Expression of Tristetraprolin (G0S24) mRNA, a Regulator of Tumor Necrosis Factor-α Production, in Synovial Tissues of Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To determine the significance of tristetraprolin (TTP) gene expression in synovial tissues of patients with rheumatoid arthritis (RA).

Methods. Gene expression was examined in synovial tissue and peripheral blood lymphocytes of a patient with RA by differential display-polymerase chain reaction (PCR). One of the identified genes, TTP, was selected for further analysis. cDNA was prepared from synovial tissues of 22 patients with RA and 22 with osteoarthritis (OA). Expression of TTP and tumor necrosis factor-α (TNF-α) genes was measured by TaqMan real-time semiquantification PCR. In RA samples, expression of TTP mRNA was compared with TNF-α mRNA, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and steroid and/or disease modifying antirheumatic drug use.

Results. Expression of TTP gene was significantly higher in synovial tissues of RA patients than in OA. There was no apparent relationship between expression of TTP and TNF-α genes. TTP gene expression had a tendency to be inversely correlated with serum CRP, measured immediately before surgery. In addition, CRP was higher in patients with a low TTP/TNF-α gene expression ratio ($p = 0.0071$, Spearman rank correlation).

Conclusion. A low TTP/TNF-α gene expression ratio could indicate failure of RA patients to produce adequate amounts of TTP in response to increased TNF-α production. Inappropriate TTP production may be one factor that contributes to higher RA disease activity. (J Rheumatol 2004;31:1044–9)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
DIFFERENTIAL DISPLAY
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Rheumatoid arthritis (RA) is a chronic disorder of unknown pathogenesis associated with polyarthropathy. Persisting inflammation in the joints may lead to total destruction of joints, causing a great reduction of quality of life of patients with RA. Among a number of cytokines involved in the pathogenesis of arthritis in RA, tumor necrosis factor-α (TNF-α) is one of the most important. TNF-α is produced by macrophages and synovial cells, and induces an array of inflammatory cytokines, chemokines, adhesive molecules, and proteinases. TNF-α is present in synovial fluids of patients with RA[1-3], but not in those of patients with osteoarthritis (OA)[3]. Interestingly, while transgenic mice carrying the complete human TNF-α gene develop normally, transgenic mice carrying a 3' modified human TNF-α transgene have dysregulated TNF-α expression and develop chronic inflammatory arthritis resembling human RA, which could be completely prevented by treatment with anti-TNF-α antibodies[4]. Thus, dysregulated TNF-α production is responsible for arthritis in this animal model, and the 3' region of the TNF-α gene is important for properly controlled production of this cytokine. Subsequent studies and the recent introduction of anti-TNF-α therapies have shown that this cytokine plays a key role in the pathogenesis of human RA, and is a potential target for therapy. However, a better understanding of the disease is needed to develop new methods of therapies, and to prevent total destruction of the joints.

To investigate the molecular mechanisms involved in the pathogenesis of RA, and to search for possible targets for RA-specific therapies, we focused on identifying genes that are specifically expressed in synovial tissues of patients with RA. We used the differential display-polymerase chain reaction (DD-PCR) method to identify known and unknown genes specifically expressed in a given tissue[5,6]. Among a

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number of candidate genes identified, we focused on tristetraprolin (TTP), since TTP is an intracellular protein involved in the degradation of TNF-α mRNA. TTP binds to the 3' untranslated region (3'-UTR) of the TNF-α mRNA, and induces its instability, as reviewed by Blackshear[7]. Thus, TTP is a natural regulator of TNF-α production[8], and theoretically may have a protective role in the inflammatory process of RA. We report that TTP mRNA is highly expressed in RA synovial tissues, compared to its expression in OA synovial tissues. We also examined the relationships between TTP gene expression and TNF-α gene expression, serum C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and rheumatoid factor (RF) in patients with RA before surgery.

MATERIALS AND METHODS

Differential display-PCR. Samples from a patient who fulfilled the American College of Rheumatology criteria for classification of RA[9] were used with written informed consent. Total RNA was extracted from synovial tissue from the operated knee joint and Ficoll (Amersham Bioscience Japan, Tokyo, Japan) separated peripheral blood mononuclear cells (PBMC), using Isogen (Nippon Gene, Tokyo, Japan), following the manufacturer's recommended protocol. RNA was reverse transcribed to cDNA using a RevertAid first-strand cDNA synthesis kit (Fermentas, Hanover, MD, USA) following the manufacturer's instructions. Gene expression in synovial and PBMC samples was compared by DD-PCR. DD-PCR was carried out using the delta-differential display kit (Clontech, Palo Alto, CA, USA) as described in the manual, with the exception that we visualized the bands on the polyacrylamide gel by silver stain. Bands visible only in amplified products from the synovial sample were excised from the gel, and were cloned into pCRII vectors (Invitrogen Japan, Tokyo, Japan) and sequenced using standard protocols by a sequencer (ABI Prism 310 gene analyzer; Perkin Elmer, Wellesley, MA, USA). A total of 105 genes considered to be specifically expressed by the synovial tissue sample were analyzed in this way. Sequences were searched for homologies by the NCBI BLAST system on the Internet [cited January 20, 2004; available from http://www.ncbi.nih.gov/blast/].

Semiquantitative PCR. Genes identified by DD-PCR were studied in the literature for possible relationships with cell proliferation or survival, inflammation, and immunological functions such as antibody or cytokine production. TTP and several other genes that were considered of possible interest were selected and their expression in synovial tissues from 22 patients with RA and 22 with OA were measured using the TaqMan PCR real-time semiquantification method. All samples were taken with donors' written informed consent, and the study was approved by the local ethical committee. Sera from these patients were obtained 0–2 days before surgery. At the time of serum sampling, patients were taking 0–10 mg/day prednisolone and 0–3 disease modifying antirheumatic drugs (DMARD) including methotrexate (8 mg/week maximum), salazosulfapyridine (1000 mg/day maximum), and 100–200 mg/day bucillamine, a DMARD commonly used in Japan. Total RNA was extracted from synovial tissues from operated knee joints, and cDNA was synthesized using the RevertAid first-strand cDNA synthesis kit. Synthesized cDNA samples were amplified with specific primers and fluorescence-labeled specific probes for the gene of interest, and accumulation of amplified products was monitored with an ABI 7700 sequence detector (Applied Biosystems Japan, Tokyo, Japan). PCR mixture (qPCR Mastermix) was purchased from Eurogentec (Seraing, Belgium); magnesium concentration was 5 mM final, primer concentrations 200 nM final, and the probe concentration was 100 nM final. Thermal cycler conditions were 50°C for 2 min, 95°C for 10 min, then 55 cycles of 95°C for 15 s, and 60°C for 1 min. Standard samples were included and standard curves for the gene of interest and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were generated in every assay. All measurements were done in triplicate. The level of gene expression was calculated from the standard curve, compensated with that of GAPDH gene, and was expressed as a ratio. The sequences of specific primers and probes are as follows: TTP forward: 5'GGCGACTCCCCATCTTCAAT3', TTP probe: 5'TCTGAGTGACAAAGTGACTGCCCGGTC3', TTP reverse: 5'CAGTGCAAGAGACGTGGCTC3'; mortality factor 4 forward: 5'TGCCGAAATTCTTG CAGATT3', mortality factor 4 probe: 5'TCCCGATGCACCCATGTCCC3', mortality factor 4 reverse: 5'AGATGTGGCACTCCATACACC3'; CD63 forward: 5'TTCTTGCTCTACGTCCTCCTG3', CD63 probe: TGGCCTTTTGCGCCTGTGC, CD63 reverse: 5'CACGGCAATCAGTCCCAC3', Ki autoantigen forward: 5'AAAGCCGCAGACCCTGG3', Ki autoantigen probe: 5'CTCTGGTGGCTAGGGATGTACTCATGCTCA3', Ki autoantigen reverse: 5'TGTCCAAGCGTGCACACAT3'; TNF-α forward: 5'TGGAGAAGGGTGACCGACTC3', TNF-α probe: 5'CGCTGAGATCAATCGGCCCGACTAT3', and TNF-α reverse: 5'TCCTCACAGGGCAATGATCC3'. Primers and the probe for GAPDH were purchased from Applied Biosystems.

The Mann-Whitney U test was used to compare gene expressions in RA and OA samples. Spearman's rank coefficient was used to examine the relationship between expressions of 2 different genes, and the relationship between gene expressions and CRP, ESR, or RF. $P < 0.05$ was considered significant.

RESULTS

High expression of TTP gene in RA synovial tissue. Using DD-PCR, we selected and sequenced 105 genes from samples of one patient with RA. Identified genes were: complement C1r, ferritin L chain, collagen type 1, chitinase, TTP (G0S24), epididymal secretory protein, cytosolic selenium-dependent glutathione peroxidase, ubiquinol-cytochrome c reductase binding protein, NADH dehydrogenase subunit 2, 17-beta-hydroxysteroid dehydrogenase, IgG1 heavy chain, Ki autoantigen, CD63, sphingolipid activator, mortality factor 4, p47, cytochrome P450 IIIA4, and immunoglobulin-binding protein 1, others being either genes of unknown function or not in the databases. Among these genes, we focused on TTP, originally reported as a member of a set of genes (putative G0/G1 switch regulatory genes) that are expressed transiently on human PBMC, after addition of lectin or cycloheximide[10]. CD63, immunoglobulin binding protein 1, mortality factor 4, and Ki nuclear autoantigen genes were arbitrarily chosen for real-time PCR analyses, since we considered that the functions of these genes may also be related to the pathogenesis of RA. To determine whether TTP gene expression is indeed enhanced in RA synovial tissues, expression of TTP gene in synovial tissues of 22 RA and 22 OA patients was measured by TaqMan real-time semiquantification PCR. Expression of TTP gene was significantly higher in RA synovial tissues compared to OA synovial tissues ($p = 0.0128$, Mann-Whitney U test; Figure 1). Expression of CD63, immunoglobulin binding protein 1, mortality factor 4, and Ki nuclear autoantigen genes showed no significant differences between RA and OA synovium samples (data not shown).

TTP/TNF-α gene expression ratio correlates inversely with CRP. To determine whether the expression level of TTP

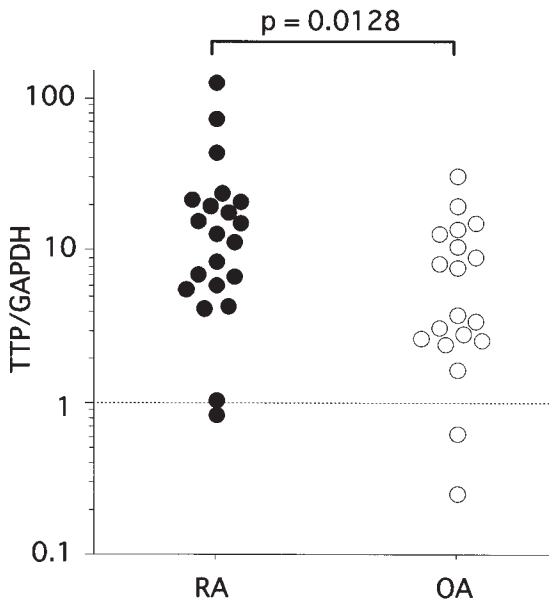

Figure 1. Expression of TTP mRNA in synovial tissues of patients with RA and OA. P value calculated by Mann-Whitney U test.

gene directly correlated with TNF-α gene, we analyzed expression of TNF-α gene using real-time semiquantification PCR, and the results were compared to the expression of TTP gene. Standard curves were generated that indicated the validity of the real-time semiquantification PCR used in this study (Figure 2). There was no significant correlation between the expression of TNF-α and TTP genes (Figure 3). To further examine the relevance of TTP gene expression in RA, we determined the relationships between TTP gene expression and CRP, ESR, or RF. CRP tended to be higher in patients with higher TNF-α gene expression, but without statistical significance (r = 0.306, p = 0.2071, Spearman rank correlation; Figure 4A). CRP also tended to be higher in patients with lower TTP gene expression, although this was statistically insignificant (r = –0.429, p = 0.0771, Spearman rank correlation; Figure 4B). However, when the ratio of TNF-α and TTP gene expression was calculated, and compared with CRP, a significant inverse relationship was observed (r = –0.653, p = 0.0071, Spearman rank correlation; Figure 5A). ESR showed a similar trend, although statistically insignificant (r = –0.441, p = 0.0692, Spearman

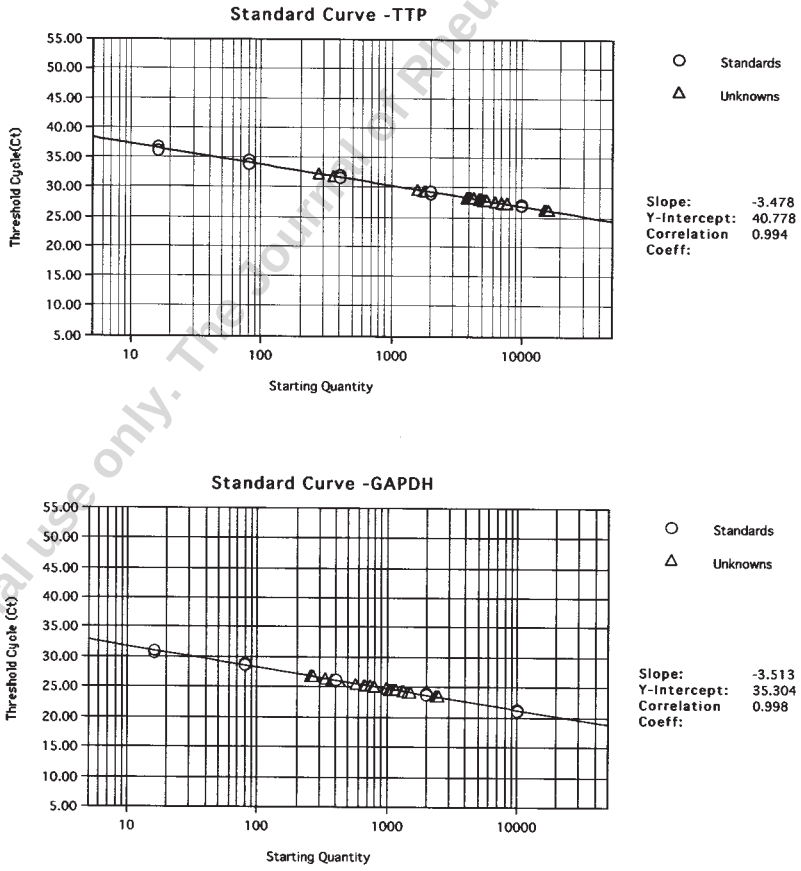

Figure 2. Standard curves generated for real-time semiquantification PCR. Upper panel: TTP; lower panel: GAPDH. All measurements were in triplicate, and sample values that were not within the standard curve were diluted and remeasured. Similar standard curves were obtained for all PCR analyses (data not shown).

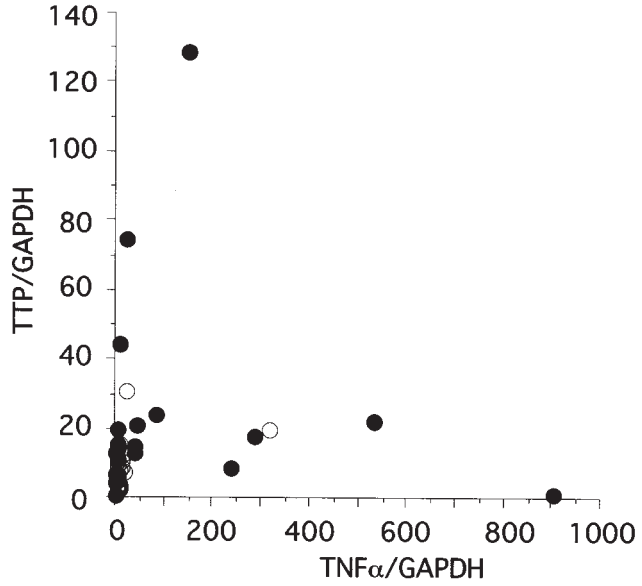

Figure 3. Relationship between expression of TTP mRNA and TNF-α mRNA. ●: RA, ○: OA.

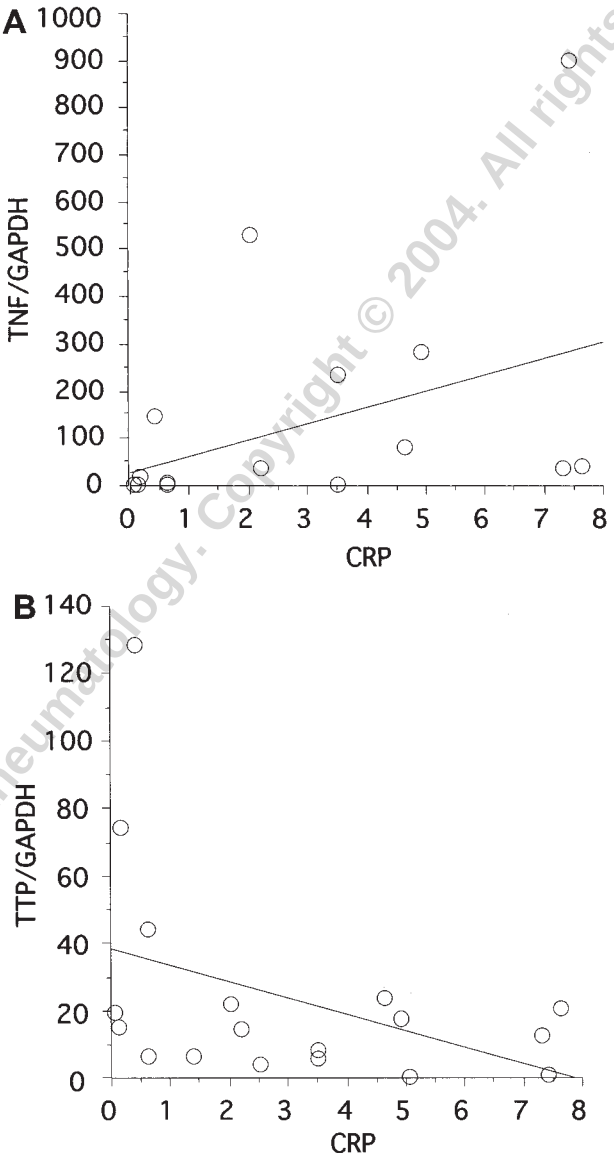

Figure 4. Relationship between serum CRP and expressions of TNF-α mRNA and TTP mRNA. A. CRP and TNF-α mRNA (p = 0.2071, Spearman rank correlation). B. CRP and TTP mRNA (p = 0.0771, Spearman rank correlation).

rank correlation; Figure 5B). Steroid and/or DMARD usage and RF did not significantly correlate with TTP gene expression (data not shown).

DISCUSSION

Our results suggest that TTP gene expression may play an important role in RA disease activity. Elucidation of the role of TTP in the pathogenesis of RA may be helpful in the search for new therapies for RA.

DD-PCR is a powerful tool for identifying genes that are highly expressed in one of the 2 samples compared. Using this technique, we identified TTP as a possible candidate gene whose expression may have a role in the pathogenesis of RA. Our strategy was to compare samples from the same patient, to avoid detecting differences between individuals, then use real-time PCR to determine whether the identified genes are highly expressed in synovial tissues from RA patients compared to those from OA patients. Applying DD-PCR to synovial tissues from RA and OA samples is an another possible approach, which may give us a completely different result.

We found that the expression of TTP gene was significantly higher in synovial tissues from RA patients than those from OA patients. In addition, in RA patients the magnitude of TTP gene expression was lower in patients with higher serum CRP, an inflammation marker commonly used to monitor RA activity. Although this relationship was not quite statistically significant (p = 0.0771), a significant relationship was observed when TNF-α gene expression was taken into account (p = 0.0071). A similar relationship for ESR was also observed (p = 0.0692), although it was not statistically significant. The finding that TTP gene expression is higher in RA samples is not merely due to more inflammatory cells in RA samples than OA samples, although this may partly account for the finding. If enhanced TTP gene expression reflects only the increment of inflammatory cells in the synovium, one would expect that, in RA synovium, TTP gene expression would correlate with TNF-α gene expression and inflammatory markers. Such a relationship was not observed. Instead, TTP gene expression seemed to be lower in synovium from patients with more active inflammation.

TTP protein binds directly to the AU-rich element in the

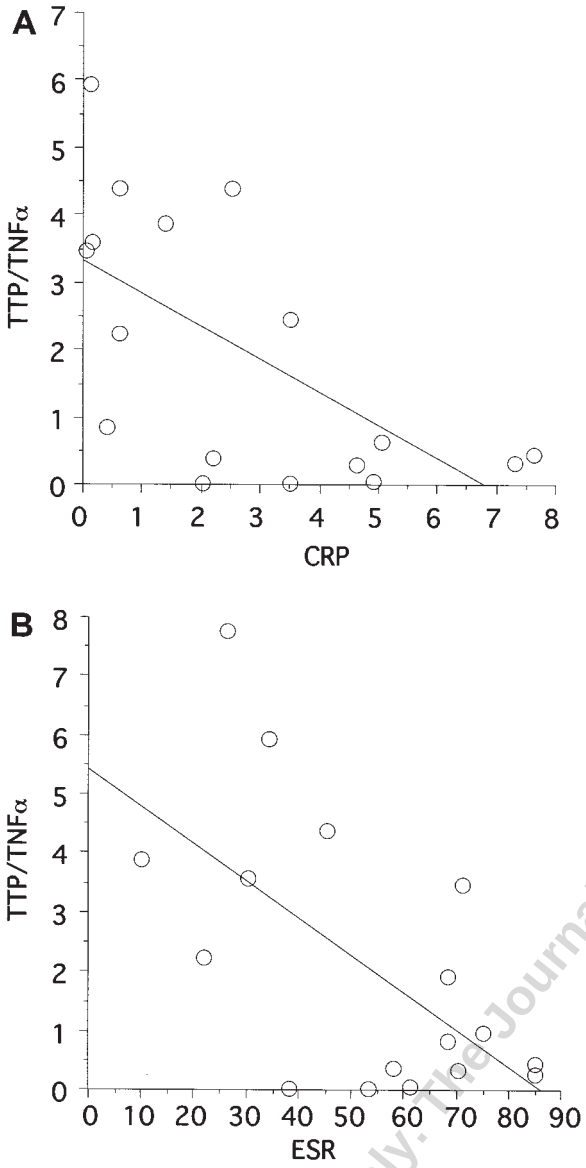

Figure 5. Relationship between expression of TTP/TNF-α gene expression ratio and CRP or ESR. A. TTP/TNF-α gene expression ratio and CRP (r = –0.653, p = 0.0071, Spearman rank correlation). B. TTP/TNF-α gene expression ratio and ESR (r = –0.441, p = 0.0692, Spearman rank correlation).

3'-UTR of TNF-α mRNA. Since binding of TTP leads to instability of TNF-α mRNA[8,11], it is conceivable that TTP is a physiological regulator of TNF-α production. It has been reported that TTP knockout mice develop erosive arthritis, dermatitis, conjunctivitis, glomerular mesangial thickening, and high titers of anti-DNA and antinuclear antibodies[12]. The pathological findings of erosive arthritis were similar to those observed in human RA. The phenotype seen in TTP knockout mice was reversed by administration of anti-TNF-α antibody[12], suggesting that TNF-α overproduction plays a major role in the pathogenesis of arthritis and other symptoms in these mice. In addition, TTP binds to AU-rich elements on mRNA of other genes such as interleukin 2 (IL-2), IL-3, granulocyte macrophage-colony stimulating factor (GM-CSF), and c-fos genes[13]. In T cells, TTP protein is increased after cell stimulation, and replaces HuA, an AU-rich element-binding protein, which stabilizes mRNA[13]. Thus, TTP is a natural suppressor of excessive cytokine production. We speculate that in patients with RA, the presence of TNF-α in synovial tissues could lead to enhancement of TTP production, and that patients who lack the potential to produce adequate amounts of TTP may develop more severe disease, leading to more intense inflammation and joint destruction. The level of TTP gene expression in the synovial tissue may be important in determining the disease activity of RA, making it a possible candidate for future therapeutic targets.

The lack of correlation between TTP mRNA and the amount of TNF-α mRNA does not necessarily indicate that the expression of TTP mRNA is not an important factor in TNF-α production in the synovial tissue of patients with RA. Physiologically, overproduction of TNF-α would lead to enhanced production of TTP, which in turn would suppress TNF-α production. On the other hand, inadequate production of TTP in RA patients would lead to overproduction of TNF-α, affecting the course of RA. Thus, the relationship between TTP mRNA production and TNF-α production may differ among individual patients. Indeed, the significant inverse relationship between TTP/TNF-α gene expression ratio and CRP suggests that an adequate TTP response could help in the control of inflammation that occurs in RA synovial tissues. Brooks, *et al*[14] reported the presence of TTP protein in RA synovial tissue. They also reported that human TTP binds to the 3'-UTR of TNF-α mRNA and reduces reporter gene expression. Their study emphasized the potential importance of posttranscriptional regulation of TNF-α production in the pathogenesis of RA. Our findings add support to their conclusions. Our study was done using synovial samples; it may be of interest to investigate whether a similar relationship can be observed in peripheral blood samples from RA patients. Studies at the protein level also should be done.

Our study does not indicate that elevated TTP gene expression is an RA-specific phenomenon. TNF-α is known to play important roles in various inflammatory diseases such as psoriatic arthritis, ankylosing spondylitis, Behçet's disease, and Crohn's disease. It is possible that interindividual differences in TTP production affect the arthritic condition in these diseases. TNF-α expression in cartilages from patients with OA has also been reported[15]. Thus, TNF-α and hence TTP may have a role in the progression of OA as well as RA. Whether TTP gene expression is elevated in OA synovium compared to healthy synovium, and whether this has implications for the severity of OA, are challenging

questions because of the difficulties of obtaining healthy synovial samples and assessing the severity of OA.

We speculate that TTP production is induced in patients with RA as a negative feedback of TNF-α overproduction, and that TTP may affect the course of RA by reducing the production of TNF-α in the synovium. Our current hypothesis is that compounds that mimic the properties of TTP or that enhance TTP gene expression, or even TTP gene therapies, may serve as a tool for controlling joint inflammation and destruction in severe RA.

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