IL-1ß stimulates IL-8 production, including prostaglandin E_2 receptor EP4-triggered pathways, in synoviocyte MH7A cells

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Abstract. Prostaglandin E_2 (PGE_2) is an important modulator of cytokine-driven inflammation. Using GeneChip analysis, we found that interleukin (IL)-1ß induces the gene expression of PTGER4, which encodes the PGE_2 receptor subtype EP4 (PGE_2EP4). This subtype is one of four PGE_2 receptors occurring in synoviocyte MH7A cells. Immunofluorescence microscopy revealed a corresponding upregulation in the production of PGE_2EP4 protein in IL-1ß-pretreated MH7A cells. PGE_2 alone has no effect on IL-8 production, but in cells pretreated with IL-1ß it markedly enhances IL-8 production. Moreover, a stimulatory effect of PGE_2 on IL-8 production in the synoviocyte MH7A cells was observed. These results indicate that, in the synovial tissues of patients with rheumatoid arthritis, PGE_2 stimulates the release of IL-8 from the fibroblastic cells classified as present, thereby exacerbating inflammation.

Introduction

Rheumatoid arthritis (RA) is an autoimmune joint disease characterized by the inflammation and destruction of the articular surfaces and bone. The pathological steps leading to RA include inflammation, the proliferation of synovial cells, and the attachment and invasion of adjacent cartilage and bone by fibroblast-like cells derived from rheumatoid synoviocytes (FLSs) (1-3). During joint movement, synovial tissues adjust to the mechanical load by changing their shape. These elastic synovial membranes are an early target of rheumatic inflammation and, together with chondrocytes, become a primary source of inflammatory factors such as cytokines, which are secreted into the synovial fluid (4,5). Levels of pro-inflammatory cytokines such as interleukin (IL)-1ß are known to be elevated in the synovial fluid of patients with RA and play important roles in the amplification and perpetuation of inflammation and joint destruction (6-8).

IL-8 is the prototypical member of a superfamily of small (8-10 kDa) inducible secreted chemoattractant cytokines (chemokines) that were originally identified as monocyte-derived factors capable of attracting and activating neutrophils (9,10). Many cell types are known to synthesize and release IL-8 (and other chemokines) in response to injury, infection, inflammation and various pathological conditions (11-14). This chemokine is overexpressed in rheumatoid synovial tissue, where its expression correlates with the degree of leucocyte infiltration (15).

Prostaglandin (PG) E_2, a product of the cyclooxygenation of arachidonic acid, is a potent mediator of immune response and inflammation (16) and contributes to the pathogenesis of RA (17). PGE_2 also displays a complex regulatory function affecting IL-8 gene expression, which is dependent on the concentration of PGE_2 and on the specific cell type involved. At physiological and pathological concentrations of up to 100 μM, PGE_2 is capable of upregulating endogenous IL-8 expression in human intestinal epithelial cells (18,19). PGE_2 alone had little detectable effect on IL-8, though a small enhancement of the mRNA and protein levels of IL-6 was observed in human synovial fibroblasts (20). A variety of transcription factors, including NF-κB, NF-IL6, activator protein-1 and octamer-1, have been shown to regulate IL-8 gene transcription (21,22). Caristi *et al* (23) previously showed that, in human T cells, PGE_2 induces IL-8 synthesis through an NF-κB-independent pathway via its EP1- and EP4-type receptors (PGE_2EP1 and PGE_2EP4, respectively).

We have demonstrated that the expression of IL-8 mRNA in the MH7A FLS cell line is markedly enhanced by stimulation with IL-1ß (22,24). Furthermore, using a DNA microarray technique, we have identified several genes, including those encoding various cytokines and signal transducers, that are upregulated in MH7A cells after stimulation with IL-1ß. In the present study, we report that PGE_2EP4 expression is upregu-

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lated in IL-1ß-stimulated MH7A cells, thereby enabling PGE_2 to enhance IL-1ß-induced IL-8 production.

Materials and methods

Synovial cell culture. The MH7A human FLS cell line (Cell Bank, Riken Bioresource Center, Ibaraki, Japan) was established from cells isolated from the knee joint of an RA patient, and retained the morphological and functional characteristics of primary synovial cells (25,26). Cells were maintained in RPMI-1640 medium (Gibco-BRL Life Technologies Inc., Rockville, MD) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin at 37˚C in a 5% CO_2 atmosphere.

RNA extraction and transcriptional profile analysis with the Affymetrix GeneChip. MH7A cells were homogenized using TRIzol reagent (Invitrogen Co., CA), then total RNA was isolated using a FasRNA kit and fastPrepFP 120 Instrument (BIO 101 Inc., CA). The quality of the extracted RNA samples was analyzed on an Agilent 2100 bioanalyzer (Agilent Technologies, CA). Samples (8 μg) of total RNA were then processed and analyzed using a DNA microarray. The Human Genome Focus Array HG-8500 GeneChip (Affymetrix Inc.), which included a test-chip, was used according to the manufacturer's protocol. The arrays were scanned using a Gene Array Scanner (Agilent Technologies, Germany), and the scanned images were analyzed using Affymetrix Microarray Suite (Version 5.0). The data generated were imported into GeneSpring 4.0 software (Silicon Genetics, CA) for the selection of induced and repressed genes in each experiment.

RT-PCR. The differential expression of genes of interest identified using the GeneChip was verified by reverse transcription-polymerase chain reaction (RT-PCR), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as an internal control. Samples (1 μg) of total RNA were reverse-transcribed using oligo-dT primer and MuLV reverse transcriptase (final volume 20 μl). Thereafter, 2 μl cDNA aliquots were used as templates for RT-PCR. To assess the effects of IL-1ß on gene transcription, PCR amplification was performed using a Palm-Cycler™ (Corbett Research, Sydney, Australia). The primers used were: IL8, 5'-ACT CCA AAC CTT TCC ACC CCA-3' (sense) and 5'-TTT CCT TGG GGT CCA GAC AGA-3' (antisense); PTGER4, 5'-ACA CCA CCT CAC TGA GGA CTT-3' (sense) and 5'-TGC TGT GTG CCA AAT ACG ACA-3' (antisense); GAPDH, 5'-ATC ACC ATC TTC CAG GAG-3' (sense) and 5'-ATG GAC TGT GGT CAT GAG-3' (antisense).

Immunoassay. In order to assay the evoked IL-8 synthesis and secretion, MH7A cells were seeded in 24-well plates at a density of $2x10^4$ cells/well and cultured for 16 h. The cells were then incubated with IL-1ß for 2 h. Subsequently, PGE_2 was added to each well and incubation was continued for an additional hour. IL-8 levels in the conditioned media were then assayed using a commercially available ELISA kit (Endogen Inc., Woburn, MA).

Immunofluorescence microscopy. For further comparison of the PGE_2EP4 expression of IL-1ß-stimulated MH7A cells, the cells were plated in Lab-Tek Chamber Slide (Nalge Nunc Int., IL), incubated for 3 h with 0.1 U/ml of IL-1ß and fixed in 4% neutral formaldehyde solution. The fixed cells were washed three times with 0.1% BSA-PBS and permeabilized using 0.1% Triton X-100 PBS. After another three washes with 0.1% BSA-PBS, the cells were incubated with rabbit anti-human PGE_2EP4 antibody (Novus Biologicals, Inc., CO) for 16 h under humid conditions, washed three times with 0.1% BSA-PBS and then incubated for 3 h with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin (Chemicon International). After a final washing, the distribution of anti-PGE_2EP4 antibody within the cells was examined using a Biorevo fluorescence microscope (Keyence, Osaka, Japan).

Results and Discussion

IL-1ß inducible genes from human synovial fibroblasts. IL-1ß-mediated pro-inflammatory synovial fluid environments have been implicated in the pathogenesis of RA. However, the cellular and molecular regulatory mechanisms underlying this process are poorly understood. This study aimed to determine the relationship between the inflammation-triggered release of IL-1ß and the induction of gene expression in rheumatoid fibroblast-like synoviocyte cells, as well as to clarify the mechanism responsible for this response. The Affymetrix Focus Array HG-8500 GeneChip was used to better understand the direct IL-1ß-mediated changes in gene expression responsibe for promoting inflammatory response in the rheumatoid synoviocyte MH7A cell line.

Six samples of total RNA from untreated control and IL-1ß-treated cells were respectively pooled for gene chip analysis. RNA integrity numbers, used as an index of the quality of the total RNA, were 8.3 and 9.0 from untreated and IL-1ß-treated MH7A cells, respectively. The harvested RNA samples were hybridized in the Affymetrix Focus Array HG-8500 GeneChip system. Thereafter, the expression data were analyzed with Affymetrix 5.0 software, which uses a complex algorithm to classify genes as present, marginal or absent, depending on the intensity of normalized gene expression. To assess the consistency of gene regulation by IL-1ß, we normalized the expression intensity of each chip to the mean expression intensity of all the chips. The mean intensities of the untreated and IL-1ß-treated cells were 194.9 and 207.2, respectively. The averages of the genes present in untreated and IL-1ß-treated cells were 54.5 and 56.4%, respectively. Of the 8746 genes on the Focus Array HG-8500 GeneChip, 4909 from untreated cells and 5073 from IL-1ß-treated cells were classified as marginal or present, respectively. Once the cut-offs for induction (≥2.0-fold) and suppression (≤0.5-fold) by IL-1ß were applied, 120 genes (74 upregulated and 46 down-regulated) were identified as being affected by IL-1ß. The expression of 74 genes was increased by 2-fold or more in the treated cells compared to the untreated cells. The top 10 genes up- and downregulated by IL-1ß are listed in Table I. The expression levels of several CXCL-type chemokines, including CXCL8 (IL-8), CXCL1 (growth-related gene α, Gro-α) and CXCL2 (Gro-ß), were upregulated by IL-1ß. IL-1ß also affected the expression of several genes in the inflammation stage, including LIMK1, FGF13 and PTGER4. The genes IL8 and PTGER4 were marked in the

Table I. Genes up- and downregulated by IL1ß-treated cells (Top 10).

Gene symbol	Affymetrix Focus Array HG-8500 GeneChip	IL-1ß/None
Upregulated by IL-1ß		
IL8	Interleukin 8	20.2
LIMK1	LIM domain kinase 1	14.5
FGF13	Fibroblast growth factor 13	9.7
PTGER4	Prostaglandin E receptor 4 (subtype EP4)	6.7
CXorf12	Chromosome X open reading frame 12	5.3
AFAP	Actin filament-associated protein	4.8
BIRC3	Baculoviral IAP repeat, containing 3	4.8
SLC4A8	Solute carrier family 4, sodium bicarbonate cotransporter, member 8	4.6
CXCL1	Chemokine (C-X-C motif) ligand 1	4.1
CXCL2	Chemokine (C-X-C motif) ligand 2	4.0
Downregulated by IL-1ß		
A2LP	Ataxin 2 related protein	0.3
POU3F1	POU domain, class 3, transcription factor 1	0.3
CUGBP2	CUG triplet repeat, RNA binding protein 2	0.3
KIAA0220	KIAA0220 protein	0.3
ZNF264	Zinc finger protein 264	0.3
PTP4A3	Protein tyrosine phosphatase type IVA, member 3	0.3
RGS17	Regulator of G-protein signalling 17	0.3
GABRR1	γ-aminobutyric acid (GABA) receptor, rho 1	0.3
EDNRA	Endothelin receptor type A	0.2
LGALS2	Lectin, galactoside-binding, soluble, 2 (galectin 2)	0.1

scatter plot microarray graph as shown in Fig. 1. IL-8 transcripts showed the largest increase compared to the control (20.8-fold), while the level of PTGER4 (4th in 74 genes) was increased 6.7-fold (Table I).

Expression of PGE_2 receptor in the IL-1ß-treated MH7A cells. The results of the microarray indicated that IL-1ß induced the expression of several genes. Of the 74 upregulated genes, the only PGE_2 receptor induced by IL-1ß was subtype EP4, PGE_2EP4, which is the protein encoded by PTGER4. Normalized intensity in the non-treated and IL-1ß-treated MH7A cells was 0.249 and 1.674, respectively. This finding was confirmed by RT-PCR analysis (Fig. 2A). Moreover, immunofluorescent staining with anti-PGE_2EP4 antibody revealed a corresponding IL-1ß-inducible production of PGE_2EP4 protein (Fig. 2B). The increase in ratio was calculated based on the DAPI stain in each cell (1.6-fold, Fig. 2C).

Stimulation of IL-8 production by PGE_2 in the IL-1ß-treated MH7A cells. Chemokines are small chemotactic proteins that play a central role in the recruitment of leucocytes in inflamed tissue (27,28). Circulating leucocytes are recruited to inflammatory sites by the local production of chemokines, such as IL-8, which promotes cell activation and proliferation. We also demonstrated that the mRNA expression of IL-8 was markedly upregulated when MH7A rheumatoid synoviocytes were stimulated with IL-1ß. This suggests the importance of these chemokines in rheumatoid FLS-mediated inflammation (22,24).

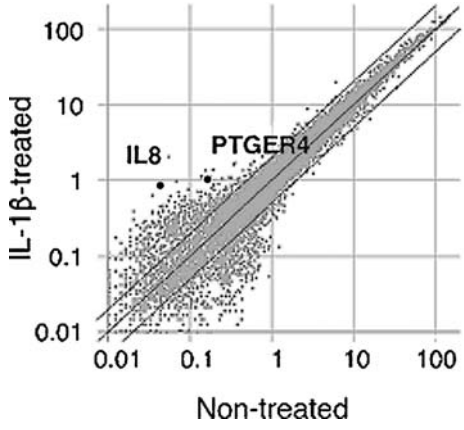

Figure 1. Scatter plot of 8793 genes. Genes whose expression ratio was changed >2-fold by IL-1ß are positioned outside the lines. The larger black dots denote the gene encoding IL-8 (gene symbol, IL8) and the PGE_2 receptor 4 subtype EP4 (gene symbol, PTGER4).

Numerous reports have indicated that PGE_2 has no effect on IL-8 production in neutrophils, alveolar macrophages or blood monocytes (29-31). As shown in Fig. 3A, PGE_2 (10^{-3} to 10^{-7} M) alone had no effect on IL-8 production in MH7A cells, although the highest concentration of PGE_2 used (10^{-2} M) slightly stimulated IL-8 production. To determine whether PGE_2 regulates IL-8 production in MH7A cells, PGE_2 was administered to the IL-1ß-induced MH7A cells and was added to MH7A cells 2 h after IL-1ß stimulation (Fig. 3B). PGE_2

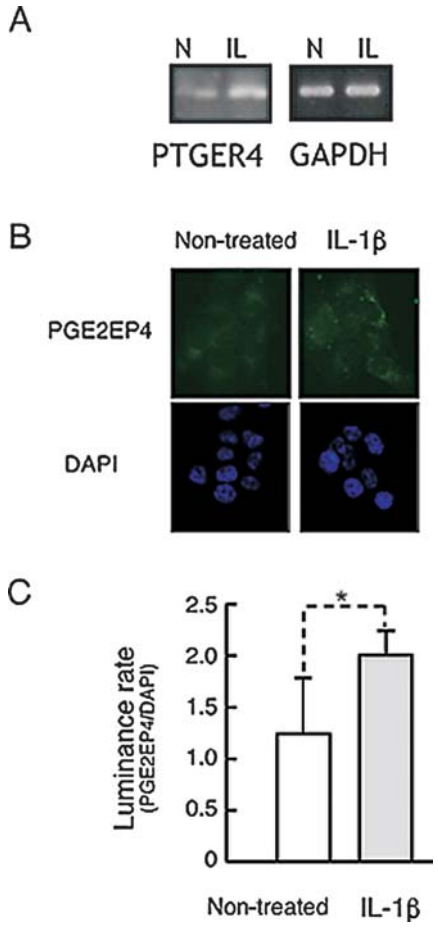

Figure 2. IL-1ß stimulates PGE_2 receptor subtype EP4 expression. (A) Comparison of the gene expression of PTGER4 (PGE_2EP4) in MH7A cells incubated for 3 h with or without 0.1 U/ml IL-1ß. (B and C) Immunofluorescent staining of PGE_2EP4. Luminance for PGE_2EP4 (FITC, green) and DAPI (blue), respectively, was measured in 6 areas. The values were indicated as FITC/DAPI. Bars indicate the means ± SD (n=6). $^*P<0.05$.

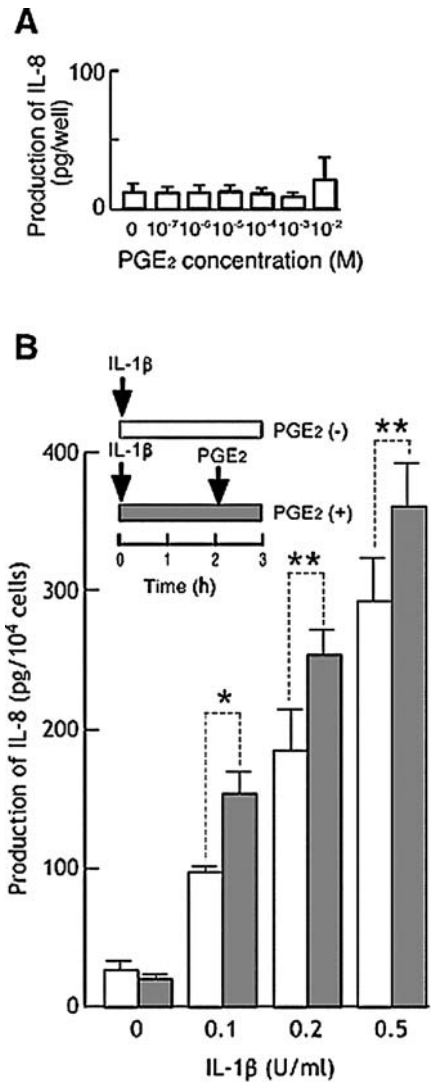

Figure 3. PGE_2 enhances the IL-1ß-mediated induction of IL-8 expression. (A) MH7A cells were incubated for 3 h with the indicated concentration of PGE_2, after which IL-8 levels in the conditioned medium were assayed. (B) MH7A cells were pretreated with IL-1ß for 2 h, after which 10^{-5} M PGE_2 was added to the well. After incubation for an additional hour, the level of IL-8 in the conditioned medium was assayed. Bars indicate the means ± SD (n=4). $^*P<0.005$, $^{**}P<0.05$.

had no effects on IL-8 production (Fig. 3A). However, an increase in IL-8 production was observed in the IL-1ß-treated MH7A cells (Fig. 3B).

In this study, we demonstrated that PGE_2 induced IL-8 production through the increased expression of PGE_2 EP4 receptors in synovial fibroblasts of RA *in vitro,* at physiological and pathological concentrations. These results are of particular interest, since the effect of PGE_2 on IL-8 production was detected in IL-1ß-pretreated MH7A cells. There are many reports that PGE_2 has no effect on neutrophil-derived IL-8 induced by lipopolysaccharides (LPSs) (29). Moreover, in response to LPSs, PGE_2 downregulates IL-8 in human alveolar macrophages and blood monocytes (30) and suppresses the production of chemokines, including IL-8, in human macrophages (31). Although in RA research PGE_2 on its own has limited effects on synovial IL-8 cell production, its effects are significant in the context of IL-1α stimulation. Thus, endogenous PGE_2 may alter the cytokines secreted by mesenchymally-derived cells.

As shown in our results, PGE_2 did not induce IL-8 release from MH7A cells, but did stimulate IL-8 release from IL-1ß-treated MH7A cells. We demonstrated that the newly expressed PGE_2 receptor is involved in this process. Therefore, we hypothesize that PGE_2 enhances the subsequent response by inducing the release of the chemokine IL-8 from synovial fibroblasts of RA. These findings show the complexity with which PGE_2 regulates IL-8 synthesis by inhibiting or enhancing its production, depending on cell type and environmental conditions. We therefore propose that, in IL-1ß-stimulated synovial fibroblasts of RA, PGE_2 induces IL-8 mRNA transcription by the activation of different signal transduction pathways, including PGE_2 receptor EP4-triggered pathways. IL-1ß enhanced the gene expression of IL8 and PTGER4. Subsequently, IL-8 production was enhanced by IL-1ß and PGE_2 from environmental neutrophils/macrophages in the synovial tissues. Our results highlight a new important role for PGE_2 in regulating IL-8 production by the synovial fibroblast cells of RA patients, confirming the pro-inflammatory activity of this prostaglandin.

This study showed that PGE_2 induced IL-8 production in an FLS model (MH7A cells) as a result of the increased expression of PGE_2EP4 induced by pre-treating the cells with IL-1ß. Newly expressed-prostaglandin E_2 receptors appear to be involved in this process. Therefore, we suggest that PGE_2 acts primarily via PGE_2EP4-triggered signaling pathways in RA patients to induce IL-8 transcription in those FLSs that already exhibit the increased expression of IL8 and PTGER4, induced by IL-1ß. We suggest that the inhibition of cellular and molecular networks activated by IL-1ß and PGE_2 in synovial tissues of RA patients enhances the beneficial effects of anti-rheumatoid treatments.

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