Coexpression of Microsomal Prostaglandin E Synthase with Cyclooxygenase-2 in Human Rheumatoid Synovial Cells

FUMIAKI KOJIMA, HIROAKI NARABA, YASUHARU SASAKI, RENZO OKAMOTO, TOMIHISA KOSHINO, and SHINICHI KAWAI

ABSTRACT. ***Objective.*** Recently, microsomal prostaglandin (PG) E synthase (mPGES) was cloned as a terminal enzyme catalyzing PGH_2 to PGE_2. We investigated mPGES as well as cyclooxygenase (COX)-2, catalyzing arachidonic acid to PGH_2, in synovial cells from patients with rheumatoid arthritis (RA). The effect of dexamethasone on mPGES expression was also studied.

Methods. Synovial cells were treated with interleukin 1ß (IL-1ß) and dexamethasone under various conditions, and expression of mPGES mRNA and protein was analyzed by Northern blot and Western blot, respectively. Conversions of arachidonic acid or PGH_2 to PGE_2 were measured by ELISA. Subcellular localization of mPGES and COX-2 was determined by immunofluorescent microscopic analysis.

Results. mPGES mRNA and protein expression were significantly upregulated by IL-1ß in synovial cells. COX-2 mRNA and protein were also upregulated by IL-1ß, but with a different time course from that of mPGES. Conversion of PGH_2 to PGE_2 was increased by IL-1ß and was correlated with mPGES expression. Increased conversion of arachidonic acid to PGE_2 was maintained when mPGES and COX-2 were coexpressed. Subcellular localization of mPGES and COX-2 overlapped in the perinuclear region in IL-1ß stimulated synovial cells. Dexamethasone inhibited mRNA and protein expression for mPGES and increased conversion of arachidonic acid to PGE_2, but inhibition of mPGES was weaker compared with that of COX-2 in IL-1ß stimulated cells.

Conclusion. The results suggest that abundant PGE_2 production at inflammation sites such as rheumatoid synovia is caused by the coordinated upregulation of mPGES and COX-2. Thus mPGES might be a potential new target for therapeutic strategies to control PGE_2 synthesis specifically in patients with RA and other inflammatory diseases. (J Rheumatol 2002;29:1836–42)

Key Indexing Terms:
PROSTAGLANDIN E SYNTHASE | CYCLOOXYGENASE | SYNOVIAL CELLS
RHEUMATOID ARTHRITIS | INTERLEUKIN 1ß | DEXAMETHASONE

Prostaglandin (PG) E_2 is one of the important mediators of inflammation associated with rheumatoid synovitis, and cytokine activated synovial cells are likely to be a primary source of this mediator in the affected joints of patients with rheumatoid arthritis (RA)[1]. Induction of PGE_2 production by cytokines coincides with the upregulation of cyclooxygenase (COX)-2 expression in synovial cells, and COX-2 has been considered to be the rate-limiting enzyme for PGE_2 production at sites of inflammation[1-3].

Microsomal prostaglandin E synthase (mPGES) catalyzes the final step of PGE_2 synthesis, which is conversion of PGH_2 to PGE_2 downstream of the site where COX-2 acts[4]. Several groups have attempted to identify the PGES molecule[5,6]. Recently, we[7] and other investigators[8–11] reported the molecular identification of mPGES molecules in several species, and it has been found to be a membrane bound, glutathione dependent, inducible enzyme. Because of its inducible nature, it is of great interest to investigate the role of mPGES in various inflammatory diseases. Thus we studied the expression of mPGES compared with that of COX-2 in interleukin 1ß (IL-1ß) stimulated synovial cells.

It is well known that glucocorticoids are highly effective for controlling chronic inflammatory diseases, such as RA. Glucocorticoids exert an antiinflammatory action at least partly by suppressing COX-2 induction in synovial cells[1,2,12]. Thus we investigated the effect of dexamethasone

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on the expression of mPGES and COX-2 in IL-1ß stimulated synovial cells.

MATERIALS AND METHODS

Materials. Rabbit anti-human mPGES polyclonal antibody, rabbit anti-human COX-2 polyclonal antibody, arachidonic acid, PGH_2 and an ELISA kit for PGE_2 were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Goat anti-human COX-2 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase conjugated goat anti-rabbit IgG, rhodamine (TRITC) conjugated donkey anti-goat IgG and fluorescein (FITC) conjugated donkey anti-rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA, USA). A megaprime DNA labeling system, [α-^{32}P]dCTP, nylon membrane (Hybond N+), and an ECL Western blot analysis system were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Recombinant human IL-1ß (Genzyme, Cambridge, MA, USA), dexamethasone (Wako Pure Chemicals, Osaka, Japan), TRIzol reagent (Life Technologies, Grand Island, NY, USA), and polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA) were purchased. Fragments of human mPGES cDNA (encoding +1 to +459) and COX-2 cDNA (encoding +590 to +1743) were prepared from human umbilical vein endothelial cells by the reverse transcription-polymerase chain reaction method. Amplified DNA fragments of mPGES and COX-2 were subcloned into the pCR3.1-uni vector (Invitrogen, Carlsbad, CA, USA) and pGEM vector (Promega, Madison, WI, USA), respectively, and digested by appropriate restriction enzymes[7].

Preparation of human synovial cells. Synovial tissues were obtained when joint replacement surgery was performed in patients with RA who gave consent to the use of their tissue specimens for our research. Synovial cells were prepared from the tissues as described[3]. Briefly, synovial tissues were minced and digested with 0.2% collagenase for 3 h, filtered through a nylon mesh, washed extensively, and cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) at 37°C under 5% CO_2. The isolated cells, referred to as first-passage, were a heterogeneous mixture of all the infiltrating mononuclear cells and connective tissue cells found in synovial tissue. The cells were incubated at 37°C under 5% CO_2 until the adherent cells reached confluence, and nonadherent cells were removed. We used the adherent cells, the second-passage synovial cells, which were homogenous, presumably fibroblasts, compared with the first-passage cells[13].

Stimulation of synovial cells by IL-1ß. Synovial cells were seeded into each well of 6 or 48 well plates at a density of 5×10^4 cells/cm^2 in culture medium containing 2% FCS, as described[3]. The cells were incubated with or without 1 ng/ml of IL-1ß for various times. In experiments involving treatment with dexamethasone, several concentrations of dexamethasone were added at 1 h before IL-1ß stimulation.

Northern blot analysis. Northern blot was performed as described[14]. Total RNA (4 μg) was extracted from synovial cells with TRIzol reagent and was electrophoresed on 1% formaldehyde/agarose gel, transferred to a Hybond N+ membrane, and hybridized with cDNA probes that had been labeled with [α-^{32}P]dCTP by random priming. After hybridization, the membranes were washed and RNA bands were visualized by autoradiography.

Western blot analysis. Cells were lysed in Tris buffered saline (TBS) containing 0.1% sodium dodecyl sulfate (SDS), and protein content was determined using the BCA protein assay reagent (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as standard. Cell lysates adjusted to 5 μg protein were applied to SDS-polyacrylamide gels (15% for mPGES and 10% for COX-2) for electrophoresis as reported[14]. Then the proteins were electroblotted onto Immobilon-P polyvinylidene difluoride membranes with a semidry blotter (Atto, Tokyo, Japan). After blocking the membranes in 10 mM TBS containing 0.1% Tween-20 (TBS-T) and 5% skim milk, rabbit anti-human mPGES antibody or rabbit anti-human COX-2 antibody was added at 1/500 or 1/1000 dilution in TBS-T, respectively, and incubation was carried out for 1.5 h. After washing the membranes with TBS-T, horseradish peroxidase conjugated goat anti-rabbit second antibody was added at 1/10,000 dilution in TBS-T and incubation was done for 1 h. After further washing with TBS-T, protein bands were visualized with an ECL Western blot analysis system.

Conversion of arachidonic acid to PGE_2. IL-1ß stimulated cells were washed with RPMI-1640 medium, and incubated an additional 30 min with 3 μM arachidonic acid, as reported[3]. Then the culture medium was harvested, and the PGE_2 concentration of the supernatant was measured with an ELISA kit.

Conversion of PGH_2 to PGE_2. PGES activity was measured by assessment of conversion of PGH_2 to PGE_2 as reported[15]. Briefly, cells were scraped off the dish and disrupted by sonication (10 s, 3 times, 1 min interval) in 100 μl of 1 M Tris-HCl (pH 8.0). After centrifugation of the sonicates at 1700 ×g for 10 min at 4°C, the supernatants were used as the enzyme source. For assessment of PGES activity, an aliquot of each lysate (40 μg protein equivalents) was incubated with 2 μg of PGH_2 for 30 s at 24°C in 0.15 ml of 1 M Tris-HCl containing 2 mM glutathione. The reaction was terminated by the addition of 100 mM $FeCl_2$. The reaction mixtures were left at room temperature for 15 min, and the PGE_2 concentration of the mixtures was measured with an ELISA kit.

Immunofluorescent microscopic analysis. Cells were seeded onto cover glasses at a density of 2.5×10^4 cells/cm^2 and incubated with or without 1 ng/ml of IL-1ß for 24 h. In experiments involving treatment with dexamethasone, 1 μM of dexamethasone was added at 1 h before IL-1ß stimulation. After removing the supernatants, the cells were fixed with 4% (w/v) paraformaldehyde in PBS for 30 min at 4°C. The cells were then permeabilized on the membranes with 0.1% Triton-X in PBS for 3 min. After blocking with 1% (w/v) BSA in PBS for 1 h at room temperature, rabbit anti-human mPGES polyclonal antibody and goat anti-human COX-2 polyclonal antibody that were premixed so that their final dilutions became 1:100 and 1:250 in PBS containing 1% albumin, respectively, were added for 1.5 h at room temperature. After washing with PBS, TRITC and FITC conjugated second antibodies were added at 1/50 dilution for each in PBS containing 1% albumin for 1 h. The coverslips were mounted on glass slides using Vectashield mounting medium (Vector, Burlingame, CA, USA) and examined using an AxioPlan-2 microscope connected to a Zeiss AxioCam digital camera. The specificities of antibodies against mPGES and/or COX-2 for these analyses were evaluated by the absorbance control experiments using antigen peptides for mPGES (Cayman Chemical Co., Ann Arbor, MI, USA) and/or COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In both cases, respective preabsorbed enzyme was not detectable in the immunofluorescent analysis (data not shown).

RESULTS

Expression of mPGES and COX-2. Expressions of the mRNA and protein for mPGES and COX-2 were assessed by Northern blot and Western blot analyses, respectively. The expression of mPGES mRNA was increased gradually from 3 to 24 h after IL-1ß stimulation and then was sustained until at least 48 h (Figure 1a). Expression of mPGES protein also showed a gradual increase to a maximum level at 24–48 h (Figure 1d). On the other hand, the COX-2 mRNA level showed a rapid increase at 3 h after IL-1ß stimulation and then gradually decreased over time until 48 h (Figure 1b). COX-2 protein showed maximal expression between 6 and 24 h, and then it decreased at 48 h (Figure 1e). COX-2 mRNA and protein were minimally expressed before culture and declined gradually in untreated cells, which may be caused by the reduction in serum concentration before the experiment.

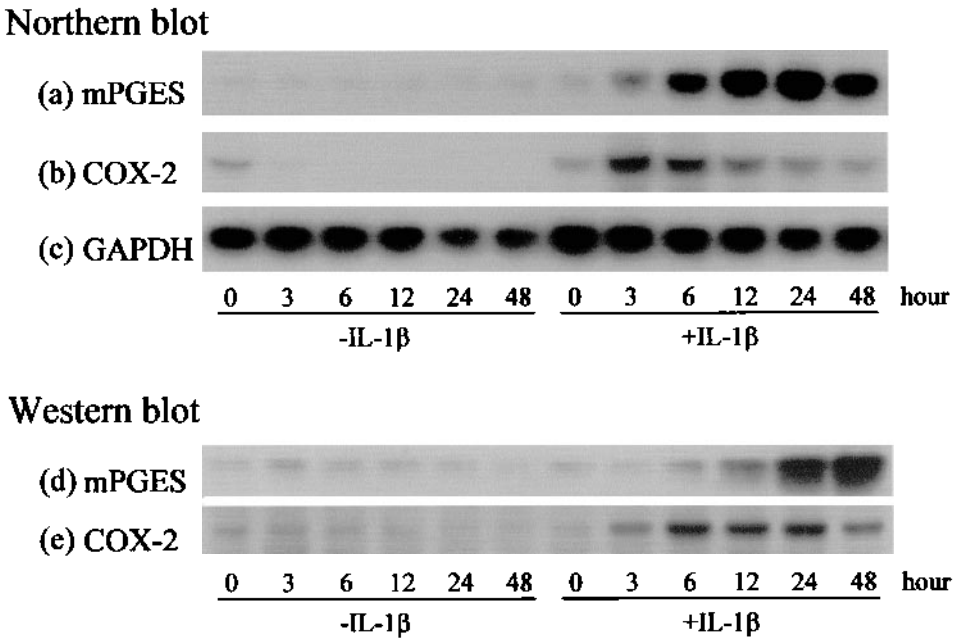

Figure 1. Effect of IL-1ß on COX-2 and mPGES expression in rheumatoid synovial cells. Total RNA and lysates were prepared from synovial cells incubated with or without 1 ng/ml of IL-1ß at the indicated times, as described in Materials and Methods. Total RNA was subjected to Northern blot analysis for mPGES (a), COX-2 (b), and GAPDH as a control (c). Lysates were subjected to Western blot analysis for mPGES (d) and COX-2 (e). The blots shown here are representative examples from 3 separate experiments.

Conversion of exogenous PGH_2 to PGE_2. To study whether PGES enzymatic activity may be upregulated by IL-1ß stimulation, we measured the conversion of PGH_2 to PGE_2 using lysates of IL-1ß stimulated cells at various times. PGES activity was increased gradually from 3 to 48 h after IL-1ß stimulation (Figure 2). The result was correlated with expression of mPGES protein (Figure 1d).

Conversion of exogenous arachidonic acid to PGE_2. To determine whether the upregulation of mPGES and/or COX-2 expression was associated with an increase of PGE_2 production, we measured the conversion of exogenous arachidonic acid to PGE_2 in IL-1ß stimulated cells at various times. PGE_2 production was observed at 3 h and it reached a maximum at 24 h after IL-1ß stimulation (Figure 3). Elevation of PGE_2 production was maintained for at least 48 h.

Effects of dexamethasone on mPGES and COX-2 expression. To determine the effect of glucocorticoid on mRNA and protein expression for mPGES and COX-2, cells were pretreated with various concentrations of dexamethasone (0.01 to 1 μM) for 1 h, followed by exposure to IL-1ß. The upregulation of COX-2 mRNA (Figure 4b) and protein (Figure 4e) induced by IL-1ß was completely inhibited by 0.01 μM dexamethasone. The upregulation of mPGES mRNA (Figure 4a) and protein (Figure 4d) was also inhibited by dexamethasone in a concentration dependent manner, but the inhibition was relatively weaker compared with those of COX-2.

Effects of dexamethasone on generation of PGE_2. Increased conversion of exogenous arachidonic acid to PGE_2 by IL-1ß stimulation, suggesting coordinated activity of mPGES and COX-2, was completely inhibited by treatment with 0.01

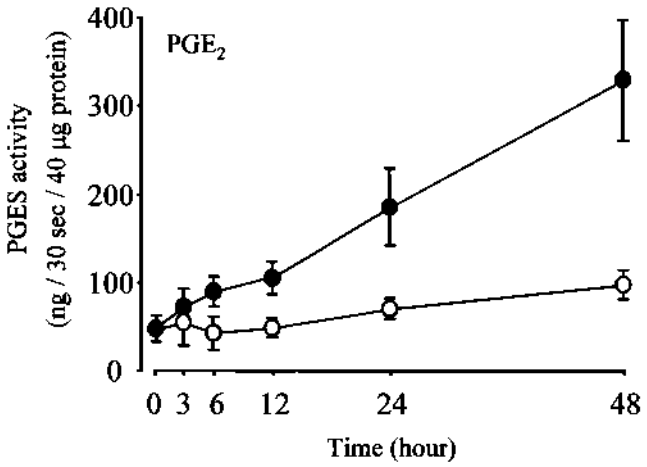

Figure 2. Induction of PGES activity in synovial cells. Synovial cells were incubated with (●) or without (○) 1 ng/ml of IL-1ß for the indicated times. Exogenous PGH_2 (2 μg) was added to cell lysates, and the tubes were incubated at 24°C for 30 s in the presence of reduced glutathione. The reaction was stopped with 100 mM $FeCl_2$, and PGE_2 concentration was measured as described in Materials and Methods. Spontaneous conversion of PGH_2 to PGE_2 obtained in the absence of cell lysates was subtracted from each value. Values are expressed as mean ± SEM from 3 separate experiments.

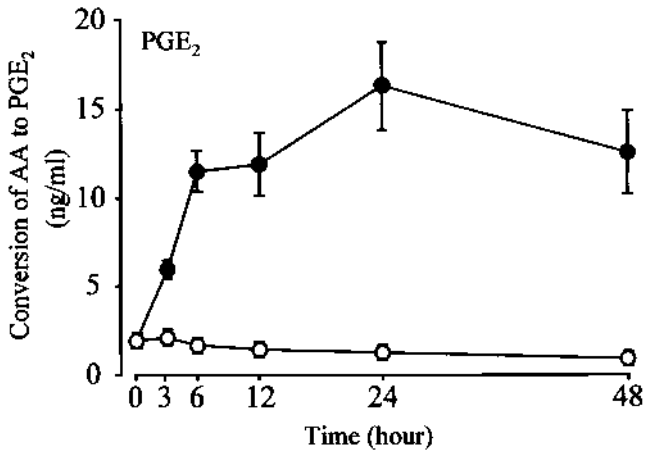

Figure 3. Conversion of exogenous arachidonic acid (AA) to PGE_2 in synovial cells. For estimation of the ability to derive PGE_2 from AA, synovial cells were incubated with (●) or without (○) 1 ng/ml of IL-1ß for the indicated times. After washing, the cells were incubated an additional 30 min with 3 μM AA, and the PGE_2 concentration of the medium was measured as described in Materials and Methods. Values are expressed as mean ± SEM from 5 separate experiments.

μM dexamethasone (Figure 5a). PGES activity, production of PGE_2 from PGH_2, was also inhibited by treatment with dexamethasone in a concentration dependent manner (Figure 5b), but the degree of inhibition was relatively weaker compared with that of conversion of arachidonic acid to PGE_2.

Localization of mPGES and COX-2 in synovial cells. To determine the subcellular localization of mPGES and COX-2 in synovial cells, double immunostaining for mPGES and COX-2 was performed. Under basal condition, mPGES immunoreactivity (Figure 6a) was observed at very low levels, and COX-2 immunoreactivity (Figure 6d) was not detectable. However, both mPGES (Figure 6b) and COX-2 (Figure 6e) immunoreactivity was expressed by IL-1ß stimulation, and the population of double immunostained cells for mPGES and COX-2 antibodies was 81%. Interestingly, subcellular localization of mPGES and COX-2 immunoreactivity overlapped at the perinuclear region. Moreover, the upregulation of mPGES (Figure 6c) and COX-2 (Figure 6f) immunoreactivity was inhibited by pretreatment of 1 μM dexamethasone.

DISCUSSION

This study showed that the expression of mRNA and protein for mPGES was significantly upregulated by IL-1ß in synovial cells. However, COX-2 showed a rapid increase of expression after IL-1ß stimulation, while expression of mPGES increased more gradually compared with COX-2. Recently, we demonstrated that both mPGES and COX-2 expression was also upregulated by lipopolysaccharide (LPS) in rat peritoneal macrophages[7,15,16]. In LPS stimulated rat peritoneal macrophages, expression of mPGES mRNA showed a marked increase from 6 to 12 h, and then decreased at 24 h. In this experiment, the time course of mPGES expression was similar to those of COX-2. Similarly, in the case of IL-1ß stimulated A549 cells, a human lung adenocarcinoma derived cell line, mPGES followed a similar time dependent expression as COX-2[17]. The reasons for the different time course of mPGES and COX-2 expression between IL-1ß stimulated human synovial cells and other reported cells have not been elucidated. Assessment of the promoter sequence of the mPGES gene and/or transcriptional factors associated with this gene may explain these differences.

To assess the regulation of PGE_2 synthesis in IL-1ß stimulated synovial cells, we measured the conversion of PGH_2 or arachidonic acid to PGE_2 at each time point. Conversion of PGH_2 to PGE_2 as PGES activity, downstream of COX-2, was gradually increased by IL-1ß stimulation and it was paralleled with the time course of mPGES expression in synovial cells. However, conversion of arachidonic acid to PGE_2, which involves 2 sequential enzymatic activities, COX-2 and mPGES, was rapidly increased and the increased level was maintained at 6 to 48 h after IL-1ß stimulation. Therefore, it seems the major factor influencing the PGE_2 increase in the early phase is dependent on rapidly expressed COX-2, while maintained PGE_2 production in the late phase is mainly dependent on slowly expressed mPGES in human synovial cells. These results suggest that the major rate-limiting enzymes involved in PGE_2 synthesis change at different phases of stimulation. Thus, coordinated expression of COX-2 and mPGES might be necessary to maintain abundant PGE_2 production.

We demonstrated the effects of dexamethasone on expression of mPGES mRNA and protein, and induction of PGES activity. The inhibitory effects of dexamethasone on mPGES expression were weaker than those on COX-2 expression at both the mRNA and protein levels. Moreover, we showed that treatment of dexamethasone completely inhibits conversion of arachidonic acid to PGE_2, which involves 2 enzyme activity, mPGES and COX-2. But its inhibitory effect on PGES activity, downstream of COX-2, was weaker than that on conversion of arachidonic acid to PGE_2. Recently, another report[18] showed that dexamethasone completely inhibited the expression of mPGES mRNA using a reverse transcription-polymerase chain reaction method in synovial cells. The reasons for the difference in degrees of inhibitory effect of dexamethasone on the expression of mPGES between their study and ours are unknown. Additional detailed examination, including assessment of the promoter sequence of the mPGES gene that interacts with the glucocorticoid receptor, remains to be done.

We previously reported that T cells and macrophages/monocytes were common in the isolated cells, referred to as first-passage synovial cells. In contrast, T cells and macrophages/monocytes were not detected in the second-

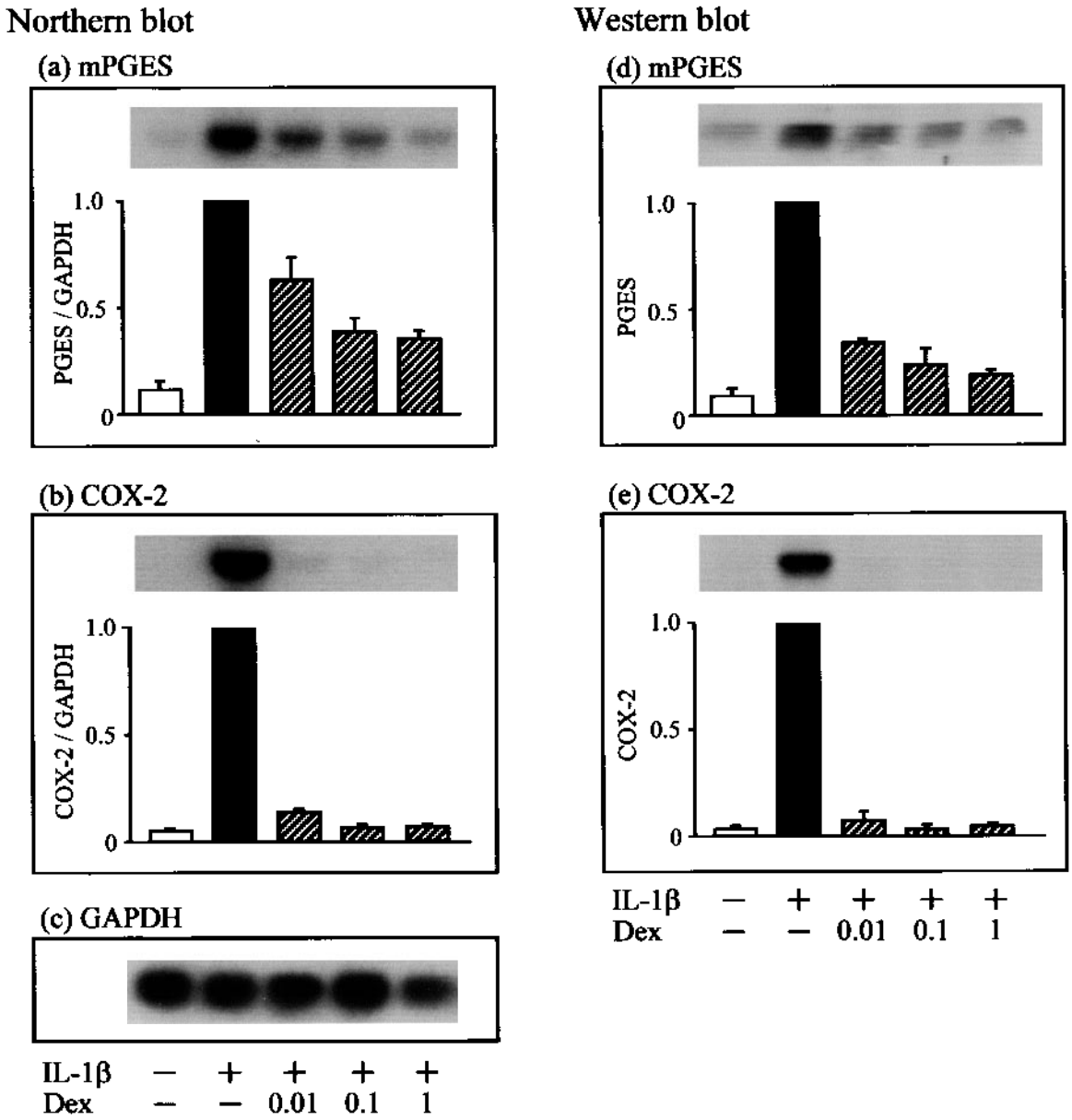

Figure 4. Effects of dexamethasone (Dex) on COX-2 and mPGES expression in IL-1ß stimulated synovial cells. Synovial cells were preincubated 1 h in the presence or absence of Dex (0.01, 0.1, 1 μM) and then were incubated 12 h with or without 1 ng/ml IL-1ß. Total RNA from the cells was subjected to Northern blot analysis for mPGES (a), COX-2 (b), and GAPDH (c), as described in Materials and Methods. mPGES and COX-2 expression was normalized to GAPDH as the average of 3 separate experiments, and results are expressed as mean ± SEM. Synovial cells were preincubated 1 h in the presence or absence of Dex (0.01, 0.1, 1 μM) and then incubated 24 h with or without 1 ng/ml of IL-1ß. Lysates from these cells were subjected to Western blot analysis for mPGES (d) and COX-2 (e), as described in Materials and Methods. Results are expressed as mean ± SEM of 3 separate experiments.

passage synovial cells by 2 color immunofluorescence and flow cytometry[13]. The major population of the second-passage synovial cells used in this study was homogenous, presumably fibroblasts. It might be suggested that synovial fibroblasts were responsible for the expression and activity of mPGES and COX-2.

The subcellular localization of COX-2 in synovial cells was similar to that of COX-2 in different kinds of cells of another report[19]. The present study revealed for the first time that mPGES protein in IL-1ß stimulated synovial cells was localized at the perinuclear region. Other reports described that mPGES protein and its activity were localized in the microsomal fraction in IL-1ß stimulated synovial cells[18] and A549 cells[17], but subcellular localizing sites of the enzyme were not defined. In this study, we found that mPGES and COX-2 were coexpressed in 81% of the synovial cells. We also revealed that subcellular localization of mPGES was overlapped with that of COX-2 at the perinuclear region in

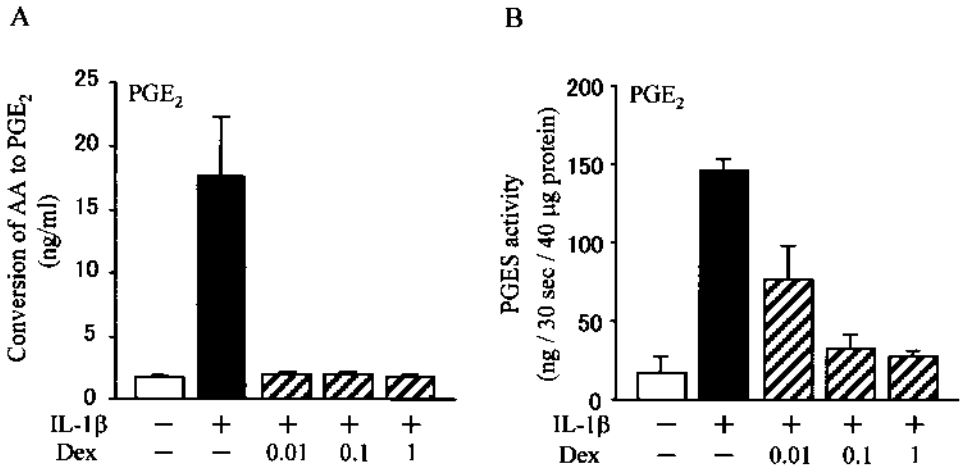

Figure 5. Effects of dexamethasone (Dex) on conversion of arachidonic acid (AA) to PGE_2 and PGES activity. A. Synovial cells were preincubated 1 h in the presence or absence of Dex (0.01, 0.1, 1 μM) and then incubated 24 h with or without 1 ng/ml IL-1ß. After washing, cells were incubated an additional 30 min with 3 μM AA and then PGE_2 concentration of medium was measured as described in Materials and Methods. B. PGES activity was determined as described in Materials and Methods. Spontaneous conversion of PGH_2 to PGE_2 obtained in the absence of cell lysates was subtracted from each value. Values are expressed as mean ± SEM from 3 separate experiments.

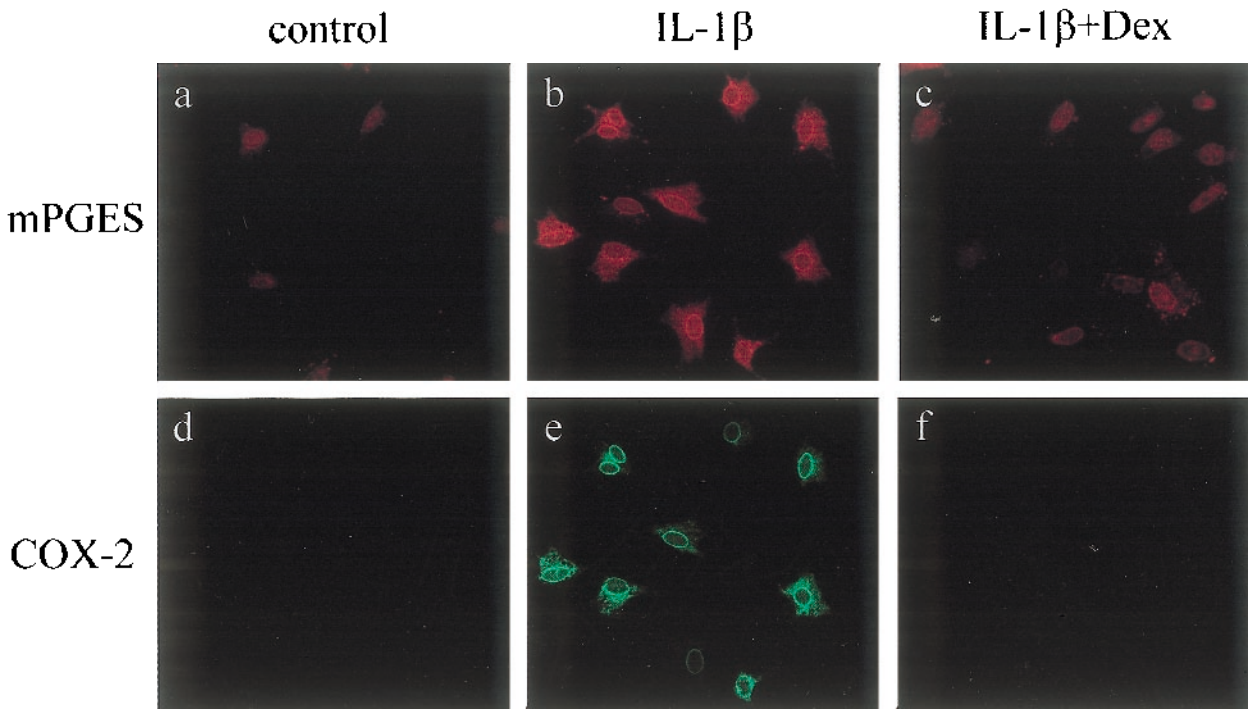

Figure 6. Localization of mPGES and COX-2 in synovial cells. Synovial cells were preincubated 1 h in the presence or absence of Dex (1 μM) and then incubated 24 h with or without 1 ng/ml IL-1ß. The cells were double immunostained with TRITC for mPGES (a, b, c) and FITC for COX-2 (d, e, f), and examined using a Fluoview laser fluorescence microscope as described in Materials and Methods.

IL-1ß stimulated cells. These data suggest that mPGES and COX-2 were functionally linked. The occurrence of this link in synovial cells was supported by our finding that increased conversion of arachidonic acid to PGE_2 was maintained during coordinated expression of mPGES and COX-2. Recently, we demonstrated that mPGES and COX-2 were functionally linked in mPGES and COX-2 transfected cell lines[7].

The role of PGE_2 in inflammation is supported by the attenuation of carrageenan induced paw edema and adjuvant arthritis in rats by neutralizing antibody against PGE_2[20]. In contrast, PGE_2 causes downregulation of a proinflammatory cytokine, tumor necrosis factor-α, whereas it causes upregulation of the antiinflammatory cytokine IL-10[21]. Because of these diverse effects of PGE_2, it is important to control its synthesis in inflammatory diseases. Since mPGES is a specific enzyme that produces PGE_2 from PGH_2, it might be a potential new target for therapeutic strategies to modulate just PGE_2 synthesis.

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