Involvement of cAMP responsive element binding protein (CREB) in the synovial cell hyperfunction in patients with rheumatoid arthritis

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Abstract

Objective

To elucidate a possible role of cAMP responsive element binding protein (CREB) in rheumatoid arthritis (RA) synovial cell function, we have studied CREB expression of synovial cells and the effects of an inhibitor of the cAMP/CREB signal pathway on synovial cell function in patients with RA.

Methods

We examined CREB expression by immunohistochemical staining, immunocytochemical staining, and gel shift assays. Effects of cAMP/CREB inhibitor on the proliferation of RA synovial cells were assessed by [^{3}H]-TdR incorporation, and those on proinflammatory cytokine and matrix metalloproteinase (MMP) production by reverse transcription PCR and ELISAs.

Results

Immunohistochemical staining of synovial tissue revealed that CREB is expressed mainly in the lining and sublining layers of synovium in patients with RA. DNA binding activity of CREB was ascertained by a gel shift assay. We also confirmed nuclear translocation and phosphorylation of CREB in TNF-α stimulated RA fibroblast-like synovial cells by immunocytochemical staining. Modulators of cAMP/CREB signaling pathway, such as Rp-cAMP, had an inhibitory potential on RA synovial cell proliferation in vitro. Rp-cAMP also inhibited the proinflammatory cytokine and MMP production.

Conclusion

CREB is involved in the synovial cell activity in patients with RA. Inhibition of CREB activity by its inhibitor brings about the correction of aberrant synovial cell functions in patients with RA, thus suggesting a possible clinical application of cAMP/CREB inhibitors.

Key words

Rheumatoid arthritis, synovial cells, cyclic adenosine 3', 5'-monophosphate (cAMP), cAMP responsive element binding protein (CREB).

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by joint swelling, synovial inflammation, and joint destruction. Hypergammaglobulinemia and autoantibody production against the IgG Fc portion are common in RA patients, suggesting autoimmune components of this disease (1-4). Hyperplasia of the synovial lining cell layer and damage of articular cartilage are major morphologic features of RA and represent an important determinant of disease progression (1, 4). In particular, activation and proliferation of fibroblast-like synovial cells contribute to pannus formation, which then leads to joint destruction in patients with RA (4). In addition, proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor- (TNF-), matrix metalloproteinase (MMP)s, reactive oxygen species and neutrophil-derived elastase play important roles in the cartilage erosion observed in the affected joints of patients with RA (5-8).

Cyclic adenosine 3', 5'-monophosphate (cAMP) is one of the intracellular signaling molecules of many cell types. Elevation of intracellular cAMP levels can result in either stimulation or repression of specific gene expression, and most of these genes contain one or more cAMP responsive element (CRE). cAMP binds to the regulatory subunit of protein kinase A (PKA) and releases the active catalytic subunit (9). This subunit phosphorylates the transactivation domain of CRE-binding protein (CREB), which then induces the expression of genes containing CREs. In addition, it has been demonstrated that CREB can be phosphorylated and transcriptionally activated by Ca^{2+} signals acting through Ca^{2+}/calmodulin-dependent protein kinases I and II and protein kinase C (10-12).

A number of CRE binding proteins have been described, including CREB, CRE modulator (CREM), and several activating transcription factors (ATFs). The CREB proteins are basic leucine zipper transcription factors and are active as either homo- or heterodimers. Some of the ATFs heterodimerize with members of the Jun/Fos family of proteins (13-15). Although the exact cause of the patho-logical processes in this chronic inflammatory joint disease is not fully understood, elucidation of the transcription factors which are responsible for abnormal proliferation of and excessive production of cytokines and MMPs by synovial cells are important for the analysis of pathological processes of RA, and such transcription factors are potential targets for therapeutic intervention in patients with RA (16). It has been reported that NF-kB and AP-1 (including c-fos and c-jun) play important roles in the pathological processes in patients with RA (16, 17). Thus, it is of interest to test whether CREB also plays an important role in the pathological responses of patients with RA. We have recently found that multiple transcription factors, including CREB, are simultaneously expressed in RA, but not OA synovial tissue (18).

In the present study, we have examined *in vitro* the involvement of CREB in the synovial cell function of patients with RA. We confirmed that CREB are involved in the inflammatory responses of RA synovial cells, and thus it is suggested that inhibitors of the cAMP/CREB pathway may have a clinical application in the treatment of patients with RA.

Patients and methods

Patients and materials

Ten patients with RA, as defined by the revised criteria of the American College of Rheumatology (19), were recruited into the study. The characteristics of the patients were as follows: 2 were male and 8 were female; their mean age ± SD was 58.4 ± 12.8 years; all were and/or had been positive for rheumatoid factors. Most of the patients were receiving non-steroidal anti-inflammatory and/or disease modifying anti-rheumatic drugs (Table I). None had been treated with high doses of corticosteroids, cytotoxic drugs nor immunosuppressants. Approval for the study was obtained from the human studies committee and individual informed consent from each patient before we began this study.

Synovial tissues were obtained from the joints of patients undergoing joint replacement or synovectomy of various joints. The procedures included knee synovectomy in 2 patients, wrist syn-

Table I. Characteristics of the RA patients at the time of the present study.

Pt.	Sex	Age (yrs.)	Onset (yrs.)	RF (U/ml)	CRP (mg/dl)	ESR (mm/hr)	RA stage	Treatment
1	F	52	12	5	1.65	10	4	SASP
2	F	70	22	541	2.81	45	4	SASP
3	M	57	20	12	3.23	28	4	Steroids*
4	F	55	15	38	6.88	110	4	BUC
5	F	49	7	138	10.13	97	4	BUC
6	F	71	20	747	5.78	84	4	Gold
7	F	66	8	306	1.65	112	4	MTX, steroids
8	F	55	3	150	3.89	77	4	Steroids
9	F	51	20	15	5.17	25	4	MTX, steroids
10	M	66	24	77	0.18	60	4	Gold

RF: rheumatoid factor; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; RA: rheumatoid arthritis; MTX: methotrexate; SASP: sarazosulfapyridine; BUC: bucillamine; NSAID: nonsteroidal antiinflammatory drugs. *steroids = prednisolone (< 10 mg/day).

ovectomy in 3, and total knee replacement in 5.

The medium used in this study was Iscove-modified Dulbecco medium (IMDM; Flow Laboratories Inc., Rockville, MD) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) (Life Technologies Inc., Tokyo, Japan) and 10% fetal calf serum (FCS) (Life Technologies, Inc).

Normal rabbit IgG, normal mouse IgG, anti-CREB antibody (Upstate Biotechnology, New York, NY), and anti-serine-133 phosphorylated CREB specific antibody (BioLabs, Beverly, MA) were used in this study. Recombinant IL-1 and TNF-α (both from Boehringer Mannheim GMBH, Mannheim, Germany) were also used. Adenosine-3', 5'-cyclic monophosphorothioate, Rp-isomer (MW, 446.5; Rp-cAMP) were obtained from Biolog Life Science Institute (Bremen, Germany). Rp-cAMP was initially diluted in PBS to 1 mM, filter sterilized, and stored at -20°C until use. To obtain fibroblast-like synovial cell lines, synovial tissue cells were isolated by enzymatic dispersion of synovial tissues obtained from patients with RA undergoing joint surgery (18, 20). The tissues were minced into 2-3 mm pieces and digested with 1 mg/ml of collagenase for 3-4 hr at 37 °C in serum-free IMDM, followed by trypsin treatment for 1 hr. The resultant cell suspension was filtered to remove fragments of undigested synovium. Thereafter, the cells were cultured at 2 x 10^4 cells/ml in the medium overnight (20). Non-adherent cells were re-

moved and adherent cells were subsequently harvested from the plates using trypsin/EDTA. The cells were further cultured, and at confluence were trypsinized, split at a 1:3 ratio, and recultured in medium. Fibroblast-like synovial cells were used from passages 4 through 8 in these experiments, during which time they represented a homogeneous population of fibroblast-like synovial cells.

Assays

Nuclear extracts and gel shift assays. Nuclear proteins were prepared as described previously (21, 22). Briefly, cells were resuspended in buffer A (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF). The samples were homogenized with a micropestle and centrifuged to sediment the nuclei. The nuclear pellet was resuspended in buffer C (20 mM HEPES [pH 7.9], 1.5 mM MgCl$_2$, 0.45 M NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 20% [vol./vol.] glycerol). After centrifugation, the supernatants were collected (22). Protein concentrations were determined by the Bradford method with a protein assay kit (Bio-Rad Laboratories, Hercules, CA). A gel shift assay was performed as previously described (18, 23). In brief, ^{32}P-labeled DNA fragments were incubated at room temperature for 15 min with 5 to 10 mg of nuclear protein. Protein-DNA complexes were separated from free probe on a 6% or 8% polyacrylamide gel. Thereafter, the gels were ex-

posed to X-ray film. We verified that a 20-fold excess of specific cold oligonucleotide did compete with the binding of the protein to the ^{32}P-labeled probe, whereas a similar excess of unlabeled irrelevant probe did not, and that anti-CREB Ab abolished the DNA binding (data not shown). A double stranded oligonucleotide of the CREB binding site gATTggCTgACgTCAgAgAgCT was obtained from Stratagene and was labeled with ^{32}P using T4 polynucleotide kinase (23).

Proliferation assays. Triplicate cultures in 96-well flat bottomed microtiter plates (Costar Data Packaging Corp., Cambridge, MA) of RA synovial cells were established in a total volume of 200 ml IMDM with 10% FCS and incubated at 37°C. Synovial cells were pre-cultured with various concentrations of cAMP/CREB inhibitor for 1 hr, followed by stimulation with or without TNF-α 2 ng/ml. The cells were further cultured for 72 hr. The proliferative response was measured by the incorporation of [^{3}H]-TdR (1 μCi/well; Amersham International, Buckinghamshire, England) during the last 16 hours of the cultures.

Limiting dilution reverse transcription polymerase chain reaction (RT-PCR) analysis. IL-6, IL-8, and MMP-1 mRNA expression of RA synovial cells were estimated by a, RT-PCR based technique (18, 20). Briefly, RA fibroblast-like synovial cells were pre-incubated for 1 hr with Rp-cAMP, followed by stimulation with TNF-α (2 ng/ml) for 16 hr. Total RNA was extracted from the RA synovial cells and was cDNA synthesized. β-actin primers were used to compare and monitor the efficiency of cDNA synthesis between different samples (18, 20). The primers used in this study have been reported (20), and the PCR products of β-actin, IL-6, and IL-8 are 314 bp, 621 bp, and 298 bp, respectively. For all reactions, temperature cycling was as follows: step 1, 94°C 1 min; step 2, 55°C 1 min; and step 3, 72°C 2 min. Steps 1 through 3 were repeated 35 times, followed by 72°C for 10 min.

We adopted RT-PCR analysis using limiting dilutions of cDNA to compare accurately the relative amounts of mRNA expression in different samples, as previously reported (20, 24, 26). In addi-

tion, RNA concentrations of the clinical samples were quantified using a Gene-Quant RNA/DNA calculator (Pharmacia Biotech Ltd, Cambridge, England).

ELISAs for cytokine and MMP-1 production. We had previously determined the optimal culture conditions for cytokine and MMP production by RA synovial cells, and used the same conditions in the present study (18, 20). RA fibroblast-like synovial cells were cultured in 48-well culture dishes. After a 3-hr culture period to induce adherence, synovial cells were stimulated for 24 hr with re-combinant TNF- 2 ng/ml. After termination of the cell culture, culture supernatants were recovered and were stored at -70°C. Production of IL-6 (BioSource International, Camarillo, CA) and IL-8 (R & D systems, Minneapolis, MN) were measured using commercial ELISA kits. MMP-1 production was estimated by a commercial ELISA kit (Amersham International).

Immunohistochemical staining. Specimens of RA synovium were embedded in compound, and sectioned onto microscope slides at a thickness of 5 μm. The specimens were fixed with cold acetone for 15 min. All subsequent procedures were performed using a DAKO LSAB kit (DAKO Co., Carpinteria, CA), and following the instructions of the manufacturer. Endogenous peroxidase activity was exhausted by incubation with hydrogen peroxide, and then incubated with goat serum for 30 min. The control, normal rabbit IgG or anti-CREB Ab, was applied to the tissue overnight at 4°C. The reactivity was visualized with an streptavidin-biotin complex system and counterstained with hematoxylin (18, 20).

Immunocytochemical staining. RA fibroblast-like synovial cells were cultured in micro-chamber slides (Nalge Nunc, Naperville, IL) and allowed to adhere for 7 hr. They were pre-incubated with or without cAMP/CREB inhibitor for 1 hr, followed by stimulation with TNF- for up to 24 hr. After cell culturing, the samples were fixed with cold acetone for 15 min and blocked with 2% skim milk for 30 min. The samples were incubated with the first Ab for overnight at 4°C. All subsequent procedures were performed using a DAKO LSAB kit.

Results

Immunohistochemical staining of CREB in RA synovial tissue

In order to clarify whether CREB is involved in the pathogenesis of RA, we first studied whether CREB is expressed by some cell types within the RA synovium. Sections of RA synovial tissue samples were first treated with rabbit anti-CREB Ab. The reaction was visualized using the streptavidin-biotin system with hematoxylin counterstaining. We found CREB positive cells to be prevalent within the RA synovial lining and sublining cells (Fig. 1), in accordance with our previous findings (18). Of note is the fact that the nuclei of some of the synovial cells were positive for CREB. In contrast, RA synovium infiltrating lymphocytes, RA peripheral blood lymphocytes and normal peripheral blood lymphocytes did not express CREB protein (data not shown). We have already reported that osteoarthritis synovial tissue do not express CREB (18). Thus, CREB expression was specific for RA synovial tissue.

cAMP responsive element binding activity of nuclear extracts of RA fibroblast-like synovial cells

To further confirm that CREB expressed on RA synovial cells really has DNA binding activity to the relevant DNA se-quence, we analyzed CREB by a gel shift assay of RA synovial cells. RA synovial cells were treated with TNF- for various periods. Nuclear extracts were prepared as described in the Patients and methods section, and a gel shift assay was performed with a double-stranded oligonucleotide probe containing the consensus DNA binding element for CREB. As shown in Figure 2, DNA binding activity of CREB was evident in TNF- stimulated fibroblast-like synovial cells in patients with RA; the CREB appeared immediately (1 hr) after pro-inflammatory cytokine stimulation, and thereafter CREB was gradually decreased. We have already reported that osteoarthritis synovial cells did not show any DNA binding activity of CREB in a gel shift assay (18). Thus, the DNA binding activity of CREB was specifically expressed in RA synovial tissue.

Effects of a cAMP/CREB inhibitor on nuclear translocation and phosphorylation of CREB in RA fibroblast-like synovial cells

We first examined the nuclear translocation of CREB pre-existing within the cytoplasm in response to TNF- stimulation in fibroblast-like synovial cells of RA patients. To this end, RA fibroblast-like synovial cells were incubated with TNF- 2 ng/ml for 0 to 24 hr. Nuclear
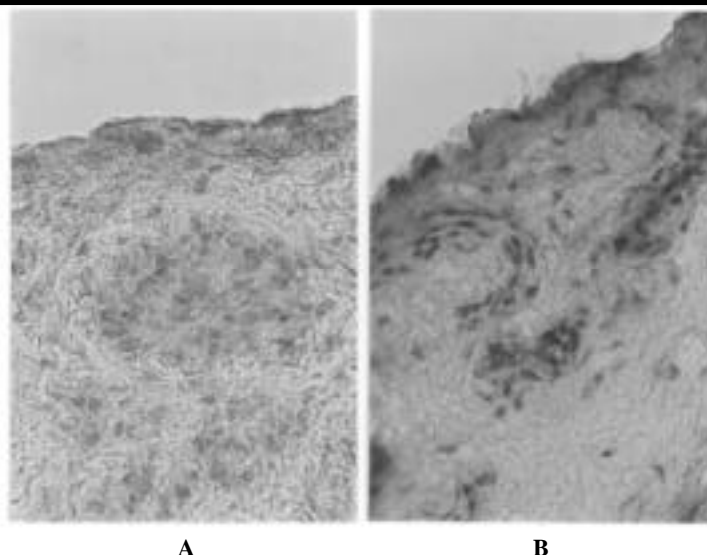

| A | B |

Fig. 1. Immunohistochemical staining of cAMP responsive element binding protein (CREB) in RA synovial tissue. Tissue samples were first treated with rabbit anti-CREB antibody. The reaction was visualized using a streptavidin-biotin system with hematoxylin counterstaining. **A**: negative control staining; **B**: CREB staining of synovial cells in RA synovial tissue (original magnification x25). Results shown are representative of 10 independent experiments.
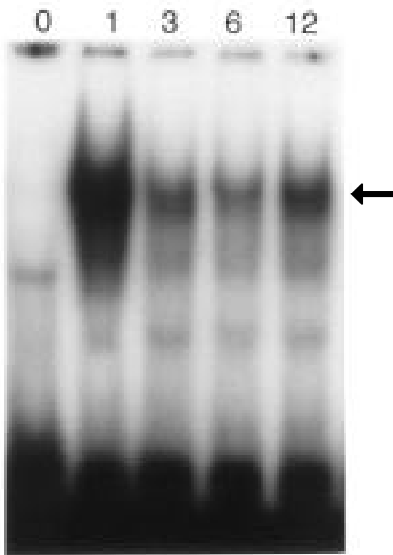
Fig. 2. A gel shift assay of CREB in nuclear extracts of TNF-α-stimulated fibroblast-like RA synovial cells.

RA synovial cells were stimulated with TNF-α for various periods of time. Nuclear extracts were prepared as described in *Patients and Methods*, and a gel shift assay was performed using a double-stranded oligonucleotide probe containing the consensus DNA binding element for CREB. The results shown are representative of 3 independent experiments. The arrow indicates the protein-DNA complex. The numbers at the top indicate the culture periods in hours.
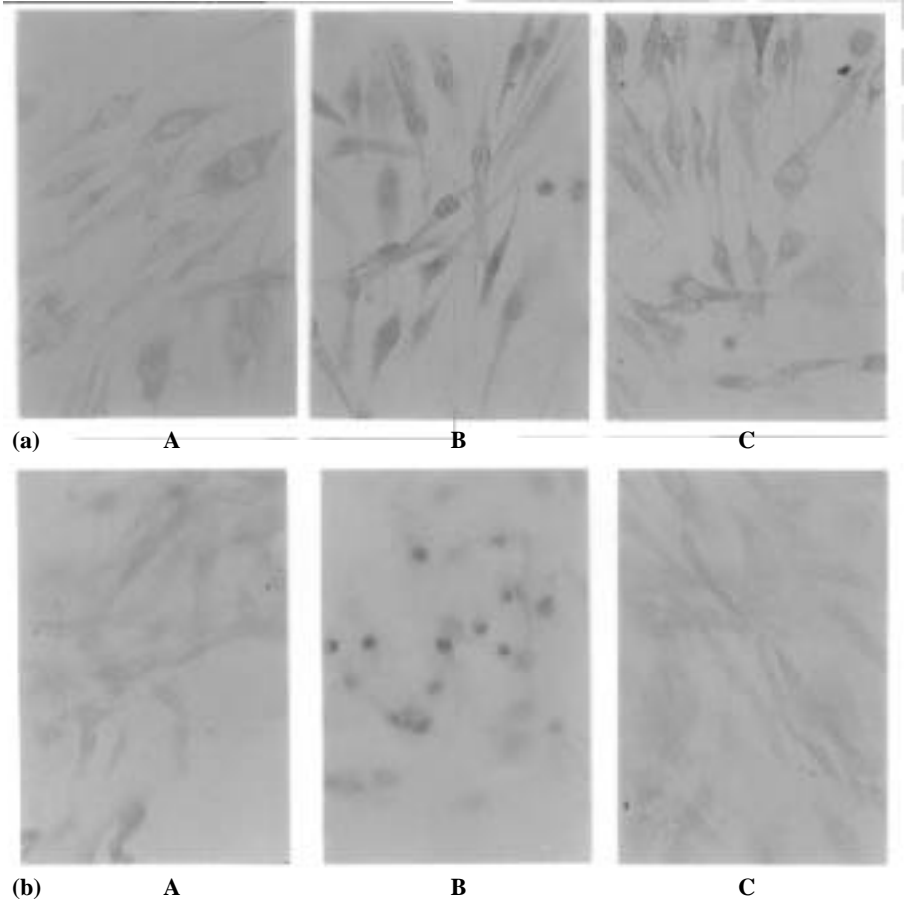

Fig. 3. Nuclear translocation and phosphorylation of CREB in fibroblast-like RA synovial cells stimulated with TNF-α; immunocytochemical analysis with anti-CREB Ab (**a**) and anti-serine-133 phosphorylated CREB Ab (**b**)

Fibroblast-like RA synovial cells (2×10^4 cells) were cultured in chamber slides. The synovial cells were preincubated with Rp-cAMP for 1 hr followed by stimulation with 5 ng/ml TNF-α for up to 24 hrs. We studied the effects of Rp-cAMP on the nuclear translocation of CREB by comparing the cellular distribution of the CREB of unstimulated synovial cells with that of synovial cells stimulated with TNF-α in the presence or absence of Rp-cAMP. In the experiments we employed anti-CREB antibody (**a**) and anti-serine-133 phosphorylated CREB antibody (**b**).

A, unstimulated culture 120 min; **B**, culture stimulated with ng/ml TNF-α 5 for 120 min; **C**, culture stimulated with 5 ng/ml TNF-α for 120 min in the presence of 1 μM Rp-cAMP. Control staining using non-immune mouse IgG showed no positively staining cells (data not shown). Results shown are representative of 5 independent experiments.

Note that CREB and phosphorylated CREB are detectable within the nuclei but not the cytoplasm of TNF-α stimulated synovial cells, whereas CREB and a lesser amount of phosphorylated CREB are present mainly within the cytoplasm of cells stimulated in the presence of Rp-cAMP.

translocation of CREB was confirmed by immunocytochemical staining of TNF-α stimulated synovial cells cultured for 2 hr in the chamber slide (Fig. 3). Thus, it is evident that CREB itself translocates from the cytoplasm into nuclei in response to stimulation with proinflammatory cytokine.

It has already been reported that a cAMP/CREB inhibitor exerts its inhibitory effects by the reduction of cAMP accumulation, inhibition of PKA activity, and suppression of CREB activity (11, 24). To test whether this is the case in synovial cells in patients with RA, we examined the effect of Rp-cAMP on the nuclear translocation of CREB pre-existing within the cytoplasm in response to TNF-α stimulation in fibroblast-like synovial cells of RA patients. The nuclear translocation of CREB protein in RA fibroblast-like synovial cells by TNF-α stimulation was inhibited by treatment of the synovial cells with the cAMP/CREB inhibitor 10^{-10} M (Fig. 3a). The inhibition of nuclear translocation and phosphorylation of CREB was confirmed when we employed anti-ser-ine-133 phosphorylated CREB Ab (Fig. 3b). Thus, it is evident that cAMP/CREB inhibitor modulates the nuclear translocation and phosphorylation of transcription factor CREB to exert their inhibitory effects.

Effects of the cAMP/CREB inhibitor on cell proliferation of RA synovial cells

We measured proliferative responses to TNF-α stimulation of RA synovial cells in the presence of various concentrations of the cAMP/CREB inhibitor to elucidate the involvement of CREB in RA synovial cell function. Synovial cells were stimulated with TNF-α for 3 days in the presence of Rp-cAMP and subsequent proliferation was measured by [^{3}H]-TdR incorporation. The proliferation was inhibited by the addition of Rp-cAMP into the cell cultures in a dose-dependent manner (Fig. 4). Thus, it is evident that Rp-cAMP inhibits the proliferation of RA synovial cells *in vitro*. Using the trypan blue dye exclusion method, we also found that the inhibition of RA synovial cell function was not due to the cytotoxicity of Rp-cAMP (data not shown), in accordance with a previous report (18).
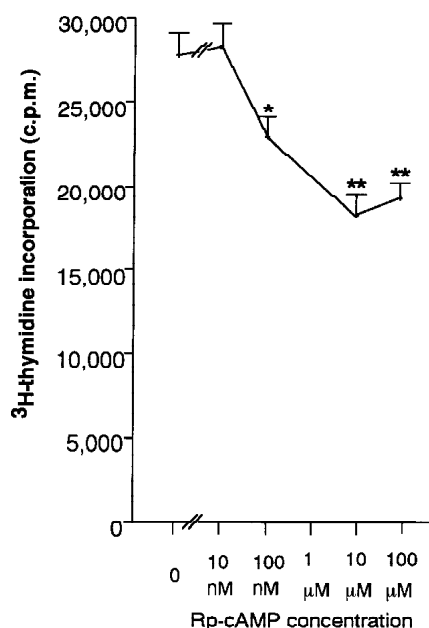
Effects of the cAMP/CREB inhibitor on cytokine mRNA expression and cytokine production of RA synovial cells

We next studied the effects of the cAMP/CREB inhibitor on proinflammatory cytokine production, including IL-6 and IL-8, of RA synovial cells. RA fibroblast-like synovial cells were pre-cultured with various concentrations of the cAMP/CREB inhibitor, followed by stimulation with TNF- 2 ng/ml for 16 hr. We examined the effects of the cAMP/CREB inhibitor on IL-6 and IL-8 mRNA expression of RA synovial cells by using limiting dilution RT-PCR. The TNF-induced IL-6 and IL-8 mRNA expres-

sion of RA fibroblast-like synovial cells was inhibited by cAMP/CREB inhibitor treatment (Fig. 5A).

In parallel experiments, we studied whether cAMP/CREB inhibitor reduces IL-6 and IL-8 protein synthesis. RA fibroblast-like synovial cells were stimulated with TNF- 2 ng/ml in the presence of the cAMP/CREB inhibitor for 24 hr and subsequent culture supernatants were recovered for the estimation of cytokine production. As shown in Figure 5B, RA synovial cells produced a large quantity of IL-6 and IL-8 in response to TNF- stimulation; treatment of RA synovial cells with the cAMP/

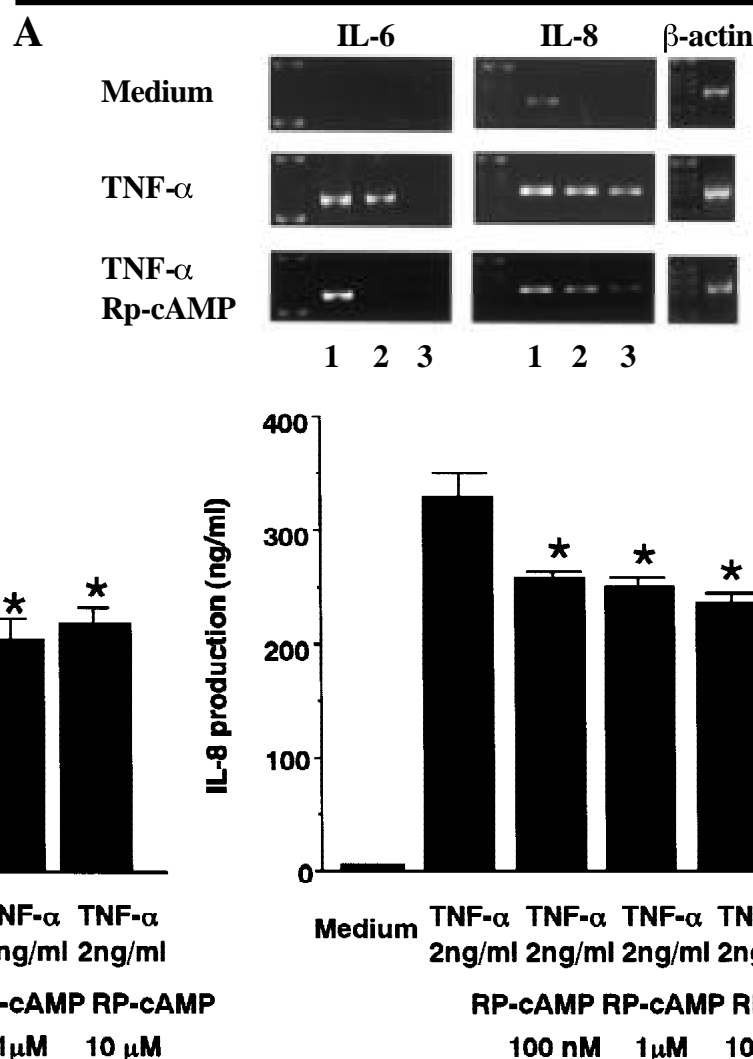**Fig. 4.** Effects of the cAMP/CREB inhibitor on cell growth of synovial cells in RA patients. Fibroblast-like synovial cells (5 x 10^3 cells) of RA patients were preincubated with or without cAMP/CREB inhibitor for 1 hr. They were then cultured for 3 days with or without 2 ng/ml TNF- . Proliferation was determined as described in *Patients and Methods*. The results shown are representative of 5 independent experiments. Mean + SE of triplicate cultures are shown (*p<0.05, **p < 0.01).

Fig. 5. Effects of the cAMP/CREB inhibitor on TNF- stimulated cytokine mRNA expression and cytokine protein production of fibroblast-like RA synovial cells. RA synovial cells were treated with cAMP/CREB inhibitor and 1 µM Rp-cAMP, followed by stimulation with 2 ng/ml TNF- for 24 hrs.
(A) The limiting dilution RT PCR technique was used, where varying dilutions of cDNAs were subjected to PCR amplification in order to more precisely determine the effects of TNF- and Rp-cAMP on the IL-6 and IL-8 mRNA expression of fibroblast-like synovial cells. Lanes 1, 2 and 3 represent 10%, 5% and 2%, respectively, of the total cDNA used for the PCR amplification. cDNA from each sample was also subjected to PCR amplification to determine the expression of the housekeeping gene -actin. A 1 kb DNA ladder from Life Technologies is included.
(B) IL-6 and IL-8 production were estimated using a cytokine-specific ELISA. The results shown are representative of 3 independent experiments. The mean + SE of triplicate estimates are shown. IL-6 and IL-8 production by Rp-cAMP-treated synovial cells were significantly reduced by Student's t-test (* p < 0.05).

CREB inhibitor reduced IL-6 and IL-8 production.

Effects of the cAMP/CREB inhibitor on MMP mRNA expression and MMP protein production of RA synovial cells

We further tested whether the cAMP/CREB inhibitor reduces MMP production of RA synovial cells. RA fibroblast-like synovial cells were stimulated with TNF- 2 ng/ml in the presence of the cAMP/CREB inhibitor for 16 hr. We used limiting dilution RT-PCR analysis to detect any change in mRNA expression. As shown in Figure 6A, cAMP/CREB inhibitor treatment clearly reduced MMP-1 mRNA expression of RA synovial cells. We also measured collagenase protein production in this study using a commercial MMP-1 ELISA. We found that cAMP/CREB inhibitor treatment reproducibly reduced MMP-1 protein synthesis of RA fibroblast-like synovial cells (Fig. 6B).

Discussion

RA is characterized by pronounced hyperplasia of the synovial lining cells and the accumulation of infiltrating T cells, macrophages, and plasma cells, and their production of proinflammatory cytokines is believed to play a major role in the pathogenesis of RA (1, 25, 26).

In many cell types, cAMP-dependent transcription regulation is mediated by CREB and related transcription factors. adrenoreceptors, G proteins and PKA are involved in the cAMP-dependent sig-nal transduction pathway (27). CREB binds to distinct consensus sequences in the promoter regions of relevant genes, activating their transcription after the phosphorylation of CREB by cAMP-dependent PKA (27, 28). It is reported that the accumulation of intracellular cAMP induces activation of the catalytic subunit of the phosphorylating enzyme PKA, resulting in the phosphorylation of serine-133 of CREB accompanied by the acquisition of transcription factor activity (27).

We found that cAMP and the subsequent activation pathway involving CREB are linked with proinflammatory cytokine and MMP production by and the proliferation of RA fibroblast-like synovial cells. However, it is generally accepted that cAMP normally activates anti-inflammatory cytokines such as IL-10 (29). In addition, -mimetics are used in allergic inflammation (30). Thus, it is possible that a cAMP-associated activation pathway positively or negatively affects inflammatory responses, depending on the target cell types and the types of inflammation (e.g., allergic reactions and cell-mediated immune responses).

We found that RA synovial lining cells express CREB by immunohistochemical staining, and that the nuclear protein of fibroblast-like synovial cells stimulated with proinflammatory cytokines showed cAMP responsive element binding activity by a gel shift assay. It is likely
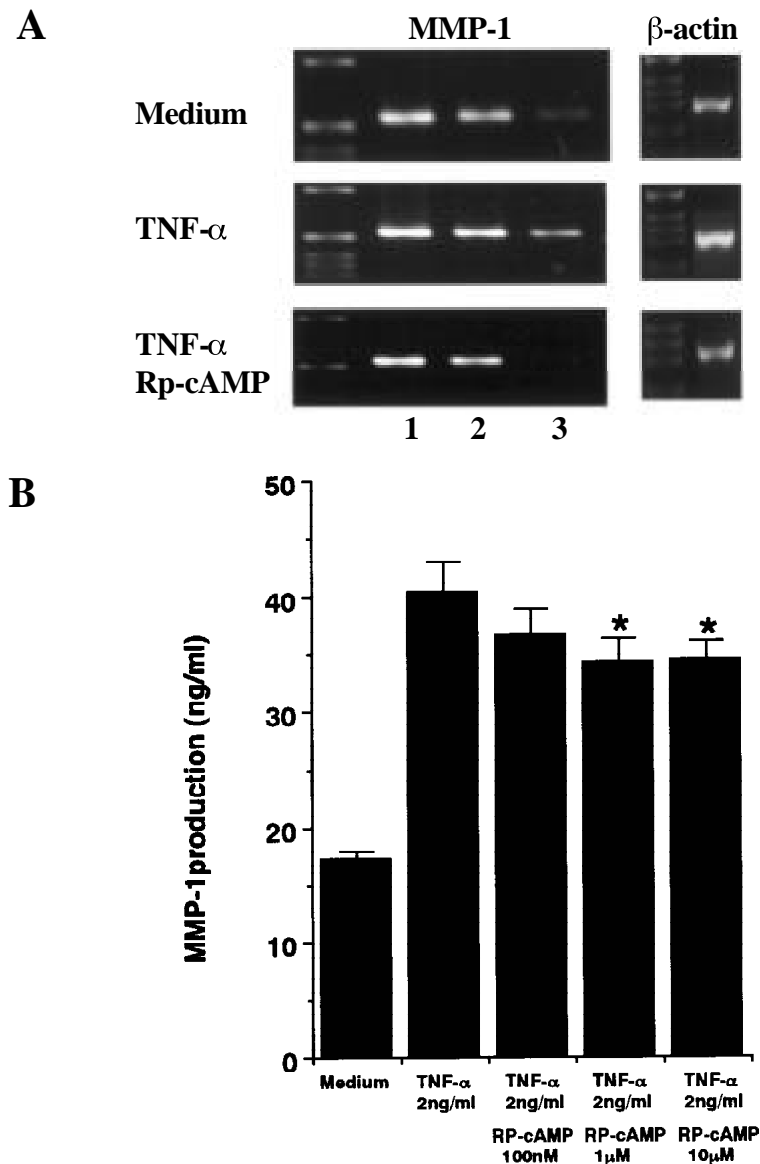

A

B

Fig. 6. Effects of the cAMP/CREB inhibitor on TNF- stimulated MMP-1 mRNA expression and cytokine protein production of fibroblast-like RA synovial cells

Fibroblast-like RA synovial cells (2.5 x 10^5 cells) were preincubated with or without the cAMP/CREB inhibitor, 1 µM Rp-cAMP, followed by stimulation with 2 ng/ml TNF- for 16 hrs (**A**) and for 24 hrs (**B**).

(**A**) The limiting dilution RT PCR technique was used to more precisely determine the effects of the cAMP/CREB inhibitor on MMP-1 mRNA expression of fibroblast-like synovial cells stimulated with TNF- . Lanes 1, 2 and 3 represent 10%, 5% and 2%, respectively, of the total cDNA used for PCR amplification. cDNA from each sample was also subjected to PCR amplification to determine the expression of -actin. Results shown are representative of 5 independent experiments. A 1 kb DNA ladder is included.

(**B**) Using an MMP-1-specific ELISA, RA synovial cell production of MMP-1 was estimated. Results shown are representative of 10 independent experiments. * p < 0.01.

that Rp-cAMP inhibits adenylate cyclase activity and then cAMP accumulation, followed by deficient PKA activity in RA synovial cells. This process may lead to deficient transcription factor CREB activity in the nuclei (27). Indeed, we found that nuclear translocation and phosphorylation of cytoplasmic CREB were induced by TNF- stimulation in RA fibroblast-like synovial cells (Figs. 4 and 5). The translocation and phosphorylation of CREB were inhibited by Rp-cAMP treatment of the RA synovial cells (Figs. 4 and 5).

The prointerleukin-1 (proIL-1) gene coding for the IL-1 precursor protein (its genomic locus name, IL-1B) has been previously reported (31) to be rapidly and transiently transcribed in monocytes by lipopolysaccharide. cAMP has been reported to be an efficient, immediate-to-early inducer of IL-1B in the presence of a costimulant (32, 33). CREB pre-exists in cells as an inactive protein which supports transcription following PKA-mediated phosphorylation at serine-133 (34, 35). CREB has been previously reported to bind to two classes of CRE as follows: (i) a perfectly symmetric CRE (symCRE) site consisting of two overlapping CGTCA palindromic half-sites, such as those found in the somatostatin (36) and achorionic gonadotropin genes (37); and (ii) asymmetrical CRE sites consisting of the sequence CGTCA, representing a single perfect half-site, such as that present in the tyrosine aminotransferase (38) and enkephalin genes (39). The symmetrical CRE can bind CREB in both the aphosphorylated and phosphorylated states, whereas the asymmetrical CRE can bind only phosphorylated CREB (40). Pseudosymmetrical CRE, consisting of one perfect and one almost perfect half-site, found in the phosphoenolpyruvate carboxykinase gene and in the IL1B gene, is transactivated by either NF-IL6 or the related C/EBPa (41) as well as CREB (42, 43). Thus, it is suggested that proinflammatory cytokine stimulation of RA synovial cells induces the phosphorylation of CREB, which would bind both the symmetrical CRE and the asymmetrical CRE, resulting in the transcription of a broader range of genes containing CRE. CREB has been reported to heterodi-

merize with CREM (44) and ATF-1 (45, 46). It is reported that CREB can bind to AP-1 sites, but with lower affinity than to CREs (47). Thus, it is possible that a diverse transcriptional regulation by members of the CREB-ATF family, one that is dependent upon the nature of the binding site, is also operating within the synovium in patients with RA (43).

Recent study have shown the involvement of cAMP and the cAMP responsive element (CRE) in the upregulation of IL-1 synthesis (42, 43). This may be the case for IL-6 synthesis by RA synovial cells in response to IL-1 stimulation; upregulation of IL-6 synthesis may involve CREB and CRE in the promoter/enhancer regions of the relevant genes. Or alternatively, reduced CREB activity by Rp-cAMP may result in the inhibition of IL-1 synthesis, and indirectly lead to reduced IL-6 production, because IL-1 promotes IL-6 and collagenase synthesis by RA synovial cells (5, 48). We found that Rp-cAMP potently inhibited proliferation and IL-6 production (Figs. 4 and 5B). Although the effects were statistically significant, IL-8 and MMP-1 production were inhibited less impressively (Figs. 5B and 6B). The less impressive effects of Rp-cAMP on IL-8 and MMP-1 production by RA synovial cell functions may suggest that c-AMP-dependent and c-AMP-independent activation pathways are operating in RA synovial cells to produce IL-8 and MMP-1. In any event, further efforts are needed to clarify the mechanisms of action of cAMP and its analogue on RA synovial cell functions.

Based on our present study, the development of cAMP analogues would appear to be quite a promising possibility for the treatment of RA patients. However, the clinical application of cAMP analogues may exert adverse effects on other cell types, because many other cells apart from the fibroblast-like synovial cells contain CREB. Thus, intra-articular administration of the cAMP analogue may have advantages over systemic administration. In addition, the development of an efficient drug delivery system specifically targeting the RA fibroblast-like synovial cells is awaited for the clinical application of cAMP analogues.

In conclusion, we have shown the potential regulatory roles of CREB in the inflammatory and destructive processes of the joints in patients with RA. On the basis of these results, we hypothesize that CREB may participate in the inflammatory reaction within the joints of RA patients. Furthermore, the antiinflammatory effects of cAMP/CREB inhibitors may be useful for treating patients with RA (32, 43, 45).

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