Prostaglandin E_2 Suppresses Nuclear Factor-κB Mediated Interleukin 15 Production in Rheumatoid Synoviocytes

SO-YOUN MIN, WAN-UK KIM, MI-LA CHO, SUE-YUN HWANG, SUNG-HWAN PARK, CHUL-SOO CHO, JONG-MAN KIM, and HO-YOUN KIM

ABSTRACT. ***Objective***. Prostaglandin E_2 (PGE_2) has a wide range of regulatory action in diverse cell types. To investigate the role of PGE_2 in rheumatoid arthritis (RA), we tested the effect of exogenous PGE_2 on the production of cytokines in synoviocytes.

Methods. Fibroblast-like synoviocytes (FLS) were prepared from the synovial tissue of patients with RA and cultured in the presence of PGE_2. The production of interleukin 15 (IL-15) and IL-10 were measured in culture supernatant by ELISA. IL-15 mRNA expression and nuclear factor-κB binding activity for IL-15 transcription were determined by reverse transcription-polymerase chain reaction and electrophoresis mobility shift assay (EMSA), respectively. The level of IL-15 production was also measured by Western blot.

Results. PGE_2 at concentrations from 10^{-11} to 10^{-5} M inhibited secretion of IL-15 by FLS, while increasing IL-10 production, in a dose dependent manner. IL-15 production stimulated by interferon-γ (IFN-γ), IL-1ß, or lipopolysaccharide were also strongly inhibited by PGE_2. This PGE_2-mediated inhibition of IL-15 production appears to be dependent in part on the increase of IL-10, since neutralizing anti-IL-10 antibodies reversed this inhibition to some extent. The EMSA of the NF-κB site in the IL-15 promoter showed that PGE_2 inhibited binding of NF-κB in a dose dependent manner. Experiments using inducers and an inhibitor of cyclic AMP (cAMP) suggest that a major intracellular signal mediates the regulatory effect of PGE_2 on the production of IL-15 and IL-10.

Conclusion. PGE_2 differentially regulates the production of IL-15 and IL-10 in FLS. The strong inhibition of PGE_2 on IL-15 production is exerted via a cAMP-dependent modulation of NF-κB activity. Our data suggest that overproduced PGE_2 in RA joints may play an antiinflammatory role. (J Rheumatol 2002;29:1366–76)

Key Indexing Terms:
PROSTAGLANDIN E_2 SYNOVIAL FIBROBLAST INTERLEUKIN 15
cAMP NUCLEAR FACTOR-κB

Rheumatoid arthritis (RA) is characterized by hyperproliferation of synovial lining cells and increased accumulation of joint fluid containing inflammatory cells. A variety of immune mediators are secreted at the site of local inflammation, among which interleukin 15 (IL-15), a novel 14~15 kDa cytokine, plays a critical role in the prolongation of the disease process[1]. Originally identified as a soluble factor with properties similar to IL-2, IL-15 possesses diverse immune regulatory functions. It promotes the activity of cytotoxic effector cells and natural killer (NK) cells, activates T cell proliferation, and supports B cell proliferation and immunoglobulin synthesis[2,3]. Previously the local environment within the RA joints was considered to be unsuitable for T cell activation and proliferation, due to lack of IL-2 and interferon-γ (IFN-γ) production. Interestingly, recent data have shown that a substantial amount of IL-15 is produced in inflamed joints by synoviocytes[3]. IL-15 recruits and expands T cells in the synovial membrane, and these T cells can produce tumor necrosis factor-α (TNF-α) directly or through cell-cell contact with macrophages[3]. TNF-α, in turn, enhances the capacity of IL-15 to induce contact mediated macrophage activation[4], generating a positive feedback loop.

Prostaglandin E_2 (PGE_2), an arachidonic acid metabolite, exerts a diverse effect on immune response and biologic outcome in a variety of inflammatory diseases including RA. It was initially considered a proinflammatory mediator causing vasodilation, hyperalgesia, and fever[5,6], but has been progressively recognized for its immune regulatory

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and antiinflammatory activities[7-11]. PGE_2 is mainly produced from antigen-presenting cells such as monocytes and dendritic cells. It suppresses the IL-12R expression in naive T cells and their differentiation into Th1 type, and strongly inhibits IL-2 and IFN-γ production by Th1 cells[7-9]. It has been shown that PGE_2 decreases IL-12 production while increasing IL-10 production in lipopolysaccharide stimulated monocytes[11]. PGE_2 suppresses IL-15 mediated NK cell functions[10]; however, not much is known about the regulatory effect of PGE_2 on IL-15 production in other cell systems.

The regulatory function of PGE_2 on target cells is exerted through receptor-coupling events. Upon binding to its receptor, PGE_2 stimulates adenylate cyclase, increasing the cytosolic level of cyclic AMP (cAMP)[12,13]. Elevated cAMP, via intracellular signal transduction cascade, induces transcription of IL-10 gene by the activation of cAMP responsive element-binding proteins (CREB)[14,15]. In the meantime, PGE_2 inhibits nuclear factor-κB (NF-κB) mediated transcription[16,17], and has been reported to block the binding activity of NF-κB to the IL-2 promoter[18]. However, little is known about the intracellular messengers or downstream transcription factors that relay the PGE_2 mediated regulation onto IL-15 production.

We analyzed the immune modulatory effect of PGE_2 on the cytokine secretion from fibroblast-like synoviocytes (FLS) in RA. We show that exogenous PGE_2 increases IL-10, but decreases IL-15 production by FLS. The inhibition of IL-15 transcription appears to be affected by the increase in IL-10, at least in part. The effects of PGE_2, including the reduced NF-κB binding activity to IL-15 promoter, were mimicked by an inducer of cAMP but were completely abrogated by a cAMP inhibitor. Collectively, these results suggest that PGE_2 strongly inhibits NF-κB mediated activation of IL-15 transcription in FLS via a cAMP-dependent pathway.

MATERIALS AND METHODS

Reagents and antibodies. PGE_2 (Dinoprostone), 3-isobutyl-1- methylxanthine (IBMX), N-2-O-dibutyryl-cAMP (DBcAMP), and 2'-3'-dideoxyadenosine (DDA) were purchased from Sigma (St. Louis, MO, USA). Lipopolysaccharide (LPS) was also obtained from Sigma. Cyclooxygenase (COX) inhibitors, indomethacin (IDC) and NS-398, were obtained from Calbiochem (La Jolla, CA, USA) and Cayman Chemicals (Ann Arbor, MI, USA), respectively. Recombinant IL-1ß and IFN-γ were purchased from Endogen (Woburn, MA, USA) and R&D systems (Minneapolis, MN, USA), respectively. For ELISA, recombinant human IL-10 and IL-15, together with antibodies to respective cytokines, were purchased from R&D systems. [γ-^{32}P]ATP was purchased from Amersham Pharmacia (Uppsala, Sweden). Reagents used for culture were found to contain < 200 pg/ml of endotoxin as determined by the Limulus amebocyte cell lysate assay.

Isolation of synoviocytes. FLS cell lines were prepared from synovial tissues of 6 RA patients (5 female, 1 male) undergoing total joint replacement surgery. The mean age of patients was 52.6 years, and the mean disease duration was 81.4 months. Five of 6 patients had positive rheumatoid factor. All had erosions on hand radiographs. Synovial tissues were minced into 2–3 mm pieces and treated for 4 h with 4 mg/ml of type I collagenase (Worthington Biochemical, Freehold, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM) at 37°C in 5% CO_2. Dissociated cells were centrifuged at 500 × g, resuspended in DMEM supplemented with 10% FCS (Life Technologies, Grand Island, NY, USA), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), and plated in 75 cm^2 flasks. Cultures were kept at 37°C in 5% CO_2 and the medium was replaced every 3 days. FLS were split weekly once primary cultures reached confluence. Cells were used between the 4th and the 8th passages, during which time they comprised a homogenous population of FLS (< 2.5% CD14, < 1% CD3, and < 1% CD19 positive by flow cytometry analysis).

Culture conditions. FLS cells were seeded in 24 well plates at a concentration of 6 × 10^4 cells per well in 1 ml DMEM/5% FCS, and incubated at 37°C for 24 h in the presence of varying concentrations of PGE_2, ranging from 10^{-11} to 10^{-5} M. To induce the synthesis of IL-15 from FLS, IFN-γ, IL-1ß, and LPS were added to wells at the onset of culture. To investigate the effect of increased IL-10 synthesis, endogenous IL-10 was neutralized by addition of monoclonal antibodies (Mab) to IL-10 (R&D systems). Various concentrations of cAMP inducers such as IBMX and DBcAMP were used to determine whether they could mimic the effect of PGE_2. A cAMP inhibitor, DDA, was used to block cAMP accumulation. IBMX, DBcAMP, and DDA were treated to each well at the onset of culture. After 24 h incubation, unless otherwise stated, cell-free media were collected and stored at –20°C until assayed. All cultures were set up in either triplicate or quadruplicate.

Immunoassays of IL-15 and IL-10. IL-15 and IL-10 in culture supernatants were measured by sandwich ELISA, as described[19]. Briefly, 4 μg/ml of Mab to human cytokines were added to each of 96 wells and incubated overnight at 4°C. After incubating the plate with blocking solution (PBS containing 1% BSA and 0.05% Tween 20) for 2 h at room temperature, test samples and the standard recombinant cytokines were added to the 96 well plate and incubated at room temperature for 2 h. Subsequently 500 ng/ml of biotinylated Mab to human cytokines were added and the reactions were allowed to proceed for 2 h at room temperature. Next, streptavidin-conjugated alkaline-phosphate (Sigma) diluted to 1:2000 was added, and the reaction was again allowed to proceed for another 2 h. Finally, 1 mg/ml of p-nitrophenylphosphate (Sigma) dissolved in diethanolamine (Sigma) was added to induce a color reaction, which was stopped by adding 1N NaOH. Between each step plates were washed 4 times with PBS containing 0.1% Tween 20. The automated microplate reader (Vmax, Molecular Devices, Palo Alto, CA, USA) set at 405 nm was used to measure the optical density (OD). The sensitivity limit was 10 pg/ml for IL-10 and 5 pg/ml for IL-15. Recombinant human cytokines diluted with culture medium, ranging from 10 to 2000 pg/ml, were used as calibration standards. Standard curves were drawn by plotting OD versus the log of the concentration of each recombinant cytokine.

Western blot analysis of IL-15. Whole cell lysate of FLS (100 μg) was fractionated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were incubated successively with 0.1% milk in TTBS (0.1% Tween 20 in Tris-buffered saline) at room temperature for 1 h, primary antibody to IL-15 (R&D systems) for 1 h, and horseradish peroxidase labeled anti-rabbit antibody (Amersham Pharmacia) for 30 min. Between each incubation, the membrane was washed extensively with TTBS. The labeled band was detected using ECL detection kit and developed with Hyperfilm-ECL (Amersham Pharmacia).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis. FLS were incubated with various concentrations of PGE_2 in the presence or absence of IL-1ß (10 ng/ml). After 6 h of culture, which was optimal for IL-15, mRNA was extracted using RNAzol B according to the manufacturer's instruction (Biotex Laboratories, Houston, TX, USA). RT of 5 μg total mRNA was carried out at 42°C using the Superscript™ RT system (Life Technologies). PCR amplification of cDNA aliquots was performed by adding 2.5 mM dNTPs, 2.5 U Taq DNA polymerase (TaKaRa

SHUZO Co., Shiga, Japan), and 0.25 μM each of sense and antisense primers. The reaction was done in PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl, pH 8.3) in a total volume of 25 μl. The following sense and antisense primers for each molecule were used (all written in 5'-3' direction): IL-15 sense TGGAAACCCCTTGCCATAGCCAGCTCTT, IL-15 antisense CTGTATCAT GAATACTTGCATCTCCGGACTC; GAPDH sense CGATGCTGGGCGTGAGTAC, GAPDH antisense CGTTCAGTCCAGGGATGACC. Reactions were processed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) through cycles of 30 s denaturation at 94°C, 1 min annealing at 55°C for GAPDH and at 58°C for IL-15, and 30 s elongation at 72°C. PCR rounds were repeated 25 cycles for GAPDH and 35 cycles for IL-15, which were determined to fall within the exponential phase of amplification for each molecule, as described[20]. The level of mRNA expression was presented as a ratio of cytokine PCR product over GAPDH product.

Determination of intracellular cAMP. FLS cells (1×10^5 cells per well) were stimulated with various concentration of PGE_2 for different lengths of time and extracted with absolute ethanol. The supernatant was dried in a speed vacuum and resuspended in phosphate buffer. The cAMP assay was performed using EIA kit (Cayman Chemicals) according to the manufacturer's protocol.

Electrophoresis mobility shift assay. FLS nuclear extract was prepared as described[21]. For the induction of NF-κB activity, cells were pretreated with 10 ng/ml of IL-1ß for 1 h in the presence or absence of PGE_2. Double stranded oligonucleotide probe containing the recognition site (underlined) of human IL-15 promotor (5'-GGGCT GGGGCTCCTCGATGTC-3') was generated by 5'-end labeling of the sense strand with [γ-^{32}P] dATP and T4 polynucleotide kinase (TaKaRa, Shiga, Japan), and purified using NucTrap columns (Stratagene, La Jolla, CA, USA). NF-κ binding reactions were performed with 2 μg of nuclear extract in 10 μl of binding buffer containing 400,000 cpm labeled oligonucleotide for 30 min at room temperature. A 100× excess of unlabeled oligonucleotide was used as a competitor. DNA-protein complex was analyzed on a 5% polyacrylamide gel electrophoresed in TBE buffer. The gels were then dried and exposed to BioMax-MR film (Eastman Kodak, Rochester, NY, USA) at –70°C for 24 h.

Supershift assay was performed to verify the shifted bands using specific antibodies to p65, p50, and c-rel proteins. Briefly, 1 μl of the antibody against p65, p50, and c-rel (Santa Cruz Laboratory, Santa Cruz, CA, USA) was added to the binding reaction prior to addition of the probe and incubated on ice for 30 min.

Statistical analysis. Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using the Student t test for matched pairs. Differences with a 95% confidence interval or higher were considered to be statistically significant ($p < 0.05$).

RESULTS

PGE_2 differentially affects 1L-15 and IL-10 production by rheumatoid synoviocytes. As shown in Figure 1A, unstimulated FLS constitutively produced IL-15 and IL-10 over a period of 24 h (IL-10, 94.2 ± 11.8 pg/ml; IL-15, 87.9 ± 7.4 pg/ml). Incubation of cells with PGE_2 (10^{-11} to 10^{-5} M) resulted in a dose dependent increase of IL-10 compared to the spontaneous level, while strongly suppressing the production of IL-15 by FLS (Figure 1B). For example, when PGE_2 was given at a concentration of 10^{-5} M, mean levels of cytokines were 192.6 ± 17.2 pg/ml for IL-10 (204.5% of initial response) and 41.5 ± 8.9 pg/ml for IL-15 (47.2% of initial response). Further, when added to FLS cultures stimulated by IL-1ß (10 ng/ml), IFN-γ (1000 U/ml), or LPS (10 μg/ml), a similar amount of PGE_2 reduced IL-15 production to 28.5%, 39.4%, 37.8% of initial responses, respectively.

To confirm the inhibitory effect of PGE_2 on IL-15 production, we performed Western blot analysis of FLS extract (Figure 2). A constitutive production of IL-15 was detected from an untreated control sample, which was significantly increased upon treatment with 10 ng/ml IL-1ß (lane 2), while the addition of PGE_2 dose dependently inhibited IL-15 production in FLS (lanes 3 and 4).

IL-10 is involved in the suppression of IL-15 by PGE_2. It is well known that IL-10 inhibits the production of Th1 and proinflammatory cytokines including IFN-γ, IL-2, IL-12, and TNF-α[22,23]. In this regard, it was of interest to determine whether endogenous IL-10 acted as an intermediate in the suppression of IL-15 production by PGE_2. As shown in Figure 3A, incubating FLS with neutralizing anti-IL-10 Mab in the presence of 10^{-5} M of PGE_2 resulted in a dose dependent recovery of IL-15 production. Using the constitutive level of these cytokines as reference values, 10^{-5} M of PGE_2 reduced the production of IL-15 to 42.4% without anti-IL-10 Mab, which were partially restored to 81.7% of constitutive levels when treated with 10 μg/ml of anti-IL-10. Similarly, IL-1ß stimulated production of IL-15 was also partially recovered by adding IL-10 Mab (Figure 3A). In both experiments, the equivalent concentration of isotype control antibodies (IgG1; R&D Systems) did not show significant effect.

To simulate the effect of increasing IL-10 production we observed in PGE_2 treatment, purified recombinant human IL-10 (0.2 to 50 ng/ml) was added to cultures, which resulted in a dose dependent suppression of spontaneous IL-15 production (Figure 3B). However, even at the highest concentration (50 ng/ml), which was about 200-fold excess of the level of IL-10 induced by PGE_2, the recombinant IL-10 suppressed IL-15 production only to a degree comparable to that of 10^{-5} M of PGE_2 (mean IL-15 levels when treated with IL-10 alone vs PGE_2 alone/mean values of spontaneous cytokine production: 48.9% vs 47.2%). Taken together, these observations indicate that mechanisms other than the enhancement of IL-10 production are involved in the suppression of IL-15 by PGE_2.

Effect of NSAID on PGE_2-mediated cytokine regulation. It is well known that nonsteroidal antiinflammatory drugs (NSAID) inhibit the production of COX, a key enzyme for endogenous PGE_2 function. Moreover, NSAID such as aspirin and salicylic acid repress NF-κB responsive genes in monocytes[24]. Therefore, it was of great interest to determine the effect of NSAID on PGE_2 mediated regulation of IL-15 and IL-10 production. Assays performed in the presence of a COX-2 specific inhibitor NS-398 (0.01 to 10 μM) and IDC that blocks both COX-1 and COX-2 (1 to 100 μM) showed that both agents dose dependently decreased IL-15 production while increasing IL-10 production (Figure 4A and B). Interestingly, when these NSAID were added together with 10^{-5} M of PGE_2, the production of cytokines from FLS was differentially affected depending on the dose of NSAID. At

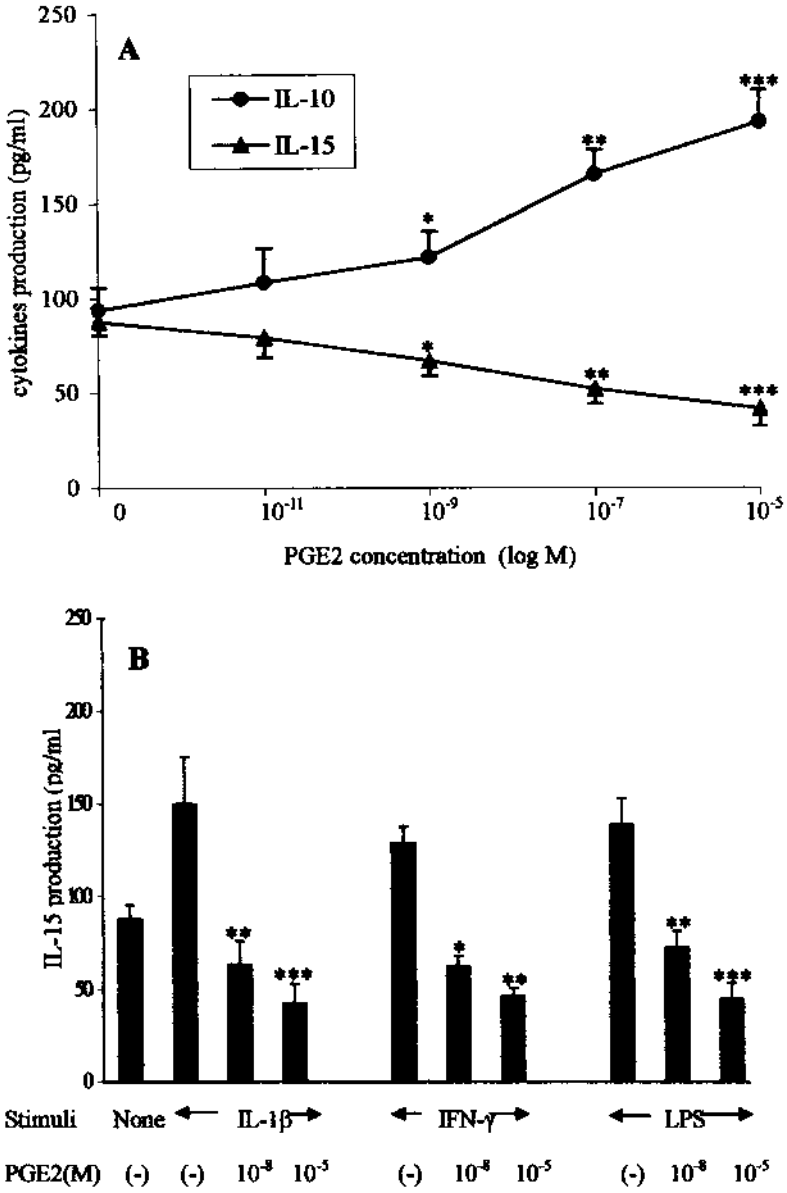

Figure 1. Effect of PGE_2 on cytokine production from synovial fibroblasts. A. Changes in the spontaneous cytokine production. Fibroblast-like synoviocytes (FLS) were cultured in triplicate for 24 h with PGE_2, given at concentrations ranging from 10^{-11} to 10^{-5} M. The production of IL-15 and IL-10 was measured in the supernatants by ELISA. B. Effect of PGE_2 on the production of IL-15 stimulated with IFN-γ (1000 U/ml), IL-1ß (10 ng/ml), or LPS (10 μg/ml). Data are expressed as mean ± SD of 6 independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 vs medium (A) or stimuli alone (B).

lower dose of NSAID (0.1 μM of NS-398 and 1 μM of IDC), combined stimuli of NSAID and 10^{-5} M PGE_2 restored changes in IL-15 and IL-10 production by PGE_2 nearly to original levels. When given at higher concentrations (10 μM of NS-398 and 100 μM of IDC), NSAID did not attenuate the downregulation of IL-15 by PGE_2, while they synergistically increased the production of IL-10 with PGE_2. These data suggest that NSAID, depending on their concentrations, may have different modulatory effects on the PGE_2 mediated production of IL-15 and IL-10.

Cytokine regulation by PGE_2 is mainly mediated via a cAMP pathway. It has been demonstrated that many effects of PGE_2 are mediated via induction of intracellular cAMP[12,13]. In this light, experiments were conducted to determine whether PGE_2 could stimulate intracellular cAMP formation in FLS. As shown in Figure 5A, a rapid increase in cAMP levels was observed as early as 30 min after treatment with 10^{-5} M PGE_2 (190% of basal level), which peaked after 1 h (238% of basal level) and remained high up to 12 h. The accumulation of cytoplasmic cAMP

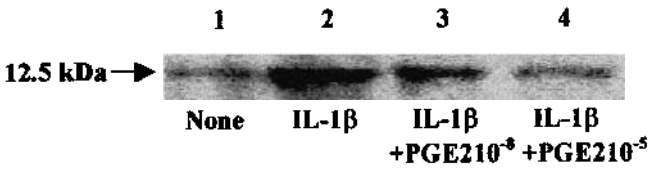

Figure 2. Western blot analysis of IL-15. FLS were incubated with PGE_2 in the presence of IL-1ß (10 ng/ml). IL-15 protein expression in the cell extract was measured after treatment without (lane 1) or with IL-1ß (lane 2), with IL-1ß plus 10^{-8} M PGE_2 (lane 3), and IL-1ß plus 10^{-5} M PGE_2 (lane 4).

occurred in a dose dependent fashion with increasing concentration of PGE_2. These results, together with previous reports[12,13], suggest that cAMP elevation is an early event in the signal transduction that mediates the regulation of PGE_2 in FLS.

Based on this knowledge, we analyzed whether agents known to enhance cAMP could also mimic PGE_2 action on the expression of IL-15 and IL-10. The result showed that IBMX, a phosphodiesterase inhibitor, increased IL-10 levels dose dependently while strongly inhibiting the production of IL-15 (Figure 5B). Similar effects were observed when

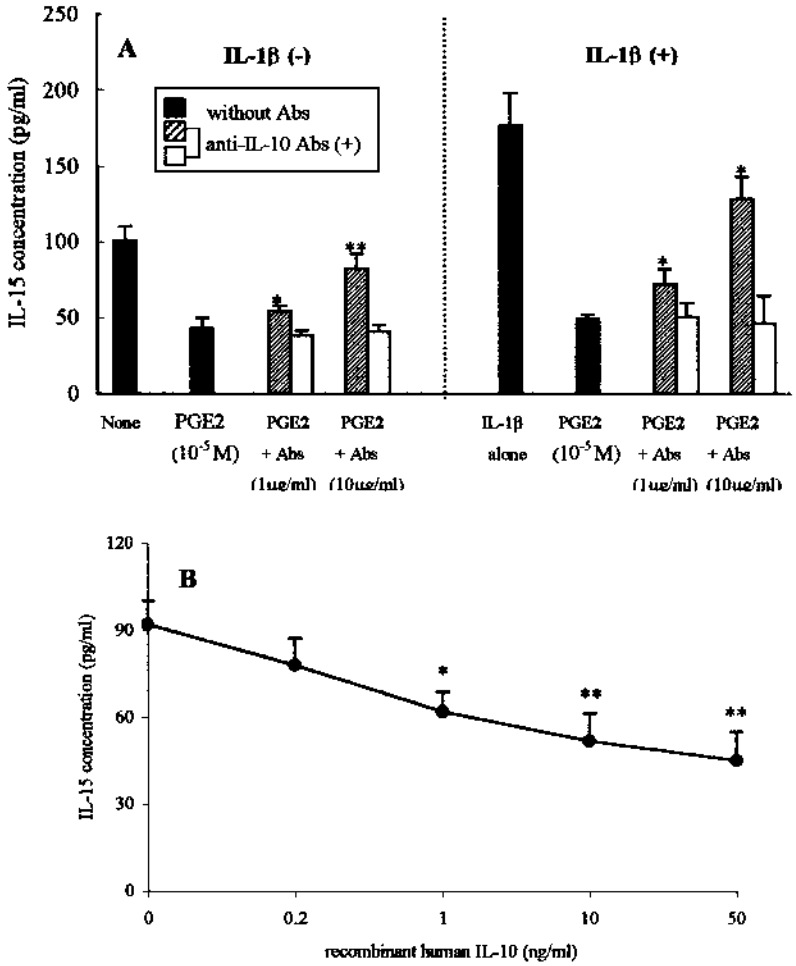

Figure 3. The suppression of IL-15 by PGE_2 is partially dependent on the increase of IL-10. A. IL-10 dependency of PGE_2 mediated IL-15 suppression. FLS were cultured with PGE_2 in the presence of neutralizing antibodies (Abs) to IL-10. The production of IL-15 from spontaneous and IL-1ß (10 ng/ml) induced cells, in the presence of 1 μg/ml or 10 μg/ml of anti-IL-10 and 10^{-5} M of PGE_2. The equivalent concentrations of isotype control antibodies were used as a control. *$p < 0.05$; **$p < 0.01$ vs the presence of PGE_2 10^{-5} M without anti-IL-10 antibodies. B. Effect of a recombinant IL-10 on IL-15 suppression. FLS were incubated with various concentrations of recombinant IL-10, ranging from 0.2 to 50 ng/ml. IL-15 production was measured in the supernatants 24 h after culturing. *$p < 0.05$; **$p < 0.01$ vs medium alone. Data are expressed as mean ± SD of 3 independent experiments.

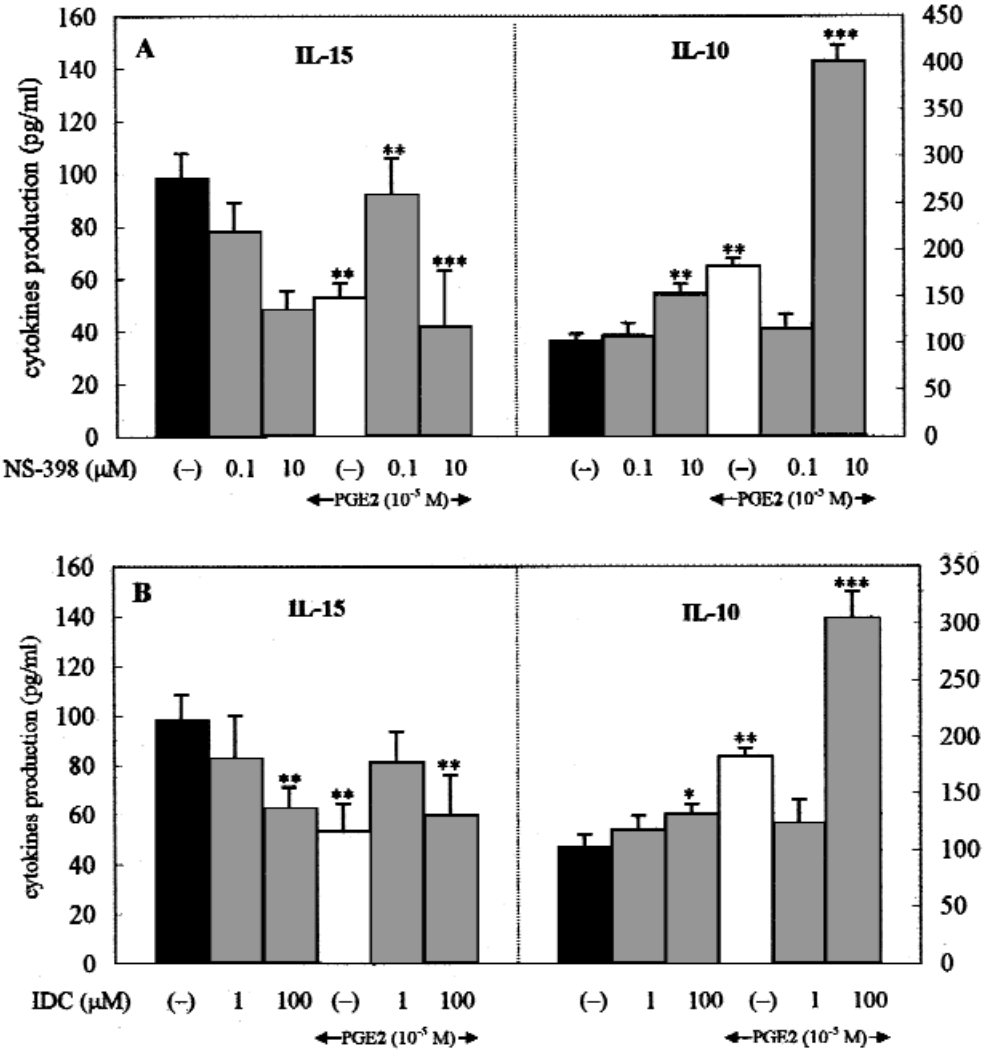

Figure 4. Effect of NSAID, NS-398 (A) and indomethacin (IDC) (B), on PGE_2 mediated cytokine regulation. FLS culture was treated with NS-398 and IDC with or without 10^{-5} M PGE_2. The production of IL-15 and IL-10 was measured by ELISA in the supernatant 24 h after culturing. Data are expressed as mean ± SD of 5 independent experiments. *p < 0.05; **p < 0.01; *** p < 0.001 vs medium alone.

DBcAMP, a membrane permeable analog of cAMP, was used (Figure 5C). On the other hand, DDA, an inhibitor of adenylate cyclase, completely abrogated the effect of PGE_2 on IL-10 production down to 0.93-fold of constitutive level (Figure 5D). DDA also reversed the inhibition of IL-15 production by PGE_2 to 98.2% of spontaneous levels. These data, combined with previous reports of increased intracellular cAMP levels upon PGE_2 treatment, imply that the regulation by PGE_2 is mainly mediated via a cAMP dependent pathway.

PGE_2 and cAMP inducer strongly inhibit IL-15 mRNA expression. We further investigated whether the suppressive effect of PGE_2 and cAMP inducer on IL-15 production involves a modulation of IL-15 transcription, using semiquantitative RT-PCR analysis (Figure 6). The addition of IL-1ß (10 ng/ml) caused a remarkable increase of IL-15 mRNA level (lane 2). Treatment of cells with PGE_2 resulted in a dose dependent suppression of IL-1ß induced IL-15 mRNA expression, where 10^{-8} M and 10^{-5} M PGE_2 resulted in partial and complete suppression, respectively (lanes 3 and 4). A sharp reduction in the level of IL-15 mRNA was also observed upon IBMX treatment (lane 5). The inhibition of IL-15 transcription by PGE_2 was reversed partly by adding 10 μg/ml anti-IL-10 Mab (lane 6), or near completely by DDA (lane 7). Similar results were obtained with 3 different FLS cell lines from independent RA patients (data not shown). Together, these data suggest that the PGE_2 mediated modulation of IL-15 production is exerted at the transcriptional level, using both cAMP and IL-10 as intermediate regulators.

Effect of PGE_2 and cAMP inducer on the NF-κB binding. The IL-15 promotor region contains binding sites of several

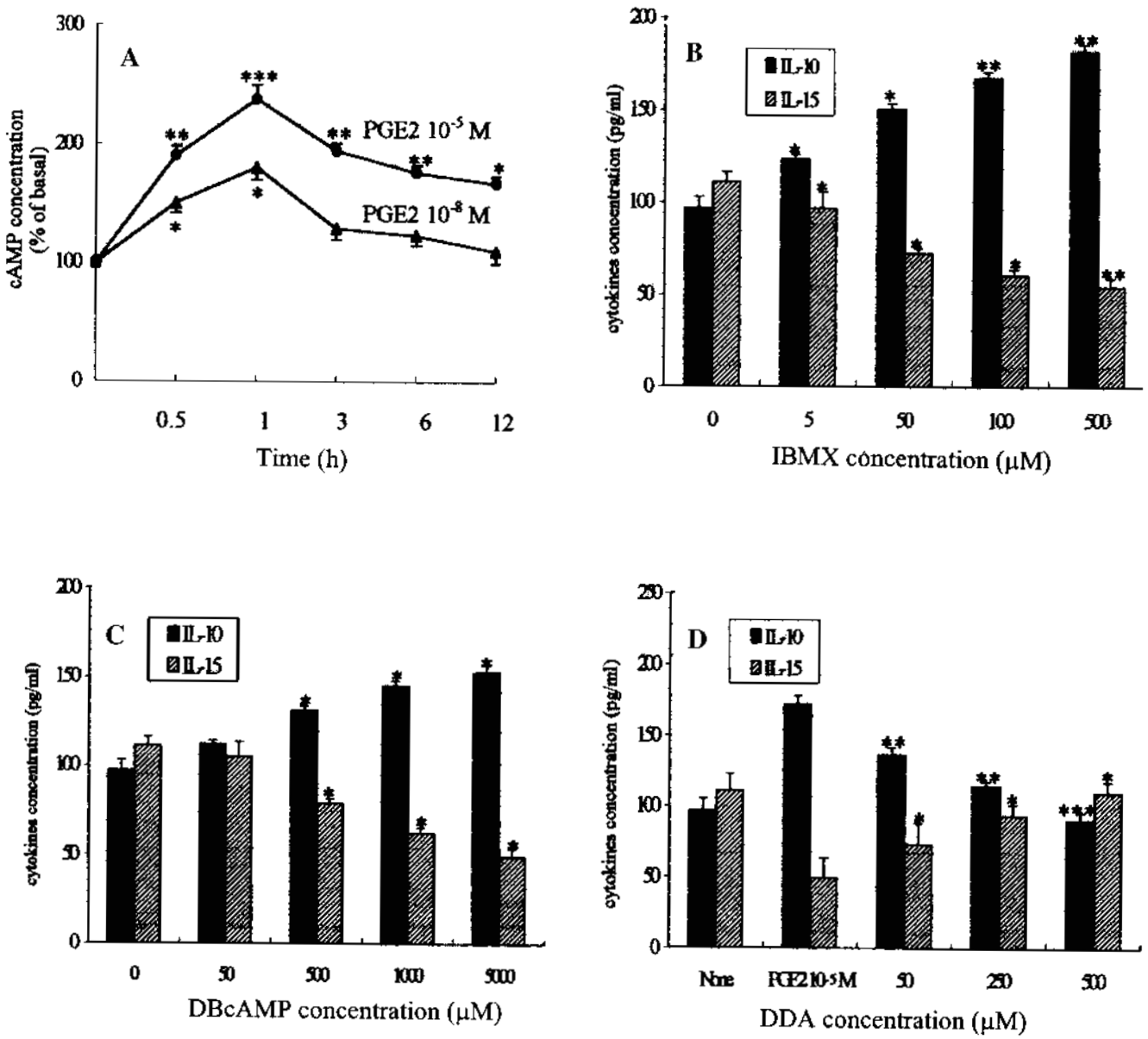

Figure 5. Intracellular cAMP mediates cytokine regulation by PGE_2. A. Changes in the intracellular cAMP concentration in FLS following PGE_2 treatment. Results are expressed as the mean of 3 independent experiments, each performed in triplicate. The cAMP concentration in basal state was 28.4 pmol/ml. B, C, and D. FLS were incubated with various concentrations of cAMP inducers, 3-isobutyl-1-methylxanthine (IBMX) and N-2-O-dibutyryl-cAMP (DBcAMP), or a cAMP inhibitor, 2'-3'-dideoxyadenosine (DDA) in the presence of 10^{-5} M of PGE_2. Amounts of IL-10 and IL-15 in supernatants were measured by ELISA. Data are expressed as the mean ± SD of 3 independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs medium alone (A, B, and C) or the presence of PGE_2 10^{-5} M (D).

transcription factors including NF-κB[25,26]. We tested if the accumulation of cAMP by PGE_2 is relayed onto the down-regulation of IL-15 promoter activation, by a mobility shift assay of the NF-κB site. Figure 7A shows that incubation of FLS with IL-1ß (10 ng/ml) strongly induced the DNA-binding activity of NF-κB (lane 3), compared to that of unstimulated cells (lane 2). The shifting of radiolabeled NF-κB element was chased by competition with 100-fold excess of unlabeled DNA (lane 1). The addition of PGE_2 to IL-1ß stimulated FLS cultures resulted in a dose dependent reduction of NF-κB binding (lanes 4 and 5). Treatment with IBMX exhibited results similar to that of PGE_2 (lane 6), whereas DDA completely abrogated the effect of PGE_2 (lane 7). A similar pattern of changes was observed among FLS extracts from 3 RA patients. These results suggest that PGE_2 inhibits IL-15 mRNA production via the elevation of cAMP, which in turn blocks NF-κB signaling through the IL-15 promoter. To verify the identity of binding NF-κB isoforms, supershift assay using antibodies to p65, p50, and *c-rel* was performed (Figure 7B). It appears that the factors binding to

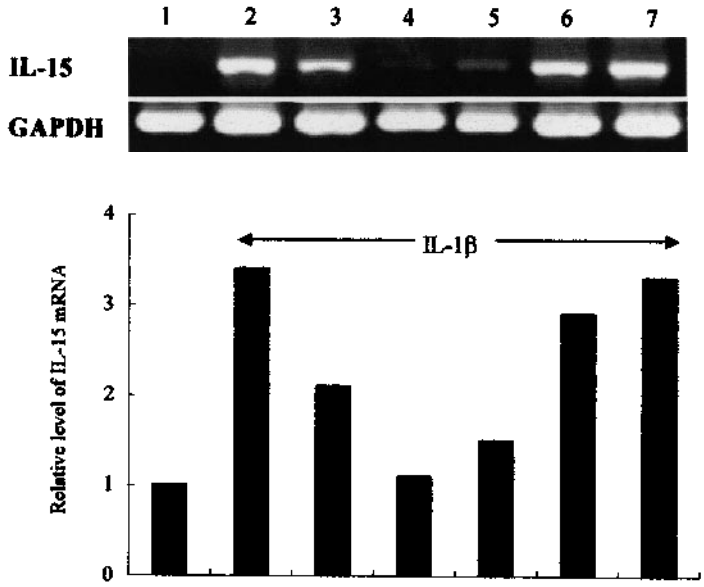

Figure 6. Messenger RNA expression of IL-15 in synovial fibroblasts. FLS were incubated with PGE_2 in the presence or absence of IL-1ß (10 ng/ml). After 6 h of culture, which is optimal for IL-15, FLS were analyzed for IL-15 mRNA expression by semiquantitative RT-PCR. Amplification of GAPDH was used as internal control. IL-15 mRNA expression after treatment without (lane 1) or with IL-1ß (lane 2), IL-1ß plus 10^{-8} M PGE_2 (lane 3), IL-1ß plus 10^{-5} M PGE_2 (lane 4), IL-1ß plus IBMX 500 μM (lane 5), and IL-1ß plus 10^{-5} M PGE_2 in the presence of 10 μg/ml of anti-IL-10 (lane 6), or DDA 500 μM (lane 7). The levels of mRNA are expressed as fold increase relative to mRNA in unstimulated control cells, and corrected for the levels of GAPDH mRNA signal. Representative data from 3 independent experiments is shown.

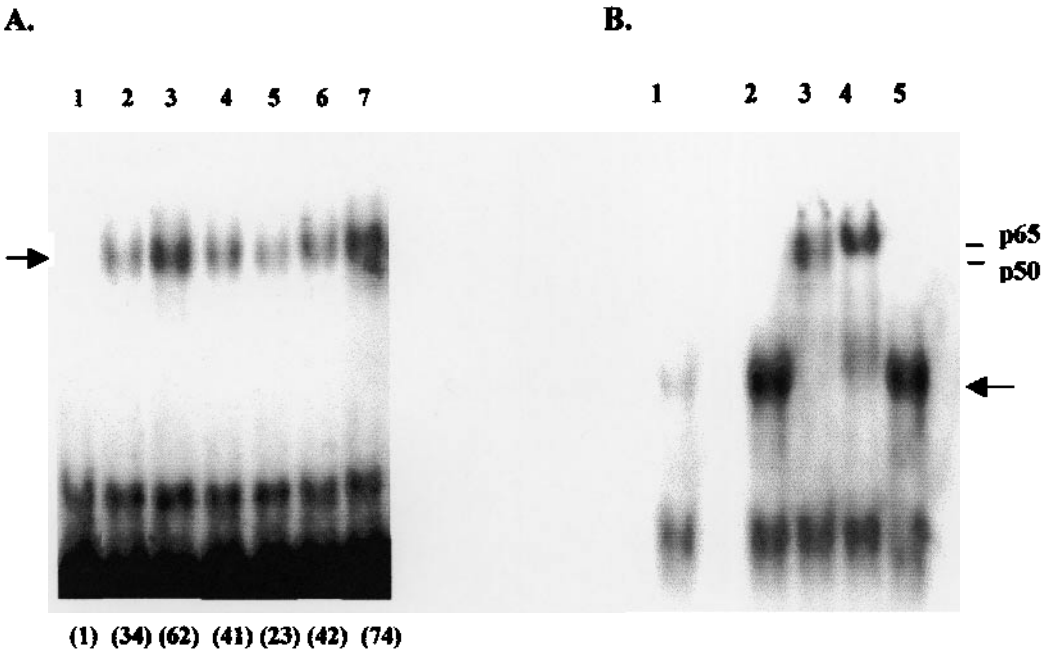

Figure 7. Effect of PGE_2 and cAMP inducer on NF-κB complex in nuclear extracts of FLS. A. Cells were pretreated with 10 ng/ml of IL-1ß for the induction of NF-κB activity for 1 h prior to the analysis. Abrogation of NF-κB activity by excess of unlabeled oligonucleotide in IL-1ß-stimulated cells (lane 1), NF-κB activity in the absence (lane 2) or presence of IL-1ß (lane 3), IL-1ß plus 10^{-8} M PGE_2 (lane 4), IL-1ß plus 10^{-5} M PGE_2 (lane 5), IL-1ß plus IBMX 500 μM (lane 6), and IL-1ß plus 10^{-5} M PGE_2 in the presence of DDA 500 μM (lane 7). Numbers in parentheses represent densitometric measurements of shifted bands. A representative result from 3 independent experiments using cell lines of different RA patients is shown. B. Supershift assay of NF-kB site using antibodies against p65 (lane 3), p50 (lane 4), and c-Rel (lane 5). Cells in all lanes, except lane 1 (medium alone), were prestimulated with IL-1ß. Arrows denote labeled oligonucleotide band shifted upon NF-κB binding.

the oligonucleotide include p65 and p50 (lanes 3, 4), but not *c-rel* (lane 5).

DISCUSSION

The role of PGE_2 in the etiopathogenesis of immune and inflammatory diseases has become the subject of recent controversy. It has been believed that PGE_2 mediates both inflammatory and destructive features of RA[27-32]. Large amounts of PGE_2 are released from rheumatoid synovial tissues[27,28], and increase the expression of matrix metalloproteinases (MMP) that are important mediators of tissue degradation[30,31]. PGE_2 is also able to stimulate the production of vascular endothelial growth factor by synoviocytes, which may contribute to angiogenesis[32]. However, many recent experiments using purified PGE_2 have conflicted with earlier reports. For example, PGE_2 suppresses Th1 responses, and shifts Th0 T cells toward a Th2-like pattern dominated by the production of IL-4 and IL-5 in RA[33]. PGE_2 can also modulate the synthesis of rheumatoid factor[34], and a strong inhibition of MMP production is observed in rheumatoid synoviocytes under similar culture conditions[35]. For *in vivo* data, the administration of PGE_2 ameliorates arthritis in the rat model of adjuvant induced arthritis[36]. In this respect, PGE_2 acts an inhibitor of Th1 response and blocks the synthesis of proinflammatory cytokines in RA[33-36].

We demonstrated that PGE_2 acts as a potent inhibitor of IL-15 production, while inducing IL-10 production by FLS. These data suggest that overproduced PGE_2 in joints may display an antiinflammatory action by increasing IL-10 production. In addition, given that IL-15 is critical for the activation of T cells and NK cells, PGE_2 may play a role in the feedback inhibition of T cell and NK cell activation, subsequently quenching ongoing inflammation. The 10^{-5} M concentration of PGE_2, which exhibited prominent regulatory effects, is much higher than the level found in RA synovial tissue[27,28]. However, the effect of PGE_2 was also evident at concentrations as low as 10^{-8} M, which is comparable to the physiologic concentration of PGE_2 in RA joints. In addition, during an inflammatory response PGE_2 is mainly produced by monocytes and macrophages through direct cell-cell contact. It is possible that through the interaction of macrophages with synovial fibroblasts or T cells, an amount of PGE_2 sufficient to cause functional modulation of FLS may be secreted in RA joints *in vivo*.

The cellular and molecular mechanisms underlying varied effects of NSAID remain unclear. Especially the paradoxical effects of low versus high concentrations of NSAID on PGE_2 mediated cytokine regulations are intriguing. One potential explanation would be the upregulation, rather than suppression, of PGE_2 receptors (EP) by lower dose of NSAID[37,38]. It was also found that LPS-stimulated production of IL-12 and TNF-α was enhanced by the treatment with 10 μM of IDC[39], again indicating that low doses of this NSAID augmented proinflammatory reactions. On the other hand, concentrations much greater than those typically observed in plasma are required in cases where NSAID demonstrate immune suppressive effects, such as inhibition of EP4 expression and production of IL-12 and TNF-α in peritoneal macrophages[40]. These data, together with our observations, suggest that adequate and consistent dosage of NSAID should be administered to achieve clinically therapeutic effects in RA patients.

The activation of adenylate cyclase and subsequent accumulation of cAMP are downstream pathways activated by PGE_2 receptor signaling[12,13]. As expected, we observed that exogenous PGE_2 increased the level of cAMP in synoviocytes in a dose dependent manner. Experiments using inducers and a blocker of cAMP formation also indicate that cAMP is a major intracellular mediator of the cytokine regulation by PGE_2. Administration of PGE_2 instigates the increased secretion of IL-10 as well, and the blocking of this process partly diminished the suppression of IL-15 synthesis. Considering these facts, a strong repression in IL-15 production by PGE_2 may have been caused by a coordinated action of at least 2 elements, i.e., the accumulation of cAMP and the induction of IL-10 synthesis. Of note, following PGE_2 stimulation cAMP accumulation was observed as early as 30 min, whereas significant changes in IL-15 and IL-10 production were not detected until 6 h (data not shown). One possibility is that the accumulation of cAMP is a prerequisite for the induction of IL-10, whose expression is under the control of CREB binding. Alternatively, the signal transduction cascades after PGE_2 stimulation may adopt different pathways for cAMP formation and the regulation of cytokine synthesis.

The upstream regulatory region of human IL-15 gene has several consensus binding sites for factors including NF-IL-6, α-interferon response element, and NF-κB. Among these, the NF-κB site located at –75 to –65 relative to the transcription start site is shown to be critical for IL-15 mRNA upregulation induced by LPS or HTLV-1 Tax protein[25,26]. Studies have shown that the elevation of intracellular cAMP levels inhibits the induction of NF-κB regulated genes[16-18]. Also, IL-10 signaling is known to block the inhibitor of κB kinase, resulting in decreased DNA binding of NF-κB[41]. These findings, together with our data, suggest that both intracellular cAMP and IL-10 contribute to the downregulation of IL-15 gene through the suppression of NF-κB activity. In this respect, cAMP seems to take a more crucial role in the downregulation of IL-15 gene than IL-10, which appears to be dependent on cAMP accumulation. In our study, both PGE_2 and cAMP inducer caused a reduction of IL-1ß induced NF-κB binding, while treatment of DDA completely restored the binding activity. These observations suggest that downregulation of IL-15 gene by PGE_2 results from the inhibitory actions of cAMP on NF-κB activity. Interestingly, we also found that PGE_2 strongly inhibited the

AP-1 binding to IL-15 promoter (data not shown). Agonists and antagonists of cAMP affected the AP-1 activity in a manner similar to that of NF-κB, in accord with reports showing that PGE_2 signaling through AP-1 is transmitted via a cAMP dependent mechanism[42]. Still, it is possible that the regulatory effect of PGE_2 on IL-15 expression involves additional mediators yet to be identified.

In summary, findings in our study demonstrate that exogenous PGE_2 regulates the production of IL-15 and IL-10 by FLS in a divergent way. The inhibitory effect of PGE_2 on IL-15 transcription is wielded via cAMP controlled modulation of NF-κB activity. The escalation of IL-10 synthesis itself appears to be involved, at least partly, in the repression of IL-15. Together, our data suggest that over-produced PGE_2 may, in part, play an antiinflammatory role in the arthritic joints, rather than being a simple mediator of RA inflammation. Further studies, especially about PGE_2 blocking agents, are necessary to determine the effect of endogenously produced PGE_2 on cytokine regulation in RA.

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