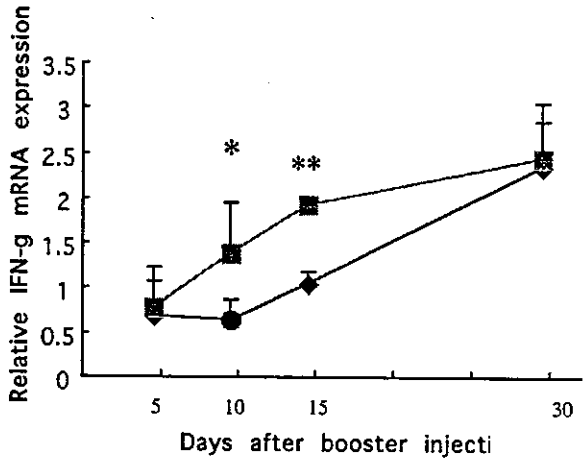
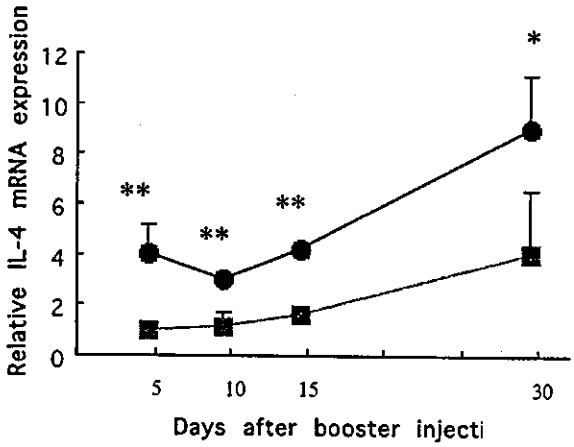
Figure 5

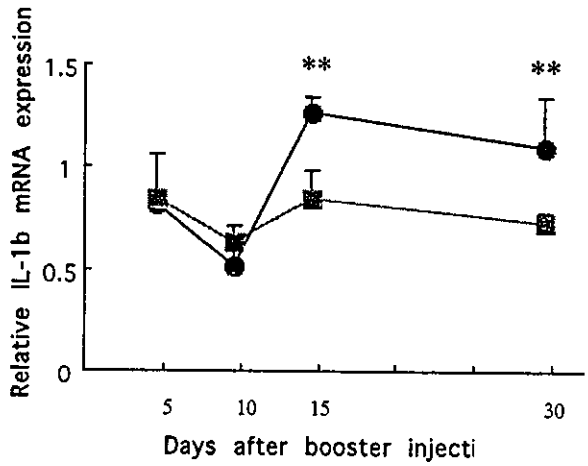
(a)


Relative IFN-g mRNA expression
3.5
3
2.5
2
1.5
1
0.5
0
*
**
5
10
15
30
Days after booster injecti

(b)


Relative IL-4 mRNA expression
12
10
8
6
4
2
0
**
**
**
*
5
10
15
30
Days after booster injecti

(c)


Relative IL-1b mRNA expression
1.5
1
0.5
0
**
**
5
10
15
30
Days after booster injecti

Analysis of abnormally expressed genes in synovium from patients with rheumatoid arthritis using a column gel electrophoresis-coupled subtractive hybridization technique

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Abstract. Rheumatoid arthritis (RA) is a chronic disease of unknown pathogenesis. To identify abnormally expressed genes in synovium from RA patients, we performed column gel electrophoresis-coupled subtractive hybridization (CGESH). CGESH is a newly developed subtractive hybridization technique to achieve sufficient enrichment of DNA sequences. CGESH was performed using restricted enzyme digested cDNA synthesized from mRNA of synovial tissues from one RA patient and one osteoarthritis (OA) patient. The obtained subtraction libraries (RA-OA) were screened by dot blot hybridization. The clones showing higher hybridization with the RA-OA probe were identified by sequence analysis and homology search. Their DNA sequencing revealed that the genes of HLA-DRB1, sequestosome 1, elongation factor 1α were included. Furthermore, a functionally unknown gene (FLJ00133) was also identified. It is reported that sequestosome 1 is a scaffold in the signal transduction of TNFα and interleukin 1, which are the important cytokines involved in the pathogenesis of RA. It is possible that other genes identified by the CGESH technique would be associated with the pathogenesis of RA, although there is no direct evidence yet. Our results imply that the CGESH technique is a useful tool to detect genes involved in the pathogenesis of RA. Further investigation of the functional roles of candidate genes should shed light on the pathogenesis of RA.

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Abbreviations: RA, rheumatoid arthritis; OA, osteoarthritis; CGESH, column gel electrophoresis-coupled subtractive hybridization; IGCR, in-gel competitive reassociation

Key words: rheumatoid arthritis, column gel electrophoresis-coupled subtractive hybridization

Introduction

Rheumatoid arthritis (RA) is a chronic disorder of unknown pathogenesis, that causes multiple joint destruction. Although new medications such as TNFα blocking agents are showing promising effects, deeper understanding of the disease is needed to overcome this possibly tragic condition. In order to understand the molecular mechanisms involved in the pathogenesis of RA, and to search for a possible target of RA-specific therapies, we wished to identify genes specifically expressed in the synovial tissues of patients with RA.

In the past, various strategies have been developed to examine tissue-specific differences in gene expression. We used column gel electrophoresis-coupled subtractive hybridization (CGESH) technique (1) for this purpose. The original technique which forms the basis of the CGESH, namely in-gel competitive reassociation (IGCR), was developed by Yokota *et al* (2,3). In this technique, electrophoresis size-separation of mixed restriction digested DNA samples to be compared and *in situ* denaturation-hybridization steps are carried out sequentially. These in-gel processes provide unique technical features that other batch subtractive hybridization techniques cannot. Firstly, size-dependent sequential fractionation of restriction digested DNA fragments results in an enormous reduction of sample complexity. This makes highly efficient hybridization possible. Secondly, closely related sequences will be physically separated and would not interfere with each other provided that they have different restriction fragment sizes. To overcome the drawbacks of this technique, such as its being time-consuming and too complex, Ozawa *et al* (1) made modifications to simplify the strategy of IGCR and developed the CGESH technique. While improving IGCR, this technique was originally used to detect differences between genome DNA samples (4,5), but if applied to cDNA samples, in addition to the advantages described above, it may

Table I. Oligonucleotides used for the CGESH experiment.

Name	Sequence	*HinP1* I site reconstitution
A20	5'-GACTGTCAAGGATCCCTTAG-3'	Yes
A10	3'-AGGGAATCGC-5'	
B20	5'-GATCGTGACAAGCTTCTGAC-3'	No
B10	3'-GAAGACTGGC-5'	
C20	5'-CAGACTCTGGAATTCGCATG-3'	Yes
C10	3'-AAGCGTACGC-5'	
Bio-C20	5'-biotin-CAGACTCTGGAATTCGCATG-3'	

CGESH, column gel electrophoresis-coupled subtractive hybridization. Underlining indicates *HinP1* I restriction enzyme site.

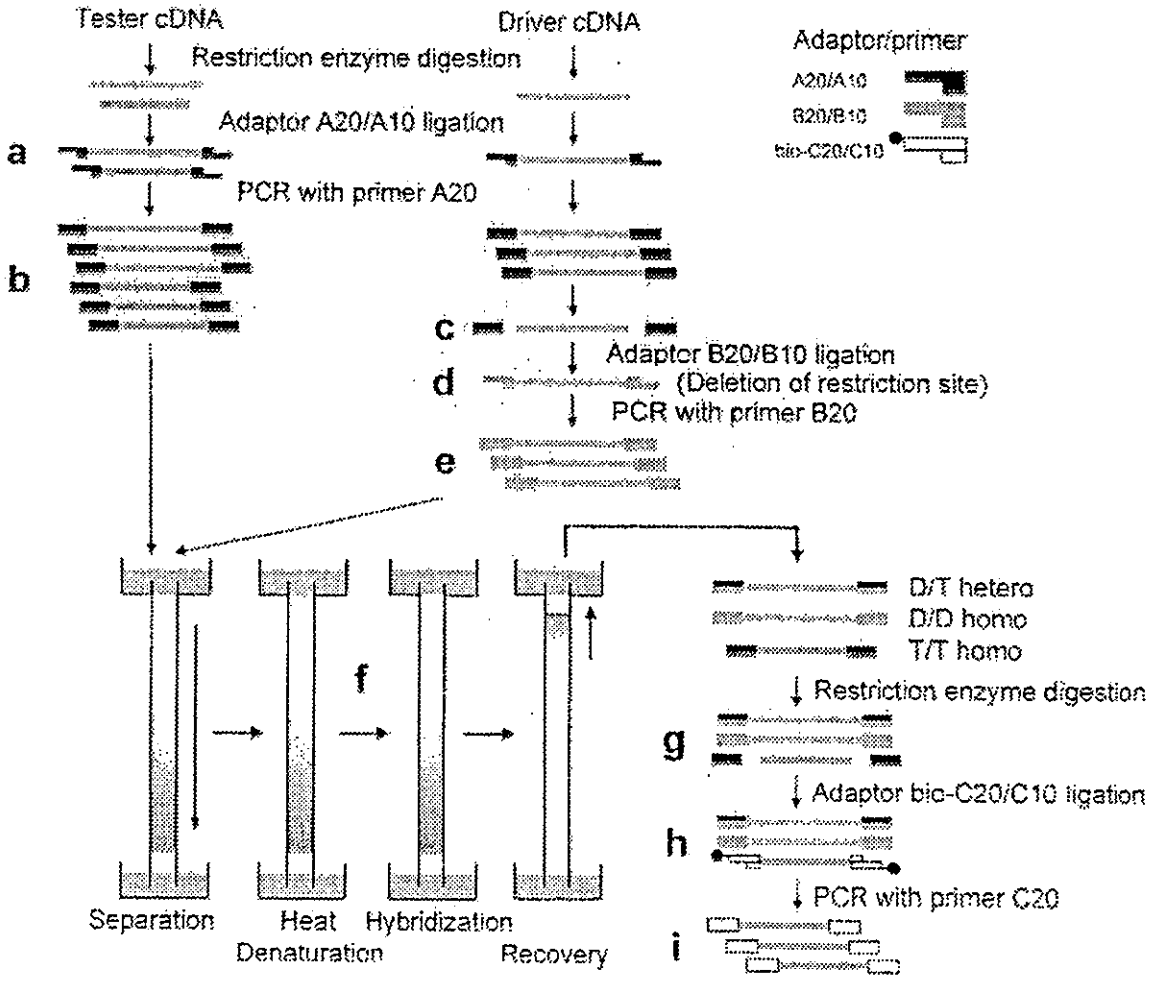

Figure 1. Summarized illustration of the steps of the column gel electrophoresis-coupled subtractive hybridization (CGESH) technique. *HinP1* I-digested driver or tester cDNA fragments were ligated with adaptor A20/A10, which preserves the *HinP1* I restriction enzyme site (a) and preamplified by PCR using primer A20 (b). Adaptor was removed from driver cDNA fragments by *HinP1* I digestion (c) and replaced with a new adaptor B20/B10, which does not reconstitute the *HinP1* I recognition site (d), and reamplified by PCR using primer B20 (e). Gel electrophoresis, denaturation, hybridization and recovery were carried out sequentially (f). Only perfectly reannealed tester-derived fragments can reconstitute the *HinP1* I site and the A20/A10 adaptor sequence are removed again by restriction digestion (g). A new adaptor, consisting of an oligonucleotide pair bio-C20/C10, is attached to both ends of the tester-derived fragments (h) and trapped by Streptavidin Sepharose beads (h). After removing unbound cDNA fragments by washing the beads, specifically captured cDNA fragments were amplified by PCR using primer C20 (i). See Table I for adaptor and primer sequences.

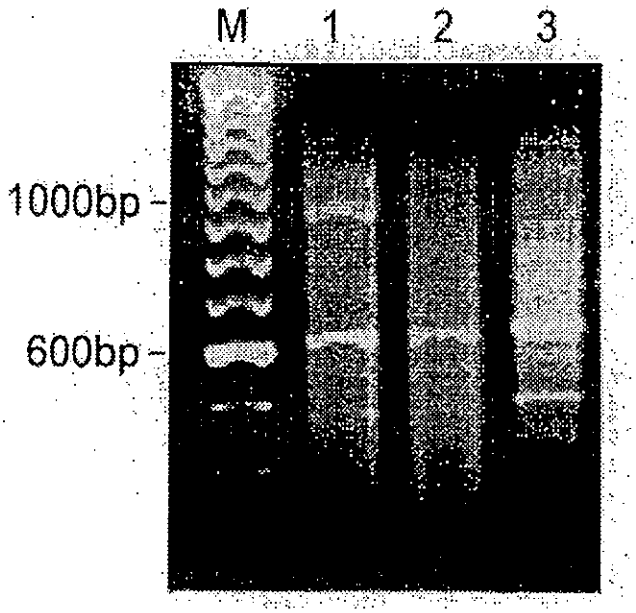

Figure 2. Electrophoresis pattern of the subtracted cDNA. Lane 1, driver cDNA amplified with primer A20, adaptor replaced and amplified with primer B20; Lane 2, tester cDNA amplified with primer A20; Lane 3, product of 1st round subtraction amplified with primer C20; Lane M, 100 bp DNA ladder.

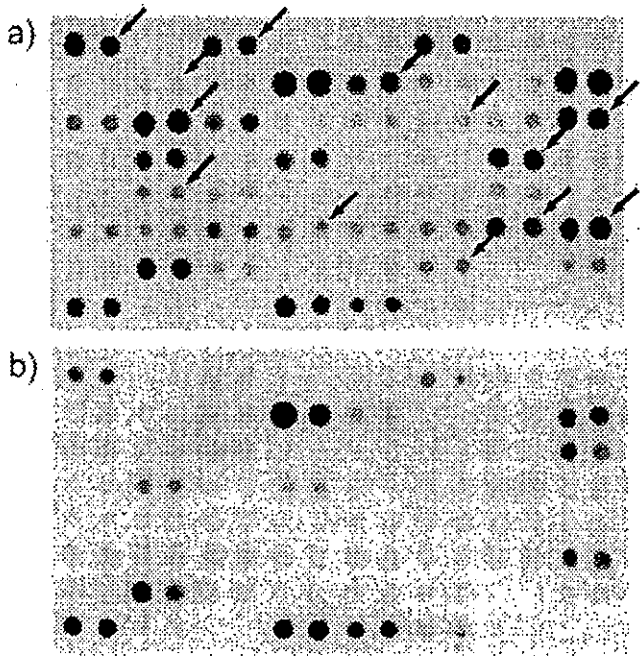

Figure 3. Screening of subtraction library by dot blot hybridization. Two identical cDNA blots of PCR-amplified inserts derived from the subtracted cDNA library were separately hybridized with DIG-labeled subtracted probe (a) and reverse-subtracted probe (b). Arrows indicate clones with a hybridization ratio of 2:1 or larger (RA-OA vs OA-RA) measured by a densitometer.

be possible to detect size variants such as those produced by alternative splicing, which are not detectable by conventional subtraction techniques. Thus, CGESH can be a powerful tool to identify known or unknown genes specifically expressed in a given tissue. Our objective was to detect genes specifically expressed in synovial tissues from RA patients using this newly developed technique.

Materials and methods

Synovial tissue was obtained under informed consent from patients with rheumatoid arthritis (RA) who met the criteria of the American Collage of Rheumatology (6), and patients with osteoarthritis (OA). Total RNA from synovial tissue was isolated using Isogen (Nippon Gene, Tokyo, Japan), and poly $(A)^+$ RNA was isolated from total RNA using a Oligotex-dt30 <Super> mRNA Purification Kit (Takara Bio, Shiga, Japan), following the protocols supplied by the manufacturers. The list of the oligonucleotide primers used in this study is provided in Table I.

CGESH technique. CGESH was performed essentially following the methods described by Ozawa *et al* (1), with some alterations (Fig. 1). Poly $(A)^+$ RNA (1 μg) from synovial tissues from RA or OA patients were subjected to cDNA synthesis. cDNA was synthesized using cDNA synthesis kit (Takara Bio) according to the manufacturer's instructions. cDNA from RA was designated as tester and that from OA as driver. Tester and driver cDNA were digested by a restriction enzyme *HinP1* I (New England Biolabs, Beverly, MA, USA), and ethanol-precipitated. Digested DNA fragments were ligated to oligonucleotide adaptor A20/A10 with T4 DNA ligase (New England Biolabs). The DNA was amplified using primer A20 by PCR using mini cycler PTC-150 (MJ Research, Waltham, MA, USA) under the following cycling conditions: 72°C for 1 min, 30 cycles of 94°C for 1 min and 68°C for 5 min, followed by 68°C for 20 min as a final extension step. Amplified driver DNA was digested with *HinP1* I, ligated with adaptor B20/B10, then amplified by PCR with primer B20 as described above. Tester DNA (10 ng) and driver DNA (1 μg) were mixed with formamide (10% final) to a final amount of 20 μl, and loaded on the top of the formamide-containing poly-acrylamide gel in a glass column (inner diameter: 5 mm; length: 130 mm), and electrophoresis was performed in the presence of a phosphate buffer. After gel separation, the column was incubated at 80°C for 10 min to denature dsDNA fragments in the gel. Then, the temperature was gradually lowered to hybridization temperature (37°C), and kept at 37°C overnight. After hybridization, DNA fragments were recovered by electrophoresis in the reverse direction, and collected by absorbing with Q-sepharose (Amersham Biosciences, Piscataway, CA, USA). Gel-recovered DNA was again digested with *HinP1* I, ligated with biotinylated adaptor bio-C20/C10. Biotinylated adaptor-ligated DNA fragments were absorbed with Streptavidin Sepharose (Amersham Bio-sciences). Amplification reaction was performed by adding the DNA-bound Streptavidin Sepharose slurry into a PCR reaction containing primer C20. The thermal cycling conditions were as described above.

Screening of differential expression. PCR products from the subtracted libraries were subcloned into pCR2.1-TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA). cDNA inserts of the cloned cDNA library were amplified by subjecting an aliquot of the bacterial culture directly to PCR.

Table II. Genes found to be up-regulated in RA compared to OA using the CGESH technique.

Clone No.	Accession No.	Identified gene
H237	NM_002124.1	Major histocompatibility complex, class II, DR ß 1 (HLA-DRB1)
H19, 180	NM_003900.2	Sequestosome 1 (SQSTM1) (p62)
H13, 167	NM_001402.4	Elongation factor 1 α 1 (EEF1A1)
H91, 129, 165, 190, 224, 252	NM_203339.1	Clusterin (CLU)
H95	NM_000852.2	Glutathione S-transferase pi (GSTP1)
H231	XM_059482.6	FLJ00133 protein (FLJ00133)

CGESH, column gel-electrophoresis subtractive hybridization.

Electrophoresis of the amplified product in agarose gels was always done to confirm that all clones were single. The amplified materials were then dot blotted onto nitrocellulose membranes in duplicates. Two identical membranes were prepared, and they were hybridized with either the subtracted probe (RA-OA) or the reverse-subtracted probe (OA-RA). Probes were labeled with a DIG DNA labeling kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's recommended protocol. Hybridization and detection via a chemiluminescence reaction were carried out employing a DIG luminescent detection kit (Roche diagnostics), according to the supplier's standard protocol. The amount of hybridization was semiquantified with a densitometer (LAS-3000; Fuji Photo Film, Tokyo, Japan). Clones with a hybridization ratio of 2:1 or larger (RA-OA vs OA-RA) were selected and plasmids were purified using a Qiagen plasmid mini kit (Qiagen, Hilden, Germany). Inserts were sequenced by chain termination reaction using an automated sequencer (ABI prism 310 genetic analyzer; Perkin Elmer, Wellesley, MA, USA). Nucleic acid homology searches were performed using the BLAST program at the National Center of Biotechnology Information (National Institutes of Health).

Results and Discussion

Using CGESH, we constructed one subtracted library using RA synovial tissue sample as tester and OA synovial sample as driver (RA-OA) (Fig. 2). In parallel, a reverse-subtracted library was prepared (OA-RA) to make a control probe for dot blot hybridization screening. This subtracted library was cloned, and a total of 273 clones were obtained. Thirteen clones with a hybridization ratio of 2:1 or larger (RA-OA vs OA-RA) measured by a densitometer in the dot blot hybridization were selected (Fig. 3). These clones were sequenced, and 6 genes (HLA-DRB1, sequestosome 1, elongation factor 1 α 1, clusterin, glutathione S-transferase pi and FLJ00133) were identified. Six clones (H91, 129, 165, 190, 224, 252) were derived from the clusterin gene, two clones (H19, 180) were sequestosome 1, and the other two clones (H13, 167) were elongation factor 1 α 1. This suggests that the dot blot hybridization worked well for selecting genes from the CGESH library, although the true frequency of this gene expression was not clarified. The sequences of the clones were identical to those submitted in the BLAST program (Table II).

HLA-DRB1, a class II MHC molecule, is important for antigen presentation, and would be important in the pathogenesis of RA (7,8). Increased expression of MHC class II molecules in RA synovial tissue is also reported (9). Sequestome 1, also known as p62, is reported to be a scaffold in the signal transduction of TNFα and interleukin-1 leading to the activation of NF-κB (10). These two important cytokines are well known to be involved in the pathogenesis of RA (11). Recently, it has been shown that p62 is an important mediator during osteoclastogenesis and induced bone remodeling (12). p62 may have a function in the joint destruction process in RA patients. Elongation factor 1 α 1 is reported to increase at the mRNA level in patients with RA synovial samples compared to OA synovial samples (13). Autoantibody against this protein has been reported to be present in patients with Felty's syndrome (14). The function of this protein is not fully understood, but may have a role in the pathogenesis of RA. Both clusterin and glutathione S-transferase pi genes are reported to be involved in the signal transduction of NF-κB (15,16). FLJ00133, which was identified in the NEDO human cDNA sequencing project at Kazusa DNA Research Institute (17), was also one of the genes identified in our study. The function of this gene product is yet to be clarified.

We used the dot hybridization technique to efficiently screen the clones obtained by CGESH. This method allows us to easily identify genes with higher expression in the tester sample than in the driver. However, it is true that dot blot hybridization cannot identify size variants such as alternatively spliced gene products. CGESH is theoretically potent for screening such variants because a size fractionation step is included in the procedure. A large number of clones that were considered non-specific in dot blot assays may contain such variants. By combining an efficient method to identify such clones, we would be able to make the most of the CGESH technique and be able to show that it is a powerful tool for identifying genes that are differently expressed between two tissues.

In summary, we have identified a number of genes possibly involved in the pathogenesis of RA by means of the CGESH technique, with RA samples as tester and OA samples as driver.

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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
TRISTETRAPROLIN
TUMOR NECROSIS FACTOR-α

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 manufucturer'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

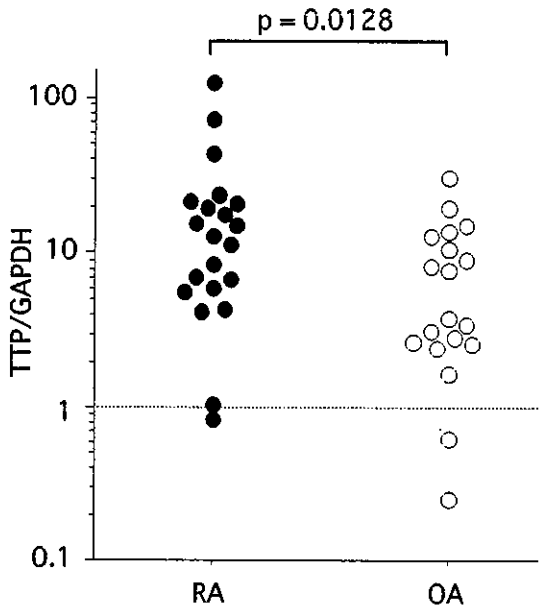

Figure 1. Expression of TTP mRNA in synovial tissues of patients with RA and OA. P value calculated by Mann-Whitney U test.

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

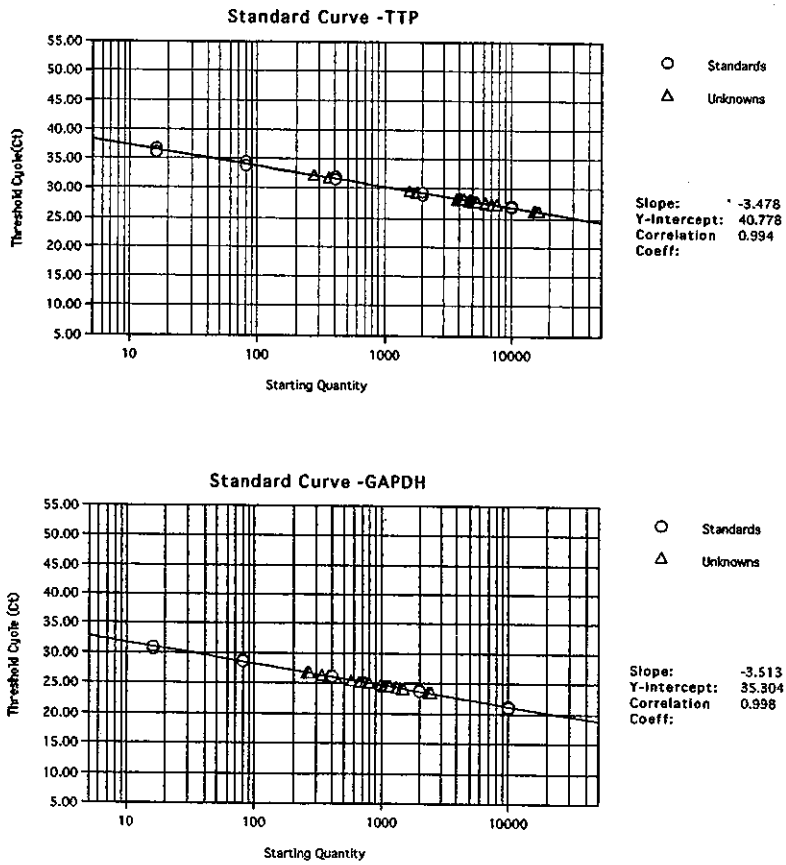

Figure 2. Standard curves generated for real-time semiquantification PCR. Upper panel: TTP; lower panel: GAPDH. All measurements were in triplicate, and sample values that were not within the standard curve were diluted and remeasured. Similar standard curves were obtained for all PCR analyses (data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

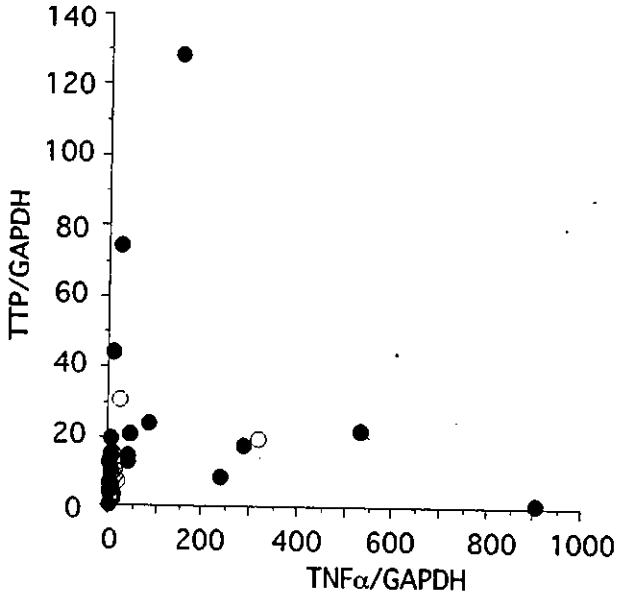

Figure 3. Relationship between expression of TTP mRNA and TNF-α mRNA. ●: RA, ○: OA.

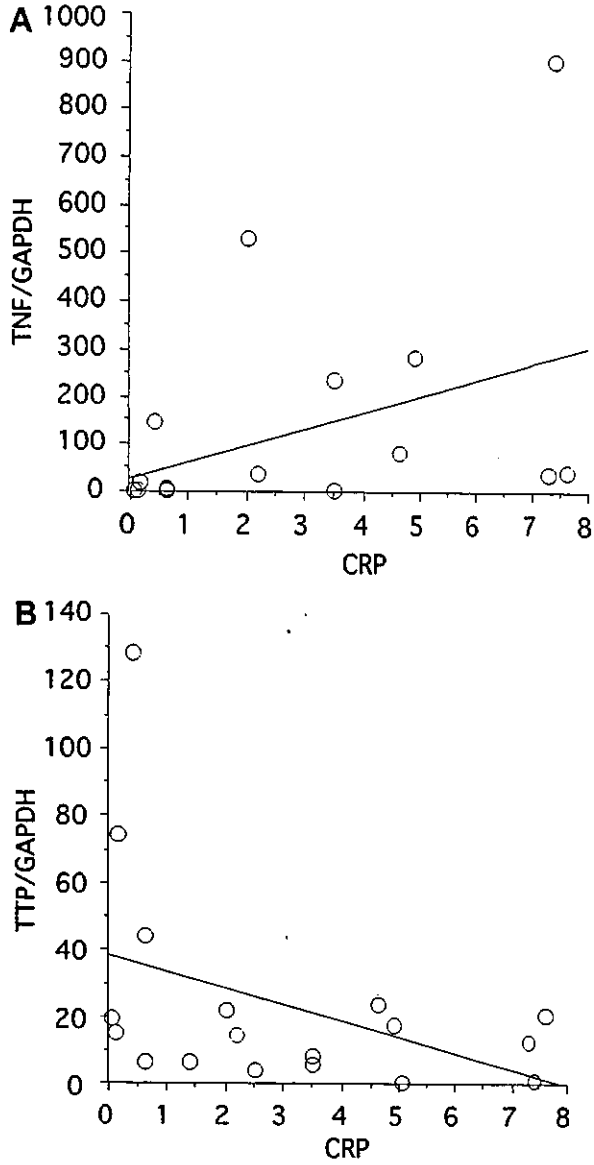

Figure 4. Relationship between serum CRP and expressions of TNF-α mRNA and TTP mRNA. A. CRP and TNF-α mRNA ($p = 0.2071$, Spearman rank correlation). B. CRP and TTP mRNA ($p = 0.0771$, Spearman rank correlation).

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

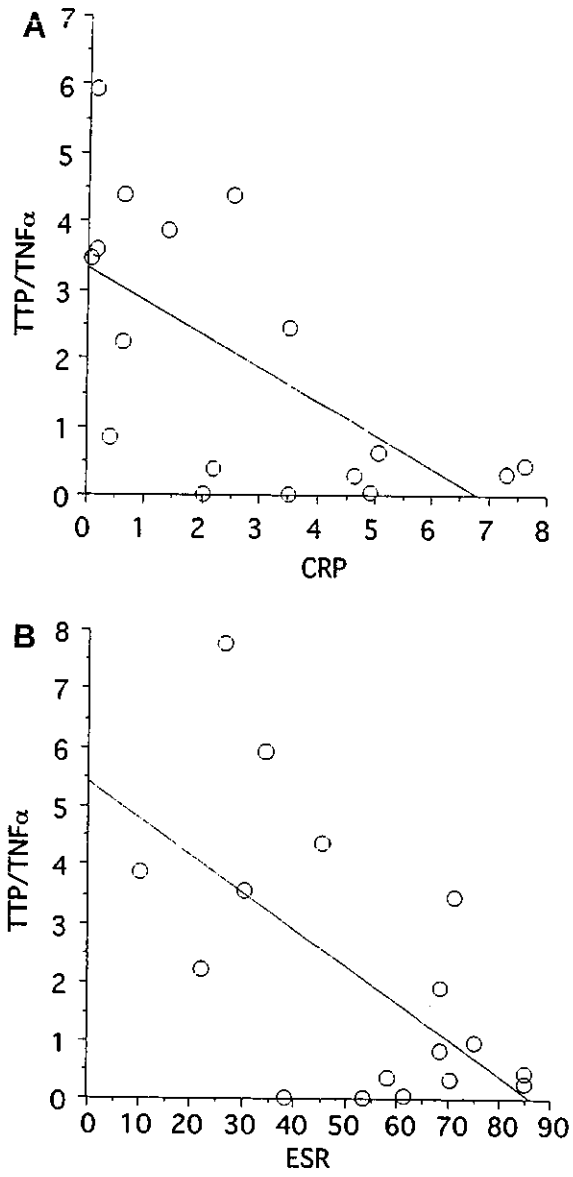

Figure 5. Relationship between expression of TTP/TNF-α gene expression ratio and CRP or ESR. A. TTP/TNF-α gene expression ratio and CRP (r = –0.653, p = 0.0071, Spearman rank correlation). B. TTP/TNF-α gene expression ratio and ESR (r = –0.441, p = 0.0692, Spearman rank correlation).

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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REVIEW

2 Takashi Okamoto

The epigenetic alteration of synovial cell gene expression in rheumatoid arthritis and the roles of nuclear factor κB and Notch signaling pathways 1

Abstract Rheumatoid arthritis (RA) is a complex process of chronic and progressive inflammation associated with activation of numerous signaling molecules and transcription factors and hyperproliferation of synoviocytes of the affected joints, although the greater part of its pathophysiological process is explained by activation of nuclear factor κB (NF-κB). For example, the self-perpetuating nature of the rheumatoid inflammation is ascribable to overexpression of the proinflammatory cytokines tumor necrosis factor α and interleukin-1β, known to elicit the activation cascade for NF-κB and activator protein-1 that are responsible for transcriptional induction of these cytokines among other target genes, which conform a positive feedback loop for continuation and expansion of the inflammatory responses. In addition, comparative gene expression profile analyses have revealed activation of a number of genes that explain the "transformed-like" phenotype of synoviocytes. Among the genes expressed in rheumatoid synoviocytes upon inflammatory stimuli, induction of gene expression of Notch proteins and its ligand have been found. Possible roles of Notch signaling in RA synoviocytes are discussed.

Key words Notch · Nuclear factor κB (NF-κB) · Rheumatoid arthritis · Signal transduction · Synoviocyte

Introduction

Rheumatoid arthritis (RA) is a common human autoimmune disease with a prevalence of about 1%.[1] While there has been progress in defining its etiology and pathogenesis, these are still incompletely understood.[1–3] Proposed causes for RA include (i) genetic predisposition, (ii) pathogenetic immunoinflammatory responses triggered by environmental agents, particularly microbes, (iii) autoimmunity directed against components of synovium and cartilage, (iv) dysregulated production of cytokines (usually upregulation of proinflammatory and inflammatory cytokines and chemokines), (v) recruitment of immunoinflammatory cells through induction of inflammatory cell adhesion molecules (such as E-selectin, intracellular adhesion molecule-1, and vascular cell adhesion molecule-1), and, last but not least, (vi) transformation of synovial cells into autonomously proliferating cells with highly invasive nature (often referred to as "transformed-like" phenotype[4–6]).

Rheumatoid arthritis is characterized by a chronic inflammation of the synovial joints associated with proliferation of synovial cells and infiltration of activated immunoinflammatory cells including memory T cells, macrophages, and plasma cells,[1,2,7] which eventually leads to progressive destruction of cartilage and bone. This process is considered to be mediated by a number of cytokines including tumor necrosis factor α(TNFα), interleukin (IL)-1, IL-6, IL-8, IL-12, IL-16, IL-18, and interferon γ(IFNγ) (reviewed in Refs. 1–3). Most of these pathophysiological features of RA can be explained by activation of limited number of transcription factor and its activation signals such as nuclear factor κB (NF-κB) and activator protein (AP)-1.[3,8] In fact, some effective anti-RA drugs are now known to inhibit NF-κB and its activation cascade (reviewed in Ref. 8). However, the mechanism by which rheumatoid synoviocytes exhibit the tumor-like nature has been yet to be clarified.

Involvement of NF-κB in RA as a primary pathogenic determinant

Among the various signaling and transcription regulation pathways, NF-κB and AP-1 are known to be the target of inflammatory responses. In fact, most of the factors involved in RA pathophysiology are under the control of these transcription factors.[3,8] Particularly, various cytokines

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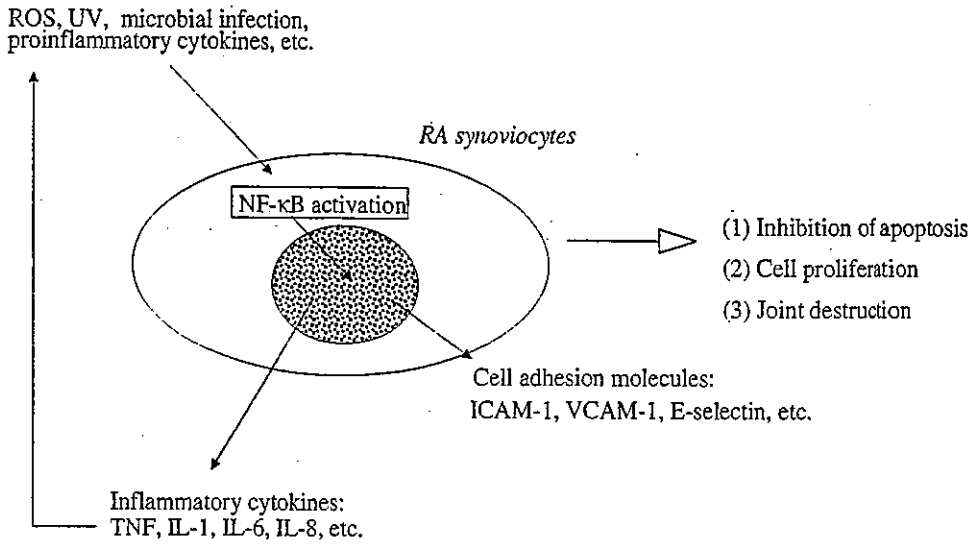

Fig. 1. Involvement of nuclear factor κB (*NF-κB*) in rheumatoid arthritis (*RA*) pathophysiology. NF-κB induces gene expression of inflammatory mediators such as cytokines and cell adhesion molecules. Since proinflammatory cytokines, tumor necrosis factor α (*TNF*), and interleukin (*IL*)-1β stimulate the NF-κB activation cascade that induces expression of these cytokines, there will be a positive feedback loop that perpetuates and expands the inflammatory responses even systemically. NF-κB also stimulates synovial proliferation by inhibiting apoptosis. See also Fig. 2. *ROS*, reactive oxygen species; *ICAM*, intracellular adhesion molecule; *VCAM*, vascular cellular adhesion molecule

and cell adhesion molecules activated in the rheumatoid joints are under the transcriptional control of NF-κB. The self-perpetuating nature of rheumatoid inflammation is ascribable to TNFα and IL-1β, known to elicit the activation cascade for NF-κB and AP-1, as they constitute another positive feedback loop in the logic of the inflammatory responses associated with RA (Fig. 1).

In addition, besides its action in upregulating inflammatory cytokines and cell adhesion molecules, NF-κB also induces gene expression of cell growth-promoting factors such as cyclin D1 and c-Myc, and physiological inhibitors of apoptosis such as cIAPs, Bcl-X_L, and cFLIP.[9,10] (Fig. 2). Moreover, it has been shown that NF-κB blocks apoptosis in the absence of de novo protein synthesis[11] through protein–protein interaction with p53 and proapoptotic protein 53BP2.[12,13] These actions of NF-κB explain not only the inflammatory responses but also the hyperproliferation of synovial tissues in RA, indicating that NF-κB acts as a major determinant for RA pathophysiology. Nuclear factor κB induces TNFα and IL-1β gene expression, and both TNFα and IL-1β stimulate NF-κB signaling, a vicious cycle formed to perpetuate and even expand the inflammatory responses.[8] The intervention therapy against using anti-TNF antibody and IL-1β receptor antagonist has been thus developed.[14,15] In addition, some of the drugs for RA have been shown to block NF-κB-activation cascade or its actions (Table 1).[15–18]

The signal transduction cascade for NF-κB activation

The members of the NF-κB family in mammalian cells include the proto-oncogene c-Rel, Rel A (p65), Rel B, NFκB1 (p50/105), and NFκB2 (p52/p100). These proteins share a conserved 300-amino-acid region known as the Rel homology domain, which is responsible for DNA binding, dimerization, and nuclear translocation of NF-κB. In most cells, Rel family members form hetero- and homodimers with distinct specificities in various combinations.[8,19,20] A common feature of the regulation of NF-κB family is their sequestration in the cytoplasm as inactive complexes with a class of inhibitory molecules known as IκBs.[20,21] Upon stimulation of the cells such as by proinflammatory cytokines, IL-1β and TNFα, IκBs are degraded, and NF-κB is translocated to the nucleus and activates expression of target genes (Fig. 2).

The IκB kinase complex capable of specifically phosphorylating Ser32 and Ser36 of IκBα was originally identified as a ~700kDa of high molecular complex.[21,22] Subsequently, two catalytic subunits (IKKα and IKKβ) and a scaffold subunit of this complex (IKKγ/NEMO/IKKAP) were identified and cloned (for review see Refs. 20–22). The IKK complex, consisting of IKKα, β, and γ, can be activated by a variety of stimuli, including TNFα, IL-1β, and LPS. Activation of the complex involves the phosphorylation of two serine residues located in the "activation loop" within the kinase domain of IKKα and IKKβ. IKK complex is stimulated by upstream kinases that belong to MAP kinase kinase kinases (MAP3Ks), including MEKK1, MEKK2, MEKK3, and NIK, capable of phosphorylating these serines in vitro, and activating NF-κB.[23,24] Phosphorylation on specific serine residues of IκBs leads to ubiquitination of IκBs and subsequent degradation by the proteasome complex.

There is accumulating evidence suggesting the involvement of additional kinases that phosphorylate the p65 (RelA) subunit of NF-κB and regulate its transcriptional

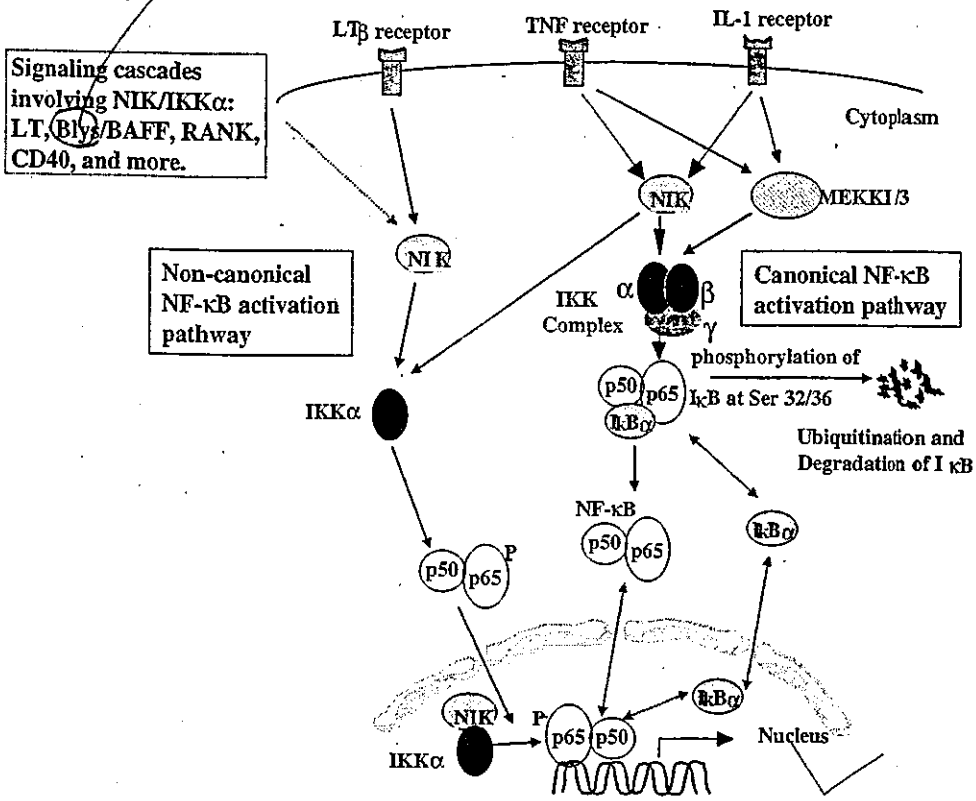

Fig. 2. NF-κB activation cascades. In addition to the canonical pathway involving IκB phosphorylation and ubiquitination followed by its proteolytic degradation in 26S proteasome within the cytoplasm, there appears to be another cascade not involving IκB phosphorylation. Lymphotoxin (*LT*) β-receptor signaling, CD40, RANK, and Blys/BAFF stimulate the NIK–IKKα cascade that leads to p100/p52 processing and p65 phosphorylation at its C-terminal transactivation (Ser536). IKKα also phosphorylates histone H3 in the nucleus and derepresses the otherwise silent nucleosome, thus reactivating the dormant genes. The effect of p65 (Ser536) phosphorylation is considered to activate the transcriptional competence of NF-κB

Table 1. List of rheumatoid arthritis drugs that inhibit nuclear factor κB

Acetylsalicylic acid
Aurothioglucose
Aurothiomalate
Auranofin
Dexamethasone
Ibuprofen
Sodium salicylate
Sulfasalazine

competence.[25–27] We recently found that IKKα is responsible for the p65 phosphorylation at Ser536 upon the lymphotoxin β receptor signaling mediated by NIK, and induces NF-κB activation independently of the IκB phosphorylation and its degradation.[28–30] Interestingly, this NIK–IKKα cascade is also involved in Blys/BAFF, RANK, and most likely CD40 signaling.[31,32] In contrast to the classical (or canonical) pathway involving IKKβ and the phosphorylation of IκB, this cascade ("non-canonical pathway") does not necessarily involve IKKβ and IκB phosphorylation but involves p100 (NFkB2) processing and p65 phosphorylation (Fig. 2). Since Blys/BAFF and CD40 signaling cascades induce B-cell activation and RANK signaling is involved in osteoclast differentiation, the NIK–IKKα cascade is considered to play important roles in disease progression of RA. The TNFα-dependent phosphorylation of Ser529 has also been demonstrated to increase the transcriptional activity of p65. For example, casein kinase II was implicated in the TNFα-dependent phosphorylation of p65 on Ser529.[33] It was shown that Ser529 and Ser536 of p65 were required for transcriptional activation of p65 by AKT and the IL-1β signaling.[30,34]

Inducible phosphorylation of p65 appears to function at many different levels, including conformational changes in the transcriptional activation domain and promoting association with coactivator proteins CBP/p300.[20] It is possible that the phosphorylation of p65 may lead to dissociation from corepressor proteins such as histone deacetylases and Groucho proteins (TLE/AES) and selective interaction with FUS/TLS coactivator protein.[35–37] Regarding the cross-talk with the camp–PKA cascade, although my group and others found that it downregulates the NF-κB-dependent gene expression presumably mediated by C/EBPβ,[38–40] it has also been reported that the catalytic subunit of PKA (PKAc), associated with the NF-κB/IκBα complex, upregulates the NF-κB-dependent gene expression by directly phosphorylating p65 on serine 276,[27] thus pending the physiological relevance.

Cytological characteristics of rheumatoid synoviocytes

Although it appears that NF-κB plays a major role in the pathophysiology of RA, there is no evidence to support the possibility that NF-κB or its signaling cascade is impaired in RA. To clarify the transformed-like nature of rheumatoid synoviocytes, my group have performed gene expression

profile analyses of synoviocytes.[41] When compared with control synoviocytes obtained from healthy individuals (upon injury) or osteoarthritis patients, we found that both platelet-derived growth factor (PDGF) receptor α and a chemokine, SDF-1, genes are activated in RA synoviocytes without any external stimulus. Gene knockout studies showed that PDGF receptor α is required for the development of limb joints. During the early developmental stages, PDGF and SDF-1 are known to act as chemotactic factors for fibroblasts[42] and macrophages,[43,44] respectively. It is possible that synovial fibroblasts (type B synoviocytes) and synovial-lining macrophage-like cells (type A) communicate with each other by producing SDF1A and PDGF, respectively, in order to form the primordial joint tissue during the early embryonic development (reviewed in Ref. 41). Thus, it is likely that rheumatoid synoviocytes may have reacquired the "revertant" phenotype of the primordial synoviocytes, like cancer cells, although the underlining mechanism is yet to be clarified.

As mentioned above, rheumatoid synovial tissues are usually under inflammatory stimuli as synovial fluid contains high concentrations of TNFα, IL-1, and oxidants (Ref. 46 and references therein). Thus, we extended the gene expression profile analysis with synoviocytes in the presence of physiological concentration (200pg/ml) of TNFα and compared the genes induced by TNFα in RA and control synoviocytes. Although well-known genes under the control of NF-κB were similarly stimulated by TNFα, we found that the genes encoding Notch-1, Notch-4, and Jagged-2, a ligand for Notch proteins, were activated only in RA synoviocytes.[49] (Fig. 3). We also found that genes encoding MMP-11 and -17, and Wee1 and cyclin B1, were induced by TNFα only in rheumatoid synoviocytes. These findings indicate that one of the effects of phenotypic reversion of rheumatoid synoviocytes, as described above, could be attributable to the induction of Notch signaling and that the activation of Notch signaling, known to be involved in cell-fate determination, may directly or indirectly cause induction of genes responsible for cell proliferation (such as induction of Wee1 and cyclin B1 genes) and tissue invasion (such as induction of MMP-11 and -17). These findings support an idea that RA synoviocytes may have reacquired the "revertant" phenotype mimicking the primordial synoviocytes, by presumably involving Notch signaling, and exhibit the hyperproliferative and invasive nature of cells.

Activation of Notch signal in RA

As TNFα induced Notch-1 and its ligand Jagged-2 in RSF, we examined if the Notch signaling is elicited by the TNFα stimulation. Rheumatoid synovial fibroblasts (RSF) and normal synovial fibroblasts (NSF) were stimulated with TNFα and the intracellular localization of Notch intracellular domain (NICD) of Notch-1 was examined by immunostaining. We found the nuclear translocation of Notch-1 NICD, a hallmark of the Notch signaling,[48,49] only in TNFα-stimulated RSF[47] (Fig. 4). These results suggested that in response to TNFα stimulation RSF expressed both Notch-1 and Jagged-2 proteins, which then interacted with each other between adjacent cells and elicited the signaling. In RA tissues we found that hyperproliferative synovial tissues were clearly stained by Notch-1, Notch-4, and Jagged-2 antibodies, and that some of the RA synovial cells showed the nuclear staining of Notch-1 and Notch-4.

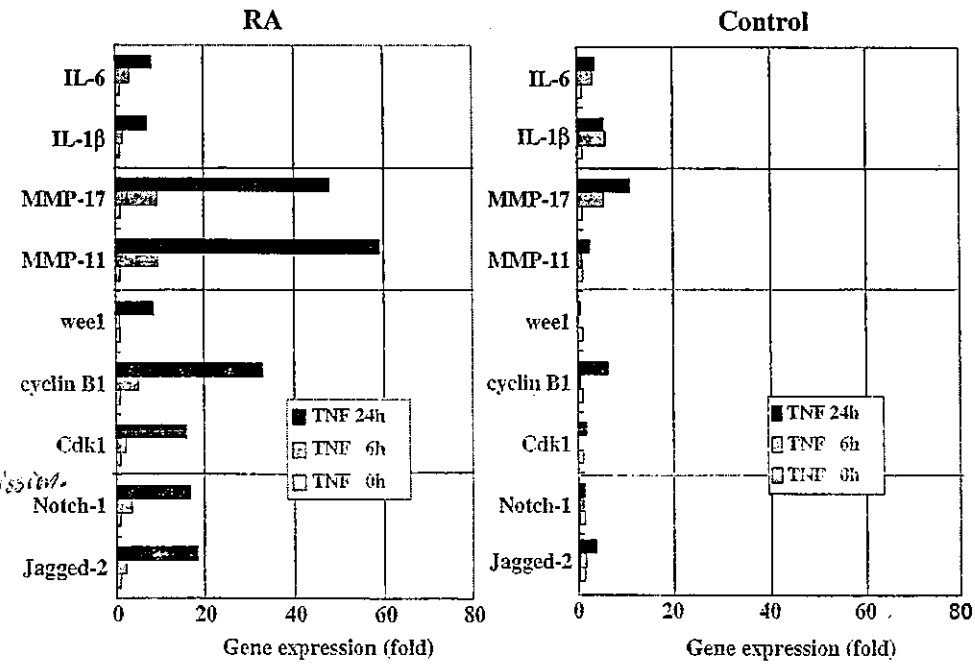

Fig. 3. Comparative gene expression profile analysis of rheumatoid and control synoviocytes upon stimulation with tumor necrosis factor α (*TNF*). Synoviocytes were cultured with or without TNF (200pg/ml). The mRNA was purified from each cell culture harvested at 0, 6, and 24h after TNF stimulation, cDNA probe was synthesized, then hybridized with a cDNA array membrane. The quantitation of gene expression level was performed and standardized based on the average levels of housekeeping genes. Based on the observation by Ando et al.[47]

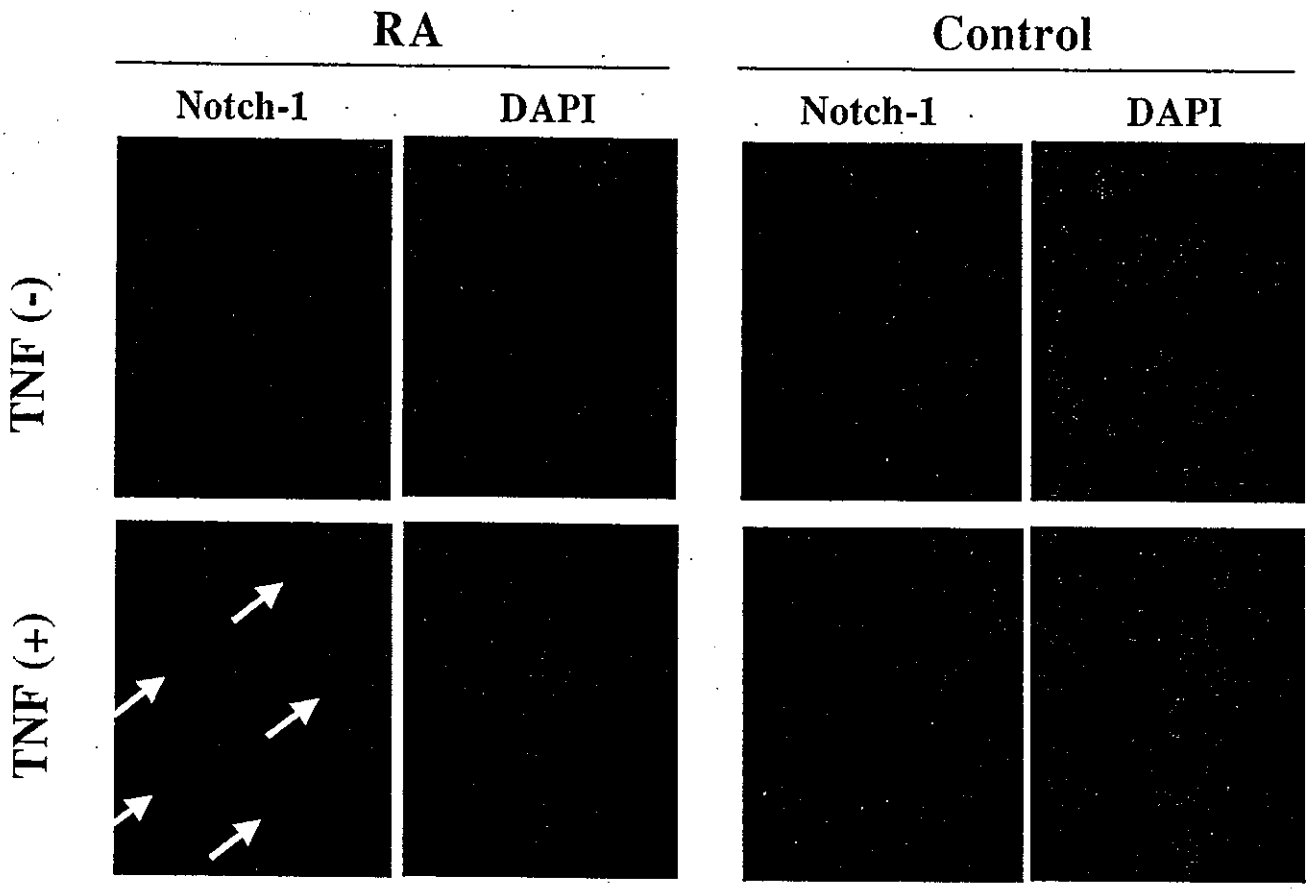

Fig. 4. Nuclear translocation of Notch-1 intracellular domain (NICD) in rheumatoid synoviocytes after tumor necrosis factor α (*TNF*) stimulation. Cells were immunostained with anti-Notch-1 C-terminus polyclonal antibody (C-20) before and after 12 h of TNF stimulation and examined by fluorescent microscopy. *Green*, Notch-1 intracellular domain (detected by fluorescein isothiocyanate-conjugated rabbit antigoat IgG as secondary antibody); *Blue*, nuclear staining with 4′,6′-diamidino-2-phenylindole hydrochloride (*DAPI*). Based on the observation by Ando et al.[47]

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We also detected expression of these proteins in the developing synovial and cartilage tissues of embryonic mice.[47] In more developed joints of newborn mice, expression of these proteins was restricted in the synovium, raising a possibility that the Notch signaling pathway might control the differentiation and development of joints.

Biological implications of Notch signal activation in RA

Notch signaling is involved in three different biological processes including (i) lateral specification, in which adjacent equipotent precursor cells coordinate each other's developmental fate, (ii) inductive signaling, in which one cell type determines the differentiation of another cell type, and (iii) cell-autonomous effects, in which a developing precursor (stem) cell regulates its own fate and maintains its status (reviewed in Refs. 50 and 51). Notch genes encode single-pass transmembrane receptors that transduce the extracellular signals responsible for cell fate determination during crucial steps of metazoan development.[52,53] The large transmembrane receptors encoded by Notch genes interact with membrane-bound ligands encoded by the Delta and Jagged (Serrate) genes at the extracellular surface of cells. The signal induced by this ligand binding leads to proteolysis of Notch, generation and nuclear translocation of NICD, and regulation of target gene expression (Fig. 5). Genes homologous to members of the Notch signaling pathway have been cloned from numerous vertebrate organisms and many have been shown to be essential for normal embryonic development. In humans, the importance of Notch signaling for growth and development is supported by the findings that T-lymphoblastic leukemia[54] and some inherited diseases involving affected organogenesis[55,56] can be ascribed to the mutations in Notch/Jagged (Delta) genes. The Notch signaling pathway is evolutionarily conserved, and mutations in its components disrupt cell fate specification and embryonic development in diverse organisms.[48,50]

Interestingly, a targeted mutation that removes a domain of the Jagged-2 protein required for the interaction with Notch-1 caused perinatal death associated with defects in craniofacial morphogenesis and syndactyly (digit fusions) of the fore- and hindlimbs, implicating that Jagged-2/Notch signaling is indispensable for the development of the joint.[57]

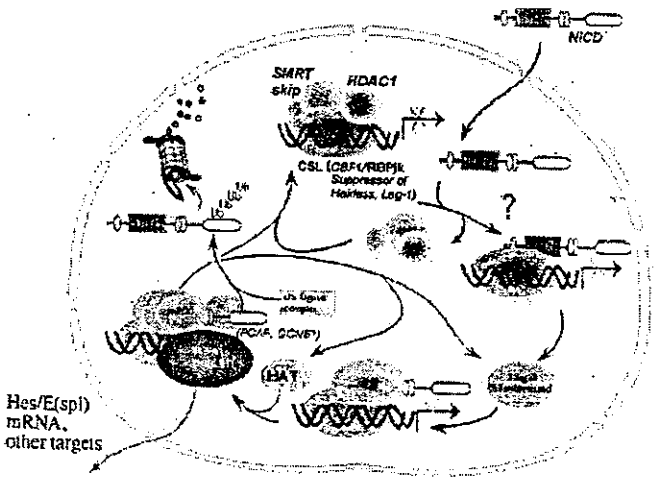

Fig. 5. Notch signaling and the transcriptional control by the Notch intracellular domain (*NICD*). Upon ligand binding, Notch protein is proteolytically cleaved by furin and presenilin to generate NICD, which is translocated to the nucleus. Once in the nucleus, NICD displaces the corepressor proteins such as *SKIP*, *SMRT*, and *HDAC-1* from the specific DNA-binding proteins *CSL* (CBP or RBP-Jκ in vertebrates, Su(H) in *Drosophila*, and Lag-1 in *Caenorhabditis elegans*; thus collectively called "CSL") and associates with the coactivator complex containing *Mastermind* (MAM) and *p300*;[62] MAM is considered to bridge the NICD/RBP-Jκ complex and p300.[63] The Notch intracellular domain is subjected to ubiquitination followed by proteasome-mediated degradation, thus terminating the transcriptional activation

A similar phenotype has been observed in mice lacking the IKKα subunit of IκB kinase complex.[58] In embryonic day 16 (E16) mutant embryos, forelimbs (but not hindlimbs) were visible but were considerably shorter than those of normal ($Ikk\alpha^{+/+}$ and $Ikk\alpha^{+/-}$) littermates and lacked separated digits. At an earlier stage, E14.5, the fore- and hindlimbs of mutant embryos were not much shorter than those of normal counterparts, but were devoid of distinct digits. Therefore, it appears that the TNF-mediated NF-κB activation through IKKα is involved in expression of Jagged-2 in the developing joints. Thus, activation of the Notch signaling found in rheumatoid synoviocytes not only confirms the phenotypic reversion of synoviocytes but also indicates its active role in pathophysiological processes of RA, which presumably involve NF-κB cascade.

Cross-talk between NF-κB activation cascade and Notch signaling

In mammals, all four known Notch family members can physically interact with recombinant signal binding protein Jκ (RBP-Jκ), a DNA-binding repressor protein, and inhibit the activity of RBP-Jκ.[59,60] Oswald et al.[61] reported that NICD overcame the RBP-Jκ-mediated repression and strongly activated NF-κB2. In the absence of Notch signaling, RBP-Jκ interacts with SKIP and SMRT that recruit transcriptional corepressor complex[62] (Fig. 5). However, upon Notch signaling NICD induces changes in the DNA-bound protein assembly containing RBP-Jκ in the nucleus, thus displacing the corepressor complex and converting it to a transcriptionally active complex. It has been shown that a non-DNA-binding transcriptional coactivator Mastermind (MAM) is essential for the Notch/RBP-Jκ complex to recruit p300 coactivator to DNA.[63] Thus, activation of Notch signaling observed in rheumatoid synoviocytes appears to stimulate the noncanonical NF-κB pathway (Fig. 5).

It is conceivable that this noncanonical NF-κB activation pathway may be responsible for the altered response to the inflammatory environment involving IKKα. It is known that IKKα is translocated, together with NF-κB, to the nuclear chromatin compartments where target genes are present, and phosphorylates Ser10 of the histone H3 component of nucleosome.[64,65] (Fig. 5). Although the histone H3 with methylated lysine 9 of H3 renders the local nucleosome to be "repressive," the adjacent serine 10-phosphorylation of H3 histone reverses this effect and derepresses the transcriptional activity of the genes located in the "derepressed" nucleosome.[66] Thus, chronic and persistent NF-κB stimulation in synoviocytes of RA patients could also lead to the change in "histone code"[66] and eventually transform synoviocytes.

Conclusion

Rheumatoid arthritis is a complex process of chronic and progressive inflammation involving numerous transcription factors and signaling molecules. Based on the unexpected transcriptomic characteristics of rheumatoid synoviocytes, suggesting the phenotypic reversion, I have explored the mechanism by which chronic inflammatory stimuli could endow normal synoviocytes with "transformed-like" phenotype and could ascribe activation of the Notch signaling to this altered cellular status. This may explain the progressive and self-perpetuating nature of the rheumatoid inflammation, at least in part. Based on these considerations, future therapeutic strategy of RA should be developed based on the action of Notch signaling on its pathophysiology, which includes the action of the noncanonical NF-κB activation pathway, its therapeutic intervention, and elucidation of the Notch target genes, particularly in synoviocytes.

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