF by acting on rheumatoid synovial cells stimulate the release of collagenase and prostaglandin E2 and induce both cartilage degradation [19] and bone resorption [20]. The probable importance of IL-1β in RA has been reinforced by the capacity of intra-articular administration of IL-1β in rabbits to reproduce many of the findings of antigen induced arthritis [21] and by the detection of IL-1β receptors on porcine chondrocytes and synovial fibroblasts [22]. TNF-α has also been shown to stimulate the production of prostaglandin E2 and collagenase by synovial fibroblasts [23] and accelerate the degradation of proteoglycans [24]. As both IL-Iβ and TNF-α have been reported to induce bone resorption [20], cartilage degradation [24] and collagenase production [25,26] they may contribute to the severity of RA.

In a study the analysis of synovial gene expression profiles prior to the start of infliximab (anti-TNF) therapy suggested that patients with features of an activated immune status in their tissue compartment are more likely to benefit from anti-TNF treatment based on ΔDAS28 response criteria [27,28]. Similar findings were reported for baseline serum markers associated with responsiveness [29]. These findings suggest that knowing the cytokine mRNA expression profile of patient may help prioritize the treatment for individual patient.

On studying the relation between mRNA expression and promoter polymorphisms, we observed the total TNF-α mRNA was expressed at significantly higher levels in RA patients that were the carriers of TNF-α -238GG genotype. In a meta-analysis by Young Ho Lee et al. an association between treatment responses to infliximab, a TNF-α blocker and the TNF-α-238 G/A polymorphism has been revealed [30] suggesting the role of polymorphism in affecting the TNF-α levels. Our study also indicated that the total IL-1β mRNA was expressed at significantly higher levels in RA patients that were the carriers of IL-1β-511 'T' allele. A previous study carried out to study the effects of IL1β-511T/C polymorphism has reported that the 'T' allele is responsible for up regulating the expression of IL-1β by about 2-3 folds than the -511'C counterpart [31]. Our results are in tune with this study by suggesting that the presence of 'T' allele in the 5′-promoter region of the IL-1β results in increased IL-1β production and hence increase susceptibility to RA.

The comparative analysis of TNF-α and IL-1β mRNA expression in the SF of OA patients revealed a 3.58 and 2.42 fold increased expression as compared to 10.70 and 5.61 fold increased expression in RA respectively. However, a mild but significant increase~1.70 was observed in the expression of IL-6 mRNA in OA as compared to 2.58

fold increased expression in RA patients. Our study revealed the difference in cytokine gene expression between RA and OA is more quantitative than qualitative suggesting more intense inflammatory nature of RA than OA. Our study is also supported by previous studies have demonstrated that synovial fluid from RA patients contains higher levels of IL-1β [32], IL-6 [33] and TNF-α [34] than synovial fluid from OA patients. Earlier immunohistochemistry studies reveal, a higher number of cells in RA synovial tissue express IL-1β, IL-6, TNF-α [35], and IL-8 [36] than in OA synovial tissue. Similar results were obtained by Sanna et al. with statistically significant differences between RA and OA synovial extracts for IL-1β, IL-6, IL-8, and TNF-α [37]. Comparative analysis of synovial tissue specimen from RA and OA patients by certain other studies have also revealed that these diseases are characterized by distinct synovial gene signatures [38-44). These studies have suggested that genes involved in the adaptive immunity (B and T cell regulation) were upregulated in RA tissues confirming increased infiltration of T cells and B cells in the rheumatoid synovium compared to OA. A study performed by Huber and colleagues identified three pathways with significantly higher variances in RA (e.g. B-cell receptor signalling and vascular endothelial growth factor signalling) compared to OA [43]. Functionally, the majority of the identified pathways are involved in the regulation of inflammation, proliferation, cell survival and angiogenesis. Additional comparative analyses of synovial biopsy tissue from patients with RA, OA and systemic lupus erythematosus (SLE) confirmed and extended observations that distinct diseases were characterized by distinct molecular synovial signatures [44,45]. Our findings also highlight the differences between the RA and OA, the fact that RA is associated with intense inflammatory nature than OA.

Conclusion

We conclude that RA is associated with the high expression of TNF-α, IL-1β and IL-6 cytokines supporting the important role played by these cytokines in the pathogenesis of RA. Also, the increased expression of TNF-α and IL-1β is associated with more severe form of disease. Moreover, the TNF-α-238 GG and IL-1β-511 TT genotypes are susceptibility markers to RA as these genotypes are associated with increased mRNA expression of the respective genes. Furthermore, the differences in cytokine gene expression between RA and OA are more quantitative than qualitative, therefore, suggesting a more intense inflammatory nature of RA than OA.

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