Interleukin-4 inhibits prostaglandin E₂ production by freshly prepared adherent rheumatoid synovial cells via inhibition of biosynthesis and gene expression of cyclo-oxygenase II but not of cyclo-oxygenase I

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Abstract

Objective—To characterise the effect of interleukin-4 (IL-4) on the biosynthesis of cyclo-oxygenases I (COX I) and II (COX II), the rate limiting enzymes of the synthesis of prostaglandin E₂ (PGE₂), in freshly prepared rheumatoid synovial cells.

Methods—Adherent synovial cells were obtained from rheumatoid synovium by collagenase digestion. The concentrations of PGE₂ in culture supernatants were determined by enzyme linked immunosorbent assay. The protein and mRNA concentrations of COX I and COX II were determined by Western blotting and reverse transcription polymerase chain reaction, respectively.

Results—Freshly prepared synovial cells produced large amounts of PGE₂. They also showed increased gene expression of COX I and COX II, and synthesised these proteins. IL-4 had suppressive effects on the production of PGE₂ by untreated or lipopolysaccharide (LPS) stimulated synovial cells. In addition, IL-4 inhibited the biosynthesis of COX II at the mRNA level. In contrast, it did not modify the protein concentration of COX I. In tests of cell specificity, IL-4 did not reduce the mRNA concentration of COX II in interleukin-1α (IL-1α) stimulated cultured synovial fibroblasts at passages 3–6, but it reduced considerably the mRNA concentrations of COX II in an LPS or IL-1α stimulated U937 monocyte/macrophage cell line.

Conclusions—These results suggest that IL-4 might inhibit overproduction of PGE₂ in rheumatoid synovia via selective inhibition of the biosynthesis of COX II, and that this inhibition might be specific to macrophage-like synovial cells.


Rheumatoid arthritis (RA) is an inflammatory joint disease in which perpetuation of the chronic synovitis leads to bone and cartilage degradation.¹ Inflammatory cytokines or soluble factors are essential in pathogenesis of RA, and rheumatoid synovia are known to be rich sources of these mediators.²,³ Among them, prostaglandin E₂ (PGE₂), actively produced by rheumatoid synovia, is widely considered to have an important role in the inflammatory process in rheumatoid joints.³,⁴ Consequently, non-steroidal anti-inflammatory drugs (NSAIDs), the inhibitors of the production of prostaglandin metabolites, are extensively used in the treatment of RA.

Cyclo-oxygenase (COX), which converts arachidonic acid to prostaglandin endoperoxides, is the rate limiting enzyme in prostanoid synthesis.⁵ At least two forms of COX have been identified, and their genes have been cloned:⁴ type I COX (COX I) is expressed constitutively in most tissues and is involved in cellular ‘housekeeping’ activities such as coordinating actions of circulating hormones and regulating vascular homeostasis.⁷ In contrast, type II COX (COX II) is highly inducible by serum, growth factors, and lipopolysaccharide (LPS) in certain cell types involved in the inflammatory process in RA, including macrophages,⁸ fibroblasts,⁹ and endothelial cells.¹⁰ In addition, it has been demonstrated that COX II is expressed in rheumatoid synovia and synovial fibroblasts, and that interleukin-1β (IL-1β) increases the mRNA concentrations of COX II in these cells.¹¹,¹² These observations suggest that COX II may play an important part in over-production of PGE₂ in rheumatoid synovia.

Interleukin-4 (IL-4), originally described as a B cell growth factor, is a 20 kDa product of activated T cells.¹³ We and other investigators previously reported that IL-4 inhibited the production of inflammatory cytokines such as IL-1β, IL-6, and IL-8 by rheumatoid synovia or freshly prepared rheumatoid synovial cells.¹⁴,¹⁵ In addition to these anti-inflammatory actions, inhibitory effects of IL-4 on PGE₂ production by monocytes/macrophages,¹⁶ fibroblasts,¹⁷ and mesangial cells¹⁸ have also been demonstrated. However, the mechanism(s) of the inhibitory action of IL-4 on PGE₂ production remains to be clarified.

We have investigated the effect of IL-4 on the production of PGE₂, and on the biosynthesis
and gene expression of COX I and COX II, in freshly prepared rheumatoid synovial cells.

**Patients and methods**

**Patient population**

Rheumatoid synovial tissues were obtained from the knee or wrist joints of patients who were undergoing surgical synovectomy or total joint replacement. The population consisted of five patients with RA (three women and two men, age range 52–63 years) who fulfilled the criteria of the American College of Rheumatology. All five patients were treated with NSAIDs; two of the five were treated with gold sodium thiomalate, and another two with bucillamine.

**Cell culture**

*Adherent rheumatoid synovial cells*—Isolated rheumatoid synovial were aseptically dissected free from surrounding tissues, minced, and enzymatically digested with 0.5–1 mg/ml clostridium collagenase (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and 5–10 μg/ml deoxyribonuclease 1 (Sigma Chemical Co St Louis, MO) for two to three hours. After digestion, the resultant single cell suspension was washed, filtered through sterile gauze and nylon mesh, washed thoroughly once again, and finally resuspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS) (ICN Biomedicals, Australia), penicillin 100 U/ml, gentamicin 60 μg/ml, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) 12.5 mmol/l, and 1-glutamine 2 mmol/l (DMEM/10% FCS). The cells were cultured overnight in a humidified 5% carbon dioxide incubator to allow them to adhere to a plastic surface. The plate was washed to remove non-adherent cells, and the remaining adherent cells were used as adherent synovial cells.

*Rheumatoid synovial fibroblasts*—The freshly prepared adherent synovial cells obtained as above were cultured with DMEM/10% FCS at 37°C. When cellular confluency was attained (five to seven days), the cells were harvested by trypsinisation, washed, and one third of them replaced in culture dishes. Thereafter, one third of the cells were passaged when 95% confluency was reached. The synovial cells at three or more passages were morphologically fibroblast-like, and all negative for CD14 antigen and HLA-DR antigen on their surface (data not shown); they were therefore used as rheumatoid synovial fibroblasts in this study.

*U937 cell line*—U937 cells, human histiocytic lymphoma, kindly provided by Japanese Cancer Research Resources Bank, were cultured in RPMI 1640 supplement with 10% FCS, HEPES, and L-glutamine. U937 cells were differentiated into monocyte/macrophage cells by 48 hours of incubation with 100 nmol/l of phorbol myristate acetate (PMA) (Sigma Chemical Co). After differentiation, the cells became adherent to the dish and expressed CD14 antigen on their surface (data not shown).

**PGE2 measurement**

PGE2 measurement on synovial cell cultures was performed using enzyme linked immunosorbent assay kit (ELISA Technology, Lexington, KY).

**Western blot analysis**

After incubation with various stimuli, freshly prepared adherent synovial cells were lysed in a buffer (50 mmol/l TRIS hydrochloric acid, pH 8.0, 1% Nonidet® P-40, 0.5% deoxycholic acid, 150 mmol/l sodium chloride, and 1 mmol/l phenyl methylsulphonyl fluoride). Proteins were separated by electrophoresis on a 7.5% sodium dodecyl sulphate-polyacrylamide gel, and transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). After blocking, the membrane was incubated with antihuman COX I or antihuman COX II rabbit polyclonal antibodies (Oxford Biomedical Research, Oxford, MI), then with antirabbit donkey antibodies conjugated with horseradish peroxidase. Specific bands were visualised by an enhanced chemiluminescence detection system (ECL, Amersham International, Buckinghamshire, UK), and exposed to Fuji new RX films (Fuji Photo Film, Kanagawa, Japan).

**Extraction of RNA, and reverse transcription polymerase chain reaction**

Freshly prepared adherent synovial cells, rheumatoid synovial fibroblasts, or PMA differentiated U937 cells were cultured with IL-1α or LPS in the presence or absence of IL-4, dexamethasone, or indomethacin for eight hours, and their total cellular RNAs were extracted by acid guanidine phenol/chloroform extraction using Isogen (Wako Pure Chemical Industries, Ltd).

Two micrograms of total RNA was reverse transcribed to cDNA after annealing with 100 pmol of oligo deoxythymine (dT)12-18 primer (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in the presence of 100 U of Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Gibco BRL Life Technologies, Inc, Gaithersburg, MD), 10 U of ribonuclease inhibitor (RNasin®, Promega Corporation, Madison, WI), 1 mmol/l dithiothreitol, 25
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SPONTANEOUS PRODUCTION OF PGE₂ BY FRESHLY PREPARED ADHERENT SYNOVIAL CELLS

To evaluate the capacity of adherent synovial cells to produce PGE₂ (the major arachidonate metabolite produced by rheumatoid synovial cells), samples of these cells were cultured in DMEM/10%FCS without stimulus, the culture supernatant was replaced with fresh medium every 24 hours, and the replaced supernatant was measured for PGE₂.

EFFECT OF IL-4 ON PRODUCTION OF PGE₂ BY ADHERENT SYNOVIAL CELLS

To examine if IL-4 inhibited the spontaneous production of PGE₂ by freshly prepared adherent synovial cells, the cells were cultured with increasing concentrations (0, 0.05, 0.5, 5, or 50 U/ml) of IL-4 for 24 hours, and the PGE₂ concentrations in the supernatants determined. Seven days after isolation, cells were cultured with or without 1 µg/ml of LPS in the presence or absence of IL-4 0, 1, 10, or 100 U/ml, dexamethasone 5 µmol/l, or indomethacin 5 µmol/l (as positive controls) for 24 hours, and the concentrations of PGE₂ in the culture supernatants determined.

Different adherent synovial cells obtained from five patients with RA were cultured with or without 10 U/ml of IL-4 (patients Nos 1–4) or 50 U/ml of IL-4 (patient No 5) for 24 hours. The supernatants were collected and the concentrations of PGE₂ determined.

EFFECT OF IL-4 ON BIOSYNTHESIS OF COX I AND COX II IN ADHERENT SYNOVIAL CELLS

To clarify the mechanism by which IL-4 inhibited the production of PGE₂, we examined the effect of IL-4 on the biosynthesis of COX I and COX II proteins. Freshly prepared adherent synovial cells were incubated with or without IL-1α 10 ng/ml or LPS 1 µg/ml in the presence or absence of IL-4 100 U/ml or dexamethasone 5 µmol/l (as positive control) for 24 hours. After incubation, the cells were lysed and the lysates analysed by western blotting using rabbit antihuman COX I and COX II. Adherent synovial cells were also cultured with LPS 1 µg/ml in the presence or absence of IL-4 1, 10, 100, or 1000 U/ml for 24 hours and the protein level of COX II in the cell lysate determined by western blotting.

EFFECT OF IL-4 ON THE GENE EXPRESSION OF COX II IN ADHERENT SYNOVIAL CELLS

To clarify further whether IL-4 might inhibit the expression of COX II at the mRNA level, we examined the effect of IL-4 on the gene expression of COX II by adherent synovial cells. Freshly prepared adherent synovial cells were cultured with or without IL-4 100 U/ml, dexamethasone 5 µmol/l, or indomethacin 5 µmol/l for 24 hours. After incubation, their total cellular RNAs were extracted, and the mRNA contents of COX I, COX II, and HPRT were determined by RT-PCR analysis. HPRT was the housekeeping gene used to assess the amount of RNA samples.

DIFFERENTIAL EFFECTS OF IL-4 ON PGE₂ PRODUCTION BY CULTURED RHEUMATOID SYNOVIAL FIBROBLASTS AND A PMA DIFFERENTIATED U937 MACROPHAGE CELL LINE AND THEIR GENE EXPRESSIONS OF COX II

To determine the cell specificity of the suppressive effect of IL-4, we examined the effect of IL-4 on the production of PGE₂ and the gene expression of COX II by macrophage-like cells or fibroblast-like cells, which comprise the two major cell populations of freshly prepared adherent synovial cells. IL-1α stimulated cultured rheumatoid synovial fibroblasts—Rheumatoid synovial fibroblasts at three to six passages were cultured at a concentration of 2 × 10⁵/well in 24 well macroplates with or without IL-1α 10 ng/ml in the presence of IL-4 100 U/ml, dexamethasone 5 µmol/l, or indomethacin 5 µmol/l for 24 hours and the concentrations of PGE₂ in the culture supernatants determined. Additional cells were cultured with or without IL-1α 10 ng/ml in the presence of IL-4 10 or 100 U/ml (experiment
Results

SPONTANEOUS PRODUCTION OF PGE2 BY FRESHLY PREPARED ADHERENT SYNOVIAL CELLS

Freshly prepared adherent synovial cells produced large amounts of PGE2 for one to two days, after which the production decreased rapidly (fig 1), suggesting that these adherent synovial cells were spontaneously activated to produce PGE2, but that the culture conditions did not continuously stimulate them to produce it. The same was observed in three different cultures of adherent synovial cells. Consequently, we carried out subsequent experiments using freshly prepared adherent synovial cells obtained within two days of the preparation of rheumatoid synovia.

SUPPRESSIVE EFFECT OF IL-4 ON PGE2 PRODUCTION BY FRESHLY PREPARED ADHERENT SYNOVIAL CELLS

IL-4 inhibited the production of PGE2 by freshly prepared adherent synovial cells in a dose dependent manner that became effective at concentrations as small as 0.05 U/ml (fig 2A). This effect was consistently observed in patients with RA: a mean (SD) inhibition of 56 (3)% was observed in the five patients studied (fig 3).

LPS markedly enhanced the production of PGE2 by these cells, and IL-4 inhibited this augmented production in a dose dependent manner (fig 2B). However, the effect of IL-4 seemed to be less potent than that of either dexamethasone or indomethacin.

EFFECT OF IL-4 ON THE BIOSYNTHESIS OF COX I AND COX II IN FRESHLY PREPARED ADHERENT SYNOVIAL CELLS

Bands of both COX proteins were detected in freshly prepared adherent synovial cells. The COX I protein consisted of a major band around 70 kDa, whereas the COX II protein was composed of three bands around 70 kDa

Figure 1. Spontaneous production of prostaglandin E2 (PGE2) by freshly prepared adherent rheumatoid synovial cells. The culture supernatants were collected on the days indicated. Values are the mean of triplicate determinations.

Figure 2. Suppressive effect of interleukin-4 (IL-4) on the production of prostaglandin E2 (PGE2) by adherent rheumatoid synovial cells. A: Cells cultured with or without increasing concentrations of IL-4 for 24 hours. Values are the mean (SD) of triplicate determinations. B: Cells cultured, seven days after isolation, with or without lipopolysaccharide (LPS) 1 μg/ml in the presence or absence of IL-4 1, 10, or 100 U/ml, dexamethasone (Dex.) 5 μmol/l, or indomethacin (Ind.) 5 μmol/l for 24 hours. Data are representative of three separate experiments.
Effect of IL-4 on biosynthesis of cyclo-oxygenase in rheumatoid synovial cells

Addition of IL-4 reduced the protein content of COX II (fig 4A). This inhibition of COX II biosynthesis by IL-4 was dose dependent and was effective at concentrations as small as 10 U/ml (fig 4B). IL-4 also reduced the remarkably increased protein content of COX II observed when the adherent synovial cells were incubated in the presence of IL-1α or LPS (fig 4A). In contrast, IL-4, LPS, or IL-1α did not alter the protein level of COX I at all. Dexamethasone was more potent than IL-4 in reducing the protein levels of COX II.

SUPPRESSIVE EFFECT OF IL-4 ON THE GENE EXPRESSION OF COX II IN FRESHLY PREPARED ADHERENT SYNOVIAL CELLS

The mRNA of COX II was spontaneously expressed in these adherent synovial cells. This gene expression of COX II was considerably reduced by both IL-4 and (as previously reported12) dexamethasone (fig 5). In contrast, IL-4 and dexamethasone did not alter the mRNA content of COX I, and indomethacin had no effect on the concentrations of either COX I or COX II mRNA (fig 5).

DIFFERENTIAL EFFECTS OF IL-4 ON PGE2 PRODUCTION BY CULTURED RHEUMATOID SYNOVIAL FIBROBLASTS AND A PMA DIFFERENTIATED U937 MACROPHAGE CELL LINE AND THEIR GENE EXPRESSIONS OF COX II

Unexpectedly, IL-4 did not inhibit PGE2 production by IL-1α stimulated rheumatoid synovial fibroblasts (fig 6A), but dexamethasone and indomethacin each had a potent inhibitory effect on its production. IL-4 also failed to alter the content of COX II mRNA in these cells (fig 6B).

When PGE2 production and the gene expression of COX II by the PMA differentiated U937 macrophage cell line were also examined, the amounts of PGE2 produced were small, but were increased by IL-1α and LPS, while addition of IL-4 reduced the enhanced amount of PGE2 to basal levels (fig 7A). In RT-PCR analysis of COX II mRNA in these cells, basal concentrations of COX II mRNA were not detectable, but the protein was markedly upregulated after stimulation with IL-1α or...
Effect of differentiated glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene). A. COX II expression was measured by reverse transcription polymerase chain reaction in RSFs stimulated with IL-4 or IL-4 + IL-1α. B. GAPDH expression was measured as a control.

Figure 7  Effect of interleukin-4 (IL-4) on production of prostaglandin E2 (PGE2) and gene expression of cyclo-oxygenase II (COX II) by a phorbol myristate acetate differentiated U937 macrophage cell line stimulated with interleukin-1α (IL-1α) 10 ng/ml or lipopolysaccharide (LPS) 1 μg/ml. A: Production of PGE2. Stimulated cells cultured with IL-4 100 U/ml or dexamethasone 5 μmol/l. Values are mean of triplicate determinations. B: Reverse transcription polymerase chain reaction analysis of gene expression of COX II. Stimulated cells cultured with IL-4 100 U/ml or dexamethasone 5 μmol/l. GAPDH = glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene). A. B—Columns or LANE S = 1 = Medium alone; 2 = LPS alone; 3 = LPS + IL-4; 4 = LPS + dexamethasone; 5 = IL-1α alone; 6 = IL-1α + IL-4; 7 = IL-1α + dexamethasone.

LPS for eight hours, and IL-4 strongly inhibited the enhanced levels of COX II mRNA, as did dexamethasone (fig 7B).

Discussion
PGE2 is a potent mediator of the pain and oedema associated with rheumatoid synovitis,14 and is involved in bone resorption.15 In the present study, freshly prepared adherent synovial cells spontaneously produced large amounts of PGE2. However, the capacity of these cells to produce PGE2 was rapidly lost during the period of culture, suggesting that these cells maintained the nature of the inflammatory process that was seen in the patient. Consequently, use of freshly prepared adherent synovial cells was appropriate in this study, though these cells were heterogeneous.

We were able to demonstrate that the protein and mRNA contents of COX II were spontaneously expressed in freshly prepared adherent synovial cells, and that IL-1α and LPS increased their protein contents. In contrast, these stimuli did not modify the protein contents of the constitutive COX I. These results are consistent with those of previous studies showing that the mRNA level of COX II was upregulated by IL-1β in cultured synovial cells from patients with RA12 or osteoarthritis,28 and suggest that overproduction of PGE2 in rheumatoid synovia might be mainly attributable to new synthesis of COX II enzyme in response to various stimuli including IL-1.

We and other investigators have previously demonstrated the anti-inflammatory effects of IL-4 on rheumatoid synovia or synovial cells by inhibiting the production of proinflammatory cytokines such as IL-1β, IL-6, and IL-8.15-17 We have now shown that IL-4 inhibited the spontaneous production of PGE2 by 56%, and that these suppressive effects were also observed in adherent synovial cells stimulated by LPS or IL-1α. In addition, IL-4 inhibited the protein and mRNA contents of COX II, but did not modify those of COX I. This is the first report of the effect of IL-4 on the expression of COX in rheumatoid synovia, while decreased concentrations of IL-4 in rheumatoid synovia have been reported at the protein27 and mRNA levels.28 Together, these findings suggest that a lack of IL-4 might be partially involved in the overexpression of COX II in the rheumatoid synovium.

The mechanism(s) by which IL-4 selectively inhibits the production of COX II mRNA are unknown, though one recent study demonstrated that IL-4 inhibited COX II mRNA at both the level of transcription and that of post-transcription.29 The human COX I and II polypeptides are 61% identical in primary
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We thank Dr Hiroshi Yamada, Takaoka City Hospital; Dr Konno, Local Government, Saiseikai Takaoka Hospital; and Dr Inoue, Pharmaceutical Co for the recombinant human IL-1α; and Ono Pharmaceutical Co for the recombinant human IL-4.