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Constitutive Transcription of the Human Interleukin-6 Gene by Rheumatoid Synoviocytes

Spontaneous Activation of NF-κB and CBF1

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The involvement of IL-6 in the pathogenesis of rheumatoid arthritis (RA) has been recently demonstrated. In the present study, we investigated the cellular and molecular mechanisms involved in the spontaneous IL-6 production by the fibroblast-like synoviocytes (FLSs) obtained from patients with RA. Cloned FLSs were established from the bulk cultures of FLSs by the limiting dilution method. Some FLS clones spontaneously produced large amounts of IL-6, whereas others produced low amounts of it. Neither anti-human TNF-α nor IL-1 antibody affected spontaneous IL-6 production of these FLS clones, suggesting that IL-6 production of the FLSs was endogenously up-regulated. A luciferase reporter plasmid containing the human IL-6 promoter region was significantly transcribed when transfected into the IL-6 high-producing clones, indicating that the rheumatoid FLSs retained constitutive transcriptional activity of the IL-6 gene. Electrophoretic mobility shift assays revealed that the binding activity of p50 and p65 NF-κB subunits and CBF1 was significantly enhanced in the IL-6 high-producing clones compared with that of IL-6 low-producing clones and cultured sarcoma cells, suggesting that spontaneous activation of NF-κB and CBF1 may lead to the constitutive transcription of the IL-6 gene by rheumatoid FLSs. *(Am J Pathol 1998, 152:793–803)*

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects systemic joints. Chronic synovitis often leads to irreversible destruction of cartilage and periarticular bone.[1] The inflamed synovium is characterized by the infiltration of monocytes/macrophages and lymphocytes.[2–4] Local proliferation of synoviocytes was also indicated.[5–7] These highly activated cells produce proinflammatory cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, and IL-6, which are involved in the pathophysiology of RA.[2,8,9]

IL-6, a pleiotropic cytokine, mediates its effects on several target cells. Specifically, IL-6 is a T-cell growth factor,[10] a B-cell differentiating factor that might contribute to rheumatoid factor production,[11,12] a major inducer of hepatic acute-phase proteins,[13] and an enhancer of IL-3-dependent proliferation of multipotential hemopoietic progenitors.[14] IL-6 also stimulates the growth of B-cell hybridomas and plasmacytomas.[15] In addition, this cytokine in cooperation with soluble IL-6 receptor (IL-6R) is involved in the proliferation of synoviocytes from RA patients.[16] Immunological disorders often associated with RA include polyclonal plasmacytosis, production of autoantibodies, increased levels of acute-phase proteins, and an increased number of peripheral blood platelets, all of which are related to the biological actions of IL-6.[17] In fact, IL-6 levels are greatly increased in the synovial fluids and in the serum obtained from RA patients.[18,19] More recently, administration of anti-IL-6R antibody dramatically improved the symptoms and clinical markers of RA patients,[20] indicating that IL-6 is one of the key cytokines for the development of RA.

Synoviocytes obtained from RA patients were demonstrated to produce biologically active IL-6 *in vitro*, which enhanced B-cell growth, immunoglobulin synthesis, and hepatic acute-phase protein production.[12] However, the mechanisms of IL-6 production by synoviocytes have been less defined. As former investigations by others used heterogeneous mixtures of adherent synoviocytes containing fibroblast-like cells (FLSs), dendritic cells, and macrophage-like cells,[8,12,21,22] it is still uncertain whether IL-6 is produced autonomously by rheumatoid synoviocytes or is produced in response to inflammatory cytokines such as TNF-α and IL-1 provided by other cell populations. Therefore, we established cloned FLSs to clarify the cellular mechanisms of IL-6 overproduction by the FLSs obtained from RA patients. Our results clearly indicate the endogenously up-regulated production of IL-6 by rheumatoid FLSs. Elucidation of the molecular mechanism of IL-6 production by rheumatoid FLSs may be a promising way to further delineate the pathophysi-

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ology of RA and to develop future effective therapeutic intervention.

Materials and Methods

Reagents

Recombinant human TNF-α, IL-1β, and IL-6 were obtained from Genzyme Corp. (Cambridge, MA). Neutralizing monoclonal antibody for TNF-α (MAB210) was purchased from R&D Systems (Minneapolis, MN). Neutralizing polyclonal antibody (P-401) for both IL-1α and IL-1β was purchased from Endogen (Woburn, MA). Polyclonal anti-p50 (sc-114X), -p52 (sc-848X), -p65 (sc-109X), -c-Rel (sc-70X), and -HMG I(Y) (sc-1564) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California).

Cells

Synovial tissue samples were obtained from patients with RA or osteoarthritis (OA) undergoing total joint replacement. All RA and OA patients were evaluated by a certified rheumatologist and were diagnosed as having RA and OA, respectively, according to the criteria of the American College of Rheumatology.[23,24] Written informed consent was obtained from each patient. Each tissue specimen was minced and then digested with 4 mg/ml collagenase for 2 hours at 37°C. Cells were plated in RPMI 1640 (Nikken, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY). When the cells had grown to confluence, they were treated with trypsin/EDTA and split at a 1:4 ratio. For experiments, FLSs were used at passages 3 to 10.

Normal dermal fibroblasts were obtained from Dr. Mori (Osaka University, Osaka, Japan). Synovial sarcoma SW982 cells and fibrosarcoma HT1080 cells were purchased from American Type Culture Collection (Rockville, MD). These cells were grown and passaged under the same conditions as described for the FLSs.

Establishment of Cloned FLSs

Synoviocytes were cloned by the limiting dilution as described previously.[25] Briefly, the cells were suspended in the RPMI 1640 medium supplemented with 20% FBS at a density of 5 cells/ml. A 100-μl aliquot of the cell suspension was then inoculated into each well of a 96-well culture plate (0.5 cells/well) and incubated at 37°C in a humidified atmosphere with 5% CO_2. No more than 10 wells/plate were positive for growth. Such growth appeared to be from a single cell, because each well was carefully examined under a microscope to ensure that only a single cell was present 2 days after the cloning of the FLSs had begun. Cloned FLSs were expanded up to the third passage under the same conditions described above.

Detection of Cytokine Production by Cultured Cells

Cells at confluence were preincubated overnight in RPMI 1640 supplemented with 0.1% bovine serum albumin (BSA) to exclude the effect of FBS. The medium was then exchanged for fresh RPMI 1640 supplemented with 0.1% BSA, and the cells were cultured for another 6 hours. The concentrations of IL-6, TNF-α, and IL-1β were measured by specific sandwich ELISAs. Briefly, supernatants or serial dilutions of recombinant cytokine standards (Genzyme) were incubated overnight at 4°C in 96-well microtiter plates (Nunc, Roskilde, Denmark) previously coated overnight at 4°C with anti-human-IL-6 monoclonal antibody (2 μg/ml; R&D Systems), anti-human-TNF-α monoclonal antibody (0.4 μg/ml; Duo Set, Genzyme), or anti-human IL-1β monoclonal antibody (0.4 μg/ml) and then for 2 hours at room temperature for saturation. After the plates had been washed, biotinylated anti-human-IL-6 polyclonal antibody (2 μg/ml; R&D Systems), biotinylated anti-human TNF-α monoclonal antibody (0.2 μg/ml; Duo Set), or goat anti-human IL-1β polyclonal antibody (2 μg/ml; R&D Systems) was added for IL-6, TNF-α, or IL-1β assay, respectively. The incubation was carried out for 4 hours at room temperature. After subsequent incubation (2 hours at room temperature) with horseradish peroxidase (HRP)-conjugated avidin (Zymed Laboratories, South San Francisco, CA) for IL-6 ELISA, HRP-conjugated avidin (Duo Set) for TNF-α ELISA, and HRP-conjugated rabbit anti-goat IgG (Zymed) for IL-1β ELISA, 3,3′,5,5′-tetramethylbenzidine (TMBZ) (Dojindo Laboratories, Kumamoto, Japan) was added to the wells. The cytokine concentration was determined by measuring the absorbance at 450 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA). The minimal detection limit of the assays was 6.25 to 12.5 pg/ml.

Northern Blotting

Cells were grown to confluence in 175-cm^2 culture flasks and then harvested. Total RNA was prepared from the cells by the method of Chomczynski and Sacchi.[26] Twenty micrograms of total RNA was loaded onto a 1.2% (w/v) agarose gel containing formaldehyde and, after electrophoresis, transferred onto Hybond N nylon membranes (Amersham, Little Chalfont, UK) by use of a VacuGene XL vacuum blotting apparatus (Pharmacia/LKB, Piscataway, NJ). Specific mRNAs were detected by hybridization with appropriate randomly primed ^{32}P-labeled cDNA probes (BcaBEST labeling kit, Takara, Osaka, Japan). The cDNA probes used were as follows: human IL-6 cDNA was a 1.16-kb *Eco*RI/*Eco*RI fragment (American Type Culture Collection),[27] and human glyceraldehyde-3-phosphate dehydrogenase cDNA was a 0.8-kb *Pst*I/*Xba*I fragment (American Type Culture Collection).[28] Autoradiography of resulting Northern blots was quantified by scanning densitometry.

Plasmid Construction

For generation of the pIL6–2BLuc plasmid, a *Sac*I/*Xho*I fragment containing a 1.2-kbp *Bam*HI/*Xho*I 5′ upstream sequence of the IL-6 gene was excised from the pGEMhIL-6 GT plasmid (Riken Gene Bank, Tsukuba, Japan)[29] and inserted into compatible sites (*Sac*I/*Xho*I) of a pGL2 basic vector plasmid (Promega Corp., Madison, WI). Furthermore, a *Sac*I/*Xho*I fragment containing the 189-bp *Bfa*I/*Xho*I 5′ upstream sequence of the IL-6 gene was inserted into the plasmid pGL3 basic vector plasmid (Promega). The IL6-κB site at positions −73 to −63, CBF1 site at positions −68 to −61, and both IL6-κB and CBF1 sites at positions −73 to −61 of the human IL-6 promoter were respectively mutated in the 189-bp 5′-flanking sequence of the human IL-6 gene by insertion of synthesized DNA. The mutant sequence used for the IL6-κB, CBF1, and both IL6-κB and CBF1 were aatATTTTCCC (mt1), TTTCCCtc (mt3), and GGGATTTTagacT (mt2), respectively. Lowercase letters represent the mutant nucleotides.

Transient Transfection and Luciferase Assay

Cells were grown to confluence in 175-cm^2 culture flasks containing RPMI 1640 medium supplemented with 10% FBS, harvested, and resuspended in RPMI 1640 medium without phenol red to give a cell concentration of 2×10^6 cells/ml. A 500-μl volume of cell suspension, 10 μg of reporter plasmid, and 1 μg of pCMV-β-galactosidase were placed into a Bio-Rad (Hercules, CA) 4-mm cuvette, and electroporation (Bio-Rad gene pulser, 0.29 kV, 960 μF) was performed. The transfected cells were cultured for 18 hours in RPMI 1640 medium without phenol red. All assays were carried out in 96-well plates (Culture Plate, Packard Instrument Co., Meriden, CT). The luminescence was measured with a Top Count (Packard) under the conditions of a 0.15-minute counting time, single-photon counting mode, and a 15-minute period of dark adaptation at 22°C. The β-galactosidase activity was measured as described earlier.[30] Briefly, cells were washed with phosphate-buffered saline (PBS) and then 100 μl of 1.5 mmol/L chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim, Mannheim, Germany) in PBS containing 20 mmol/L KCl, 2 mmol/L $MgSO_4$, 100 mmol/L 2-mercaptoethanol, and 0.5% Nonidet P-40 (Wako, Osaka, Japan) was added, and β-galactosidase activity was determined by measuring the absorbance at 570 nm by a microplate reader (Bio-Rad Laboratories).

Nuclear Run-On Assay

Cells were washed twice with ice-cold PBS and then pelleted. Their nuclei were suspended in 0.1 ml of buffer A (50 mmol/L Tris/HCl (pH 8.3), 5 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, and 40% glycerol). Elongation of nascent RNA chains was initiated by mixing the nuclear suspension with an equal volume of reaction buffer (10 mmol/L Tris/HCl (pH 8.0), 5 mmol/L $MgCl_2$, 0.3 mol/L KCl, 5 mmol/L dithiothreitol (DTT), 1.0 mmol/L concentration of each of ATP, CTP, and GTP (Pharmacia Biotech, Tokyo, Japan), and 0.1 mCi of [α-^{32}P]UTP (specific activity, 760 Ci/mmol; Amersham). After incubation at 30°C for 30 minutes, the ^{32}P-labeled RNA was purified by ISOGEN (Wako). The pBluescriptII KS (+) vector alone, pBluescript vector containing a human IL-6 cDNA insert, and pBluescript vector containing a human GAPDH cDNA insert were linearized and immobilized onto Hybond N nylon membranes (Amersham) by use of a slot-type dot blotting apparatus (Bio-Rad Laboratories). The membranes were prehybridized in a solution of 50% formamide, 5X SSPE, 2X Denhardt's reagent, 0.5% SDS, and 200 μg/ml salmon testes DNA for 18 hours at 42°C. Equal amounts of radioactivity (cpm) from control and treated samples containing ^{32}P-labeled RNA were hybridized with the immobilized plasmid DNA at 42°C for 24 hours. The membranes were washed for 60 minutes with 2X SSC/0.1% SDS at 55°C and then for 30 minutes with 0.2X SSC/0.1% SDS at 55°C and finally exposed to Fuji RxU x-ray film at −80°C.

Preparation of Nuclear Extracts

Nuclear extracts were prepared by a method previously reported[31] with slight modifications. The cells were washed with ice-cold PBS, harvested, resuspended in 400 μl of hypotonic buffer A (10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride), and kept for 15 minutes on ice. The cells were then lysed in 0.6% Nonidet P-40 by vortexing for 10 seconds. Nuclei were separated from cytosol by centrifugation at 12,000 × *g* for 30 seconds, washed with 400 μl of buffer A containing 0.6% Nonidet P-40, resuspended in buffer C (20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride), vigorously vortexed for 15 seconds, and incubated for 5 minutes on ice. This step was repeated three times. Nuclear extracts were obtained by centrifugation at 12,000 × *g* for 10 minutes. Protein concentration was measured essentially following the method of Bradford[32] using a protein dye reagent (Bio-Rad Laboratories)

Electrophoretic Mobility Shift Assays (EMSAs)

The double-stranded oligonucleotides used as probes for EMSAs were derived from the following sequences 5′ upstream of the transcriptional start site of the human IL-6 gene: activator protein 1 (AP-1), 5′-tcgacGTGCTGAGTCACTAAc-3′ (−287 to −273); multiple response element (MRE), 5′-tcgacATGCTAAAGGACGTCACATTc-3′ (−173 to −153); NF-IL6, 5′-tcgacACATTGCACAATCTTAAc-3′ (−158 to −142); IL6-κB, 5′-tcgacATGTGGGATTTTCCCATGAc-3′ (−77 to −59); IL6-κB-mt1, 5′-tcgacATGTAATATTTTCCCATGAc-3′; IL6-κB-mt2, 5′-tcgacATGTGGGATTTTAGACTGAc-3′; IL6-κB-mt3, 5′-tcgacATGTGGGATTTTCCCTCGAc-3′; immunoglobulin κ-light chain enhancer (Ig-κB), 5′-tcgacGAGGGGACTTTCCc-3′. DNA binding reaction and EMSAs were performed as described previously.[33] The 3′ ends of the oligonucleotides

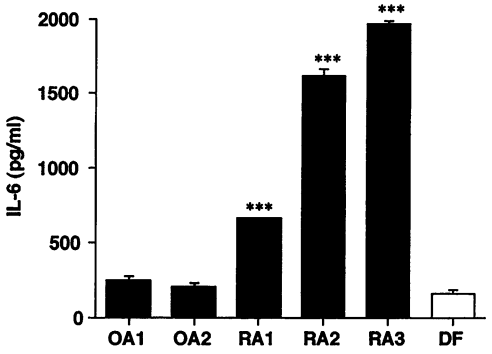

Figure 1. Spontaneous IL-6 production by FLSs obtained from patients with RA and OA, and by dermal fibroblasts (DF). FLSs obtained from two OA patients and three RA patients, as well as normal dermal fibroblasts (DF), were incubated separately in 24-well culture plates at a density of 3×10^3 cells/cm^2 for 6 hours. IL-6 concentration in the culture supernatants was assayed by a specific ELISA. IL-6 concentration in the culture medium (0.1% BSA/RPMI 1640) was below the detection limit. Data are shown as the mean of the quadruplicate cultures ± SD. ***$P < 0.001$ as compared with the FLSs from one of the OA patients (OA1).

were ^{32}P-labeled with Klenow DNA polymerase (Megaprime DNA labeling systems, Amersham). Samples of 10 μl containing 5 μg of nuclear extract were incubated with 10,000 cpm of labeled oligonucleotides and 1 μg of poly(dI-dC) in 10 mmol/L Tris (pH 7.5), 50 mmol/L NaCl, 1 mmol/L DTT, 1 mmol/L EDTA, 5% glycerol for NF-IL6, IL6-κB, and Ig-κB probes, or in 20 mmol/L HEPES (pH 7.9), 50 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L EDTA, 5 mmol/L spermidine, 5% glycerol for AP-1 and MRE probes. The samples were incubated in the presence or absence of competitor oligonucleotides for 15 minutes at room temperature and run on 4% polyacrylamide gels in 0.5X TBE (Tris-borate, EDTA) at 150 V.

Statistics

Statistical analysis was performed by analysis of variance and Scheffé's *F* test on a StatView 4.0 software program (Abacus Concepts, Berkeley, CA). A *P* value of <0.05 was considered to be significant.

Results

IL-6 Synthesis of Human FLSs and Dermal Fibroblasts

Synoviocytes were isolated from the synovial membrane of RA and OA patients as described in Materials and Methods. On the third passage, the synoviocytes became fibroblastic in appearance as determined microscopically and were thereafter referred to as fibroblast-like synoviocytes (FLSs).[34] Cells were cultured for 6 hours in RPMI 1640 containing 0.1% BSA to exclude a possible effect of serum proteins on IL-6 production, and the resulting supernatants were tested for IL-6 by the ELISA specific for it. FLSs from three RA patients spontaneously produced significant amounts of IL-6 (Figure 1). IL-6 production of FLSs obtained from two OA patients was significantly lower than that from

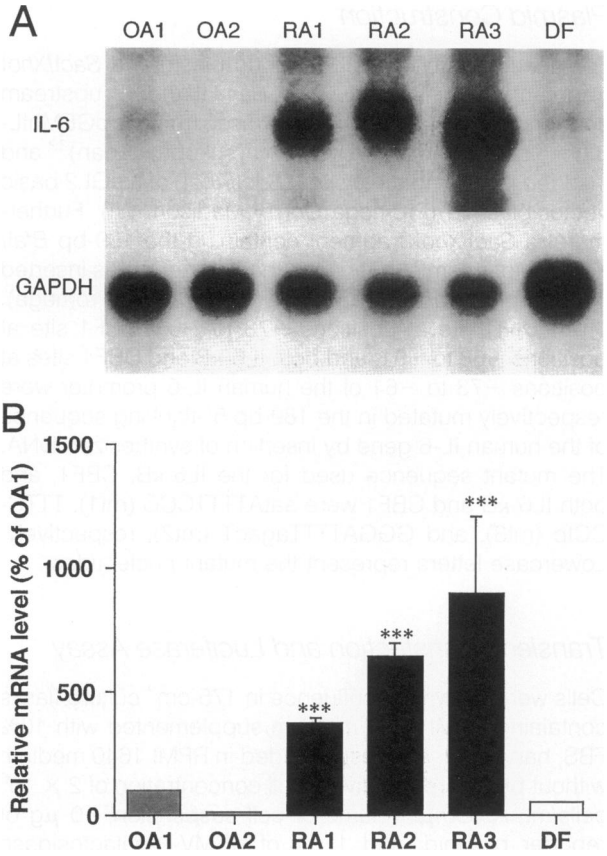

Figure 2. Enhanced IL-6 mRNA expression of RA FLSs. **A:** Total cellular RNA was extracted from FLSs obtained from two OA patients and three RA patients and from normal dermal fibroblasts (DF). Total RNA (20 μg) was fractionated on a 1.2% (w/v) agarose gel, transferred onto Hybond N nylon membranes, and then hybridized to the appropriate cDNA probes. **B:** Autoradiograms of the Northern blot shown in **A** were quantified by scanning densitometry. Relative mRNA level shows the value of IL-6 mRNA normalized with respect to GAPDH mRNA. Data are shown as the mean ± SD of three experiments. ***$P < 0.001$ as compared with the FLSs from one of the OA patients (OA1).

RA patients. Human dermal fibroblasts did not produce significant amounts of IL-6.

The findings were confirmed at the mRNA level by Northern blot analysis. The results shown in Figure 2 were obtained from the same sets of cultures indicated in Figure 1. IL-6 mRNA expression in the FLSs of RA patients was significantly greater than that in the cells of OA patients. Human dermal fibroblasts scarcely expressed IL-6 mRNA, consistent with the results obtained at the protein level.

IL-6 Synthesis of Cloned Rheumatoid FLSs

To clarify the cellular requirements for IL-6 production by rheumatoid FLSs, we established several cloned FLSs by the limiting dilution method. We first assessed the IL-6 production of these clones. Some rheumatoid FLS clones (KM01, -02, -04, -06, -07, -08, and -10; MH02 and -07; and TK01 and -09) spontaneously produced large amounts of IL-6 (Figure 3), whereas other clones (KM03, -05, and -09; MH04 and -05; and TK02 and -08) produced only small amounts of it. Commercially available

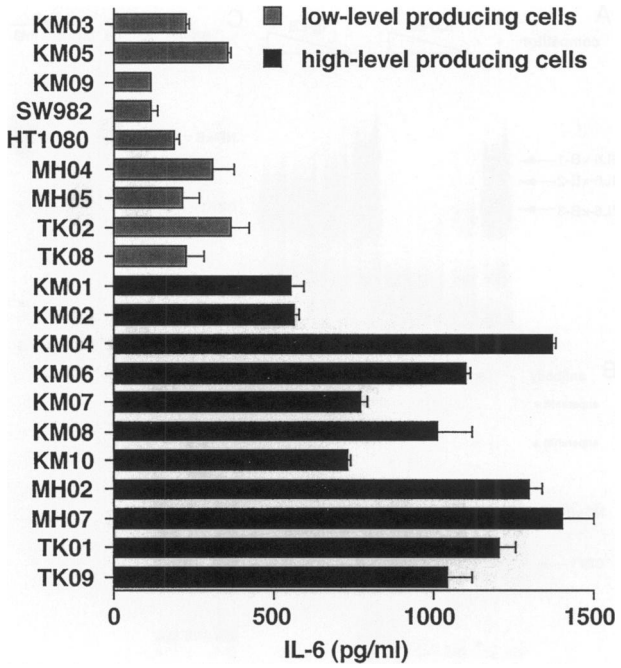

Figure 3. Spontaneous IL-6 production by cloned FLSs obtained from patients with RA, by synovial sarcoma SW982 cells, and by fibrosarcoma HT1080 cells. The FLSs from a RA patient were cloned by the limiting dilution. FLSs were incubated in 24-well culture plates at a density of 3×10^3 cells/cm^2 for 6 hours. IL-6 concentrations in the culture supernatants were assayed by a specific ELISA. IL-6 concentration in the culture medium (0.1% BSA/RPMI 1640) without cells was below the detection limit. Data are shown as the mean of quadruplicate cultures ± SD.

synovial sarcoma SW982 cells and fibrosarcoma HT1080 cells were also examined. These cells were low IL-6 producers.

Northern blot analysis was also performed, and the results indicated that IL-6 mRNA expression was significantly higher in the high-level IL-6-producing FLS clones than in the low-level IL-6-producing clones, SW982 cells, and HT1080 cells (data not shown).

Transcriptional Activities of Human IL-6 Gene Expressed in Rheumatoid FLS Clones

To determine whether IL-6 synthesis by the cloned rheumatoid FLS is up-regulated at the transcriptional level, we constructed a luciferase reporter plasmid containing the −1158 to +11 human IL-6 promoter region for the transfection of these cloned synoviocytes. The high-level IL-6-producing clones KM04, MH07, and TK01 exhibited significantly higher luciferase activity compared with the low-level IL-6-producing clones KM03, MH05, TK08, SW982 cells, and HT1080 cells (Figure 4). A basic luciferase reporter plasmid, pGL2, which contained no promoter/enhancer gene, was not transcribed by any cloned FLSs, SW982 cells, or HT1080 cells.

The findings were confirmed at the level of endogenous IL-6 gene transcription. The results shown in Figure 5 were obtained from the same sets of cultures indicated in Figure 4 (MH05, TK08, MH07, and TK01). The rate of the IL-6 gene transcription in the high-level IL-6-producing clones (MH07 and TK01) was significantly greater

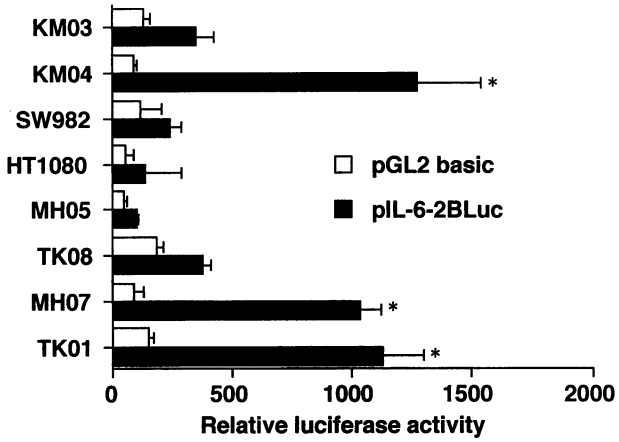

Figure 4. Transcriptional activity of the human IL-6 gene expressed in the cloned rheumatoid FLSs (KM03, KM04, MH05, TK08, MH07, and TK01), SW982 cells, and HT1080 cells. FLSs were transiently transfected with 10 μg of pGL2 basic or pIL-6–2BLuc by electroporation. FLSs were co-transfected with 1 μg of pCMV-β-galactosidase. The transfected FLSs were cultured for 18 hours and then harvested. The luciferase activity and β-galactosidase activity were analyzed. Data are shown as the mean of quadruplicate cultures ± SD. *$P < 0.001$ as compared with the cloned low-producer FLSs (KM03).

than that in the low-level IL-6-producing clones (MH05 and TK08). The rate of GAPDH gene transcription was not statistically different among the four clones. No hybridization to the vector plasmid was detected. These results clearly indicate that IL-6 synthesis of the cloned rheumatoid FLSs is enhanced at the level of gene transcription.

EMSA Analysis of Transcription Factors Expressed in the Cloned Rheumatoid FLSs

Several investigators demonstrated that the 5′ flanking region of the IL-6 gene contains several transcriptional elements such as IL6-κB site (−73 to −63), NF-IL6 site (−158 to −145), MRE (multiple response element) site (−163 to −158), and AP-1 site (−283 to −277).[35–39] To assess which elements are responsible for the constitutive transcriptional activity of human IL-6 gene expressed in the rheumatoid FLSs, we prepared nuclear extracts from low- and high-level IL-6-producing FLS clones, SW982 cells, and HT1080 cells and analyzed them by

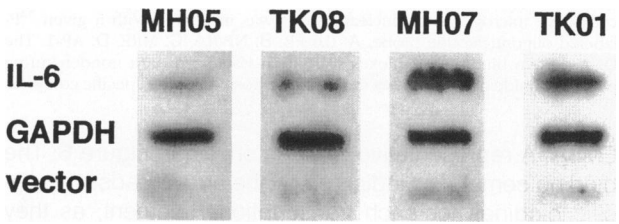

Figure 5. IL-6 gene transcription in the cloned rheumatoid FLSs (MH05, TK08, MH07, and TK01). The nuclei were isolated, and labeled transcripts were prepared by *in vitro* transcription with [α-^{32}P]UTP. Equal amounts of labeled RNA (3×10^6 cpm) were hybridized to nitrocellulose membrane containing 10 μg/lane immobilized plasmids, ie, pBluescriptII-KS(+), pBluescriptII-KS(+) with IL-6 cDNA insert, and pBluescriptII-KS(+) with GAPDH cDNA insert. Nuclear run-on assays were performed as described in Materials and Methods. An additional experiment gave essentially the same results as shown by this nuclear run-on autoradiograph.

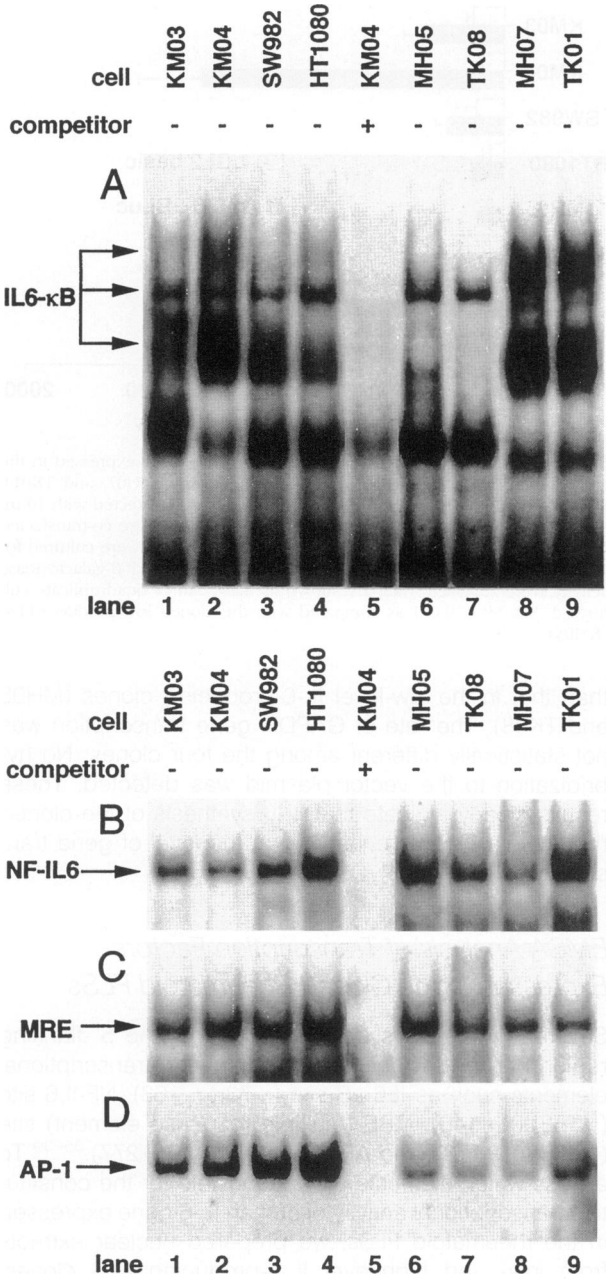

Figure 6. Electrophoretic mobility shift assays for IL6-κB, NF-IL6, MRE, and AP-1 factors in nuclear extracts obtained from cloned rheumatoid FLSs (KM03, KM04, MH05, TK08, MH07, and TK01), SW982 cells, and HT1080 cells. Five micrograms of nuclear extract was incubated with a given ^{32}P-labeled oligonucleotide probe. **A:** IL6-κB. **B:** NF-IL6. **C:** MRE. **D:** AP-1. The DNA-protein binding complexes were then visualized on a nondenaturing polyacrylamide gel. The **arrow** indicates the position of the specific complex.

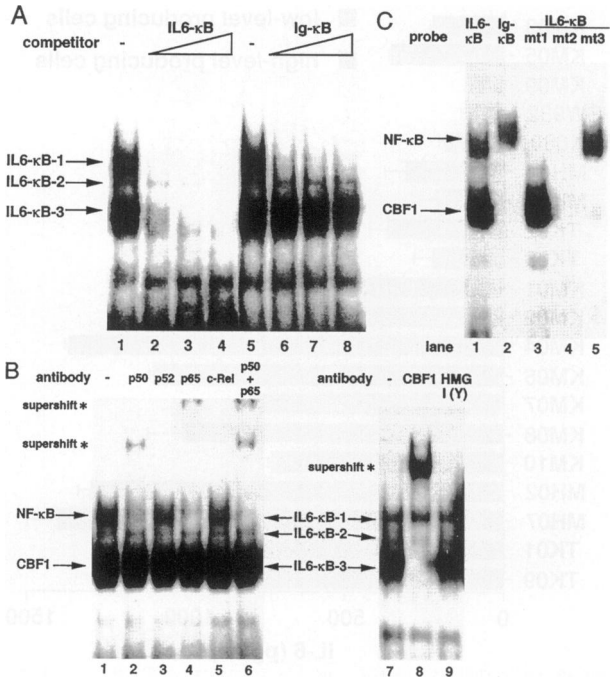

Figure 7. Electrophoretic mobility shift assays demonstrating specificity of binding to the IL6-κB site in nuclear extracts obtained from cloned rheumatoid FLSs KM04. **A:** Five micrograms of nuclear extract was incubated with a given ^{32}P-labeled oligonucleotide probe IL6-κB in the presence or absence of unlabeled IL6-κB and Ig-κB. **B:** Five micrograms of nuclear extract was incubated with a given ^{32}P-labeled oligonucleotide probe IL6-κB in the presence of anti-p50, -p52, -p65, -c-Rel, -CBF1, or -HMG I(Y) antibodies. **C:** Five micrograms of nuclear extract was incubated with a given ^{32}P-labeled oligonucleotide probe, IL6-κB, Ig-κB, or IL6-κB-mt1, -mt2, and -mt3. The DNA-protein binding complexes were then visualized on a nondenaturing polyacrylamide gel. The **asterisk** indicates a supershift. The **arrow** indicates the position of the specific complex.

EMSA. A representative result is shown in Figure 6. The binding complexes indicated by the arrowheads are specific bindings for each transcriptional element, as they were diminished by the addition of excess amounts of unlabeled oligonucleotides (lane 5). IL6-κB-binding activity of high-level IL-6-producing clones KM04, MH07, and TK01 (lanes 2, 8, and 9) was significantly higher than that of the low-level producing clones KM03, MH05, and TK08 (lanes 1, 6, and 7), SW982 cells (lane 3), or HT1080 cells (lane 4). The levels of NF-IL6-, MRE-, and AP-1-binding activities were not significantly different among these cells.

The three retarded bands binding to the IL6-κB site were designated as IL6-κB-1, IL6-κB-2, and IL6-κB-3 (Figure 7A). Only IL6-κB-1 complex was competed out by unlabeled Ig-κB oligonucleotide (lanes 6 to 8) whereas IL6-κB-2 and -3 complexes were not affected (lanes 6 to 8). We next performed supershift experiments employing antibodies against p50, p52, p65, c-Rel, CBF1, and HMG I(Y) to identify individual proteins in EMSA (Figure 7B). The IL6-κB-1 complex consisted of p50 and p65 NF-κB subunits (lanes 2, 4, and 6), as supershifts were observed with anti-p50 and anti-p65, but not with anti-p52 or anti-c-Rel, antibodies. In addition, the IL6-κB-3 binding complex contained a CBF1 molecule, as supershifts were observed with anti-CBF1, but not with anti-HMG I(Y) antibodies.

Requirement of NF-κB and CBF1 for Constitutive Transcription of the Human IL-6 Gene in Rheumatoid FLSs

To analyze functional significance of NF-κB and CBF1 bindings, we constructed luciferase vectors including the human IL-6 promoter region with site-specific mutations (mt1 to mt3). The loss of binding activity of NF-κB (mt1) or

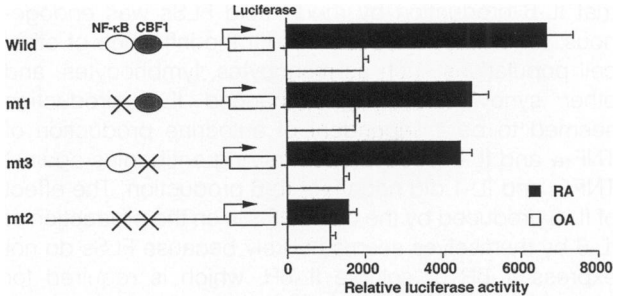

Figure 8. Functional cooperativity of IL6-κB and CBF1 motifs in the human IL-6 gene promoter. The diagram at the left shows wild-type and mutant IL-6 promoter constructs. IL6-κB and CBF1 elements are shown schematically. FLSs from RA patients (■) or from OA patients (□) were transfected with 10 μg of the designated luciferase construct. FLSs were co-transfected with 1 μg of pCMV-β-galactosidase as a transfection control. The transfected cells were cultured for 18 hours and then harvested. The luciferase activity and β-galactosidase activity were analyzed. Data are shown as the mean of quadruplicate cultures ± SD.

CBF1 (mt3) or both NF-κB and CBF1 (mt2) was confirmed by EMSA (Figure 7C). RA FLSs were transfected with each vector, and the luciferase activity was measured. As shown in Figure 8, the luciferase activity decreased by 37% in the NF-κB mutant, 43% in the CBF1 mutant, and 100% in the NF-κB and CBF1 double mutant. These results suggest that both NF-κB and CBF1 act as transcriptional up-regulators for the IL-6 gene in rheumatoid FLSs. In contrast, OA FLS, a low-level IL-6-producing FLS, did not significantly transcribe the luciferase gene driven by the IL-6 promoter.

Effects of Anti-TNF-α and Anti-IL-1 Neutralizing Antibodies on Spontaneous Production of IL-6 by Cloned Rheumatoid FLSs

As there is a possibility that spontaneous production of IL-6 may be caused by cytokines such as TNF-α, IL-1α, and IL-1β produced by the FLSs themselves, the effects of TNF-α and IL-1 neutralizing antibodies on IL-6 production were analyzed. However, neither anti-human TNF-α nor IL-1 antibody affected the IL-6 production (Table 1). Furthermore, IL-6 production by FLSs was not affected by the combination of both TNF-α and IL-1 antibodies (data not shown). The concentrations of the neutralizing antibody preparations were determined according to the manufacturers' data sheets. The amount used, 10 μg/ml, of anti-TNF-α or anti-IL-1 antibody was sufficient to block IL-6 production by FLSs induced by 1 ng/ml TNF-α or IL-1α (or IL-1β; data not shown). TNF-α and IL-1β were undetectable (<6.25 pg/ml) in the culture supernatants of FLSs obtained from either OA or RA patients, or in those of dermal fibroblasts, synovial sarcoma SW982 cells, or fibrosarcoma HT1080 cells.

Effects of Dexamethasone on Spontaneous Production of IL-6 by Cloned Rheumatoid FLSs

The effect of dexamethasone on IL-6 production by FLS clones was also investigated. A representative result is shown in Table 2. Cells were incubated for 6 hours in the presence of dexamethasone. Dexamethasone (10^{-10} to 10^{-6} mol/L) concentration-dependently inhibited IL-6 production by the FLS clones. Dexamethasone did not affect the viability of cells after 24 hours of incubation at the concentrations used in this experiment, thereby excluding nonspecific toxicity of the agent (data not shown). These results clearly indicate that dexamethasone directly acted on rheumatoid FLSs to suppress IL-6 synthesis.

Table 1. Effects of Anti-TNF-α and Anti-IL-1 Antibodies on the Spontaneous Production of IL-6 by High-Producer Clones of Rheumatoid FLSs

	IL-6 (pg/ml)		
Clone	None	Anti-TNF-α antibody*	Anti-IL-1β antibody*
KM04	1202 ± 117	1444 ± 49	1339 ± 137
KM06	1227 ± 57	1073 ± 20	1265 ± 7
KM08	1547 ± 51	1596 ± 23	1405 ± 72
MH02	1297 ± 40	1406 ± 25	1339 ± 46
MH07	1403 ± 97	1445 ± 139	1397 ± 110
TK01	1201 ± 56	1306 ± 13	1275 ± 140
TK09	1044 ± 77	1019 ± 46	975 ± 100

FLSs were preincubated in 24-well culture plates at a density of 3×10^3 cells/cm^2 for 18 hours in the presence or absence of neutralizing antibodies and then suspended in fresh medium containing the same antibodies for 6 hours. IL-6 concentrations in the culture medium were assayed by a specific ELISA. IL-6 concentration in the culture medium (0.1% BSA/RPMI 1640) was below the detection limit. Data are shown as the mean of quadruplicate cultures ± SD.

*Either 10 μg/ml anti-TNF-α or 10 μg/ml anti-IL-1 antibody was added to the designated cultures.

Discussion

Our present study clearly demonstrated that human rheumatoid FLSs spontaneously produced large amounts of IL-6 *in vitro* compared with OA FLSs and normal dermal fibroblasts (Figures 1 and 2). IL-6 production by rheumatoid FLSs was endogenously up-regulated, as several FLS clones devoid of the influence of other cell populations still produced IL-6 as efficiently as bulk synoviocytes (Figure 3), and anti-TNF-α and/or IL-1 antibodies did not affect the IL-6 production (Table 1). We also

Table 2. Effects of Dexamethasone on the Spontaneous Production of IL-6 by High-Producer Clones of Rheumatoid FLSs

	IL-6 (pg/ml)		
	KM04	MH07	TK01
Treatment			
Control	1578 ± 90	1408 ± 116	1017 ± 66
DEX 10^{-10} mol/L	1491 ± 115	1381 ± 8	966 ± 41
DEX 10^{-9} mol/L	1256 ± 42*	775 ± 77*	586 ± 28*
DEX 10^{-8} mol/L	955 ± 17*	395 ± 4*	320 ± 13*
DEX 10^{-7} mol/L	879 ± 35*	333 ± 7*	183 ± 8*
DEX 10^{-6} mol/L	868 ± 32*	317 ± 7*	151 ± 16*

FLSs were incubated with different doses of dexamethasone (DEX) for 6 hours. IL-6 concentrations in the culture medium were assayed by a specific ELISA. IL-6 concentration in the culture medium (0.1% BSA/RPMI 1640) was below the detection limit. Data are shown as the mean of quadruplicate cultures ± SD.

*$P < 0.001$ as compared with the corresponding control value.

demonstrated that the IL-6 production was up-regulated at the level of gene transcription (Figures 4 and 5). EMSAs and reporter gene assays indicated that transcriptional factors NF-κB and CBF1 were involved in the dysregulation of the IL-6 gene in these rheumatoid FLSs (Figures 6, 7, and 8).

RA is characterized by the persistent inflammation of peripheral synovial joints and the progressive destruction of cartilage and bone.[40] The cause of inflammation is yet to be clarified, but autoimmunity and infectious agents such as Epstein-Barr virus (EBV), human T-cell lymphotropic virus type I (HTLV-I), parvovirus, and cytomegalovirus have been implicated as major factors.[1,41] Collagen type II and IgG have been nominated as autoantigens.[1] Recently, it was reported that *in vivo* blockade of either TNF-α or IL-6 signals by the administration of anti-TNF-α chimeric monoclonal antibody (cA2) or anti-IL-6 monoclonal antibody, respectively, to RA patients was clinically very effective.[42,43] The clinical efficacy of these blocking antibodies strongly indicates that correction of the dysregulation in cytokine production may be a rational approach for the treatment of RA, even though the pathogenesis of RA remains obscure.

TNF-α and IL-1 were reportedly expressed in RA joints.[44] Other pro-inflammatory cytokines produced locally include chemokines such as IL-8[45] and RANTES,[46] which attract neutrophils and macrophages to the inflamed joints. IL-6 is also produced by the synovial tissue of RA patients.[8] IL-6 induces the production of acute-phase proteins by hepatocytes.[21] It also facilitates the differentiation of B cells and may contribute to the production of antibodies (hence immune complexes) that are pro-inflammatory.[17] Moreover, IL-6 is involved in FcγRI expression of monocytes through the induction of STAT family factors.[47] The excessive production of IL-6 seems to be related to immunological abnormalities associated with RA. So, in view of the above findings, IL-6 production of FLSs derived from RA joints was investigated in this study.

Although Guerne et al[12] have reported that spontaneous IL-6 production by cultured synoviocytes is not different between RA and OA, our present study is the first to demonstrate that the amount of IL-6 production by RA FLSs was significantly higher than that by OA FLSs (Figure 1). The discrepancy between our results and theirs might be due to several experimental conditions as follows: 1) we compared IL-6 production by FLSs without any stimulation by factors such as cytokines, growth factors, and serum, and 2) we directly measured IL-6 protein by a specific ELISA. There still remains a possibility that, in the case of Guerne et al, IL-6 was produced in response to other cytokines, such as TNF-α and IL-1 secreted from other synoviocyte populations, as these cytokines are strong inducers of IL-6 synthesis *in vitro*.[48–50] Our present investigation employing cloned rheumatoid FLSs is the first study to elucidate the cellular and molecular mechanisms of IL-6 overproduction at the single-cell population level.

The most outstanding finding of our present study is that IL-6 production of the FLSs is enhanced at the clonal level, at the single-cell level. Our findings clearly indicate that IL-6 production by rheumatoid FLSs was endogenous; ie, it occurred even without the influence of other cell populations such as monocytes, lymphocytes, and other synoviocytes. The enhanced IL-6 production seemed to be independent of autocrine production of TNF-α and IL-1 because neutralizing antibodies against TNF-α and IL-1 did not affect IL-6 production. The effect of IL-6 produced by the cloned FLSs on the production of IL-6 by themselves seems unlikely because FLSs do not express IL-6R or soluble IL-6R, which is required for mediating the IL-6 signal.[16,51] Establishment of the cloned FLSs from OA patients was not successful, probably because OA synoviocytes proliferated less vigorously than RA synoviocytes. Although IL-6 production *ex vivo* might not always be indicative of processes relevant *in vivo*,[52] IL-6 production of the rheumatoid FLSs was significantly enhanced compared with that of OA synoviocytes (Figure 1), suggesting that the autonomously up-regulated IL-6 production by rheumatoid FLSs might be involved in the rheumatoid pathogenesis.

Northern analysis showed that IL-6 mRNA expression of the high-level IL-6-producing FLS clones was significantly enhanced compared with that of the low-level IL-6-producing clones (data not shown), suggesting that IL-6 synthesis is up-regulated at the transcriptional level. Indeed, a luciferase reporter plasmid containing the −1158 to +11 human IL-6 promoter region was highly transcribed when transfected into the high-level IL-6-producing FLS clones (Figure 4). Furthermore, this finding was confirmed at the level of endogenous gene transcription by the nuclear run-on assay (Figure 5). These data indicate that the spontaneous production of IL-6 by rheumatoid FLSs results from the endogenous up-regulation of IL-6 gene transcription. Therefore, we performed EMSAs to examine which *cis*-acting transcription factors contribute to the enhanced IL-6 gene transcription of rheumatoid FLSs.

The 350-bp gene segment 5′ upstream of the transcription start site is highly homologous between human and mouse IL-6 genes.[53] Several regulatory elements, such as IL6-κB, NF-IL6, MRE, and AP-1, have been identified within the conserved IL-6 promoter region. The IL-1-responsive element was mapped within the IL-6 promoter region (−180 to −123), including the NF-IL6 site.[35] Ray et al[36] showed that an IL-6 MRE (−173 to −151) is responsible for the induction of the IL-6 gene by TNF-α and IL-1β as well as by activators of protein kinase A (forskolin) and protein kinase C (phorbol ester). A cAMP-responsive element (CRE) motif is also located in the MRE region. It has also been reported that an IL6-κB binding motif (−73 to −64) is responsible for IL-6 gene induction.[37–39] The results of EMSAs shown in Figure 6 clearly demonstrated that IL6-κB-binding activity was enhanced in the high-level IL-6-producing clones, whereas NF-IL6-, MRE-, and AP-1-binding activities were not significantly different among high- and low-level IL-6-producing cells. Supershift experiments identified that the IL6-κB binding complex observed in the high-level IL-6-producing clone comprised p50 and p65 NF-κB subunits (Figure 7A). Only very faint expression of p50 subunit and no expression of p65 subunit was observed in low-level

IL-6-producing cells (K. Miyazawa, unpublished results). Previous studies have established that the p65 subunit of NF-κB is responsible for the transactivation due to the existence of a potent transactivation domain located in its carboxyl-terminal portion.[54–56] We assume that constitutive expression of the p65 subunit NF-κB may lead to the enhanced IL-6 transcriptional activity of rheumatoid synoviocytes. Our finding is consistent with the report that p65 NF-κB is activated in the synovium biopsy specimens obtained from RA patients.[57,58]

CBF1, another transcription factor, might also be involved in the increased IL-6 gene transcription of RA FLSs, as CBF1-binding activity was also enhanced in the high-level IL-6-producing clone. The mutation introduced in the CBF-1 motif reduced the transcriptional activity of the IL-6 promoter gene. CBF1 (EBV C-promoter binding factor 1) is a DNA-binding protein that is targeted by the viral transactivator EBNA2 (EBV nuclear antigen 2) on EBV infection of human B lymphocytes.[59] CBF1 is also known as RBP-Jκ (recombination binding protein Jκ) involved in the rearrangement of the immunoglubulin V(D)J gene,[60] although conflicting data were reported more recently.[61] Constitutively active human Notch1 binds to CBF1 and activates transcription through the CBF-1-responsive element.[62] On the other hand, CBF1 also functions as a negative regulator of the IL-6 gene in a mouse cell line.[63] However, our data clearly indicate that CBF1 acts as a positive regulator of human IL-6 gene transcription in rheumatoid FLSs (Figure 8). The differences in cell species and/or phenotypes used in each study may account for the discrepancy of the CBF1 function. In addition, the double mutant of NF-κB- and CBF1-binding elements exhibited further loss of transcriptional activity compared with the single mutants of either the NF-κB- or CBF1-binding element. These results suggest that the cooperation of these two factors plays an important role in the transcription of the IL-6 gene. Additional studies are required to determine whether CBF1 is activated in the rheumatoid synovium tissues *in vivo*.

IL-1β, a potent inducer of IL-6, further stimulated IL-6 production by cloned FLSs over the level found for the unstimulated FLSs (data not shown). In addition, dexamethasone significantly inhibited IL-6 production by the high-level IL-6-producing FLS clones (Table 2). Thus, IL-6 production by these rheumatoid FLS clones was functionally regulated in response to both an IL-6 inducer and an inhibitor. The NF-κB element in the IL-6 promoter might be the target of the regulation, as molecular interaction between the glucocorticoid receptor and NF-κB have been reported.[64,65] Thorough elucidation of glucocorticoid action on the IL-6 gene in these RA FLSs is warranted, perhaps by investigations including various mutagenesis experiments.

It has been postulated that FLSs have two possible routes for activation.[7] FLSs could respond passively to factors produced by macrophages and T cells, or alternatively, could be irreversibly altered in rheumatoid status to autonomous activation, as RA FLSs, in some circumstance, proliferate in an anchorage-independent manner.[66] The present findings seem to largely extend our knowledge as to the pathophysiology of rheumatoid synovium in showing that the rheumatoid FLSs were endogenously activated in the absence of an inflammatory environment, although they still may respond to signals such as cytokines and adhesion molecules provided by other cell types. Thorough understanding of the cellular and molecular mechanisms involved in the spontaneous activation of NF-κB and CBF1 warrants further investigation and will provide promising potential for the novel treatment of RA.

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