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Regulation by PGE_2 of the production of interleukin-6, macrophage colony stimulating factor, and vascular endothelial growth factor in human synovial fibroblasts

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1 We examined the effects of endogenous prostaglandin E_2 (PGE_2) on the production of interleukin-6 (IL-6), macrophage colony stimulating factor (M-CSF), and vascular endothelial growth factor (VEGF) by interleukin-1β (IL-1β)-stimulated human synovial fibroblasts.

2 NS-398 (1 μM), a cyclo-oxygenase-2 (COX-2) inhibitor, inhibited IL-6 and VEGF production ($35\pm4\%$ and $26\pm2\%$, respectively) but enhanced M-CSF production ($38\pm4\%$) by IL-1β (1 ng ml^{-1}) in synovial fibroblasts isolated from patients with osteoarthritis (OA) and rheumatoid arthritis (RA). Exogenous PGE_2 completely abolished the effects of NS-398 on the production of each mediator by OA fibroblasts stimulated with IL-1β.

3 8-Bromo cyclic AMP and dibutyryl cyclic AMP, cyclic AMP analogues, mimicked the effects of PGE_2 on IL-6, M-CSF, and VEGF production by OA fibroblasts.

4 The EP_2 selective receptor agonist ONO-AE1-259 (2 nM) and the EP_4 selective receptor agonist ONO-AE1-329 (2 or 20 nM), but not the EP_1 selective receptor agonist ONO-DI-004 (1 μM) and the EP_3 selective receptor agonist ONO-AE-248 (1 μM), replaced the effects of PGE_2 on IL-6, M-CSF, and VEGF production by OA and RA fibroblasts stimulated with IL-1β in the presence of NS-398.

5 Both OA and RA fibroblasts expressed mRNA encoding EP_2 and EP_4 but not EP_1 receptors. In addition, up-regulation of EP_2 and EP_4 receptor mRNAs was observed at 3 h after IL-1β treatment.

6 These results suggest that endogenous PGE_2 regulates the production of IL-6, M-CSF, and VEGF by IL-1β-stimulated human synovial fibroblasts through the activation of EP_2 and EP_4 receptors with increase in cyclic AMP.

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Abbreviations: COX, cyclo-oxygenase; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; M-CSF, macrophage colony stimulating factor; OA, osteoarthritis; PGE_2, prostaglandin E_2; RA, rheumatoid arthritis; RT–PCR, reverse transcription–polymerase chain reaction; TNF-α, tumour necrosis factor-α; VEGF, vascular endothelial growth factor; α-MEM, α-minimum essential medium

Introduction

The inflammatory process is a complex mechanism, orchestrated by both infiltrating cells and mesenchymally derived cells. Pro-inflammatory cytokines are considered to play important roles in the initiation and development of joint diseases, including RA and OA (Feldmann *et al*., 1996; Westacott & Sharif, 1996; Isomaki & Punnonen, 1997). IL-1, as well as tumour necrosis factor-α (TNF-α), is the most prominent cytokine for developing synovial inflammation and plays a predominant role in the etiopathology of joint disease. It has been established that IL-1 enhances synovial fibroblast DNA synthesis (Butler *et al*., 1988) and activates synoviocytes to secret soluble mediators such as collagenase (Dayer *et al*., 1986), PGE_2 (Dayer *et al*., 1986), IL-6 (Guerne *et al*., 1989), and VEGF (Jackson *et al*., 1997). These mediators are involved in inflammatory response, joint destruction, and angiogenesis. PGs and related eicosanoids also contribute to inflammatory responses in joint diseases (Robinson *et al*., 1975). PG production is mediated by two isoforms of cyclo-oxygenase (COX) (DeWitt, 1991; Xie *et al*., 1991), a constitutive form (COX-1), and an inducible form (COX-2). The induction of COX-2 by IL-1β in synovial cells is associated with an increase in PGE_2 production (Crofford *et al*., 1994; Hulkower *et al*., 1994). In addition, the expression of COX-2 is elevated in a disease-related pattern of synovial tissue from patients with arthritis (Siegle *et al*., 1998).

PGE_2 is able to control the production of diverse chemical mediators in various cells. While PGE_2 induces VEGF

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expression in RA synovial fibroblasts (Ben-Av *et al*., 1995) and human prostate cancer cells (Liu *et al*., 1999), it reduces TNF-α production by rodent macrophages (Kunkel *et al*., 1988) and M-CSF production by human monocytes (Lee *et al*., 1990). We previously reported that PGE_2 induces the production of parathyroid hormone-related peptide in OA synovial fibroblasts (Yoshida *et al*., 1998). Furthermore, PGE_2 is suggested to regulate the production of cytokines such as IL-6, IL-8, IL-11, and granulocyte macrophage colony stimulating factor (GM-CSF) by IL-1-stimulated synovial fibroblasts (Agro *et al*., 1996; Mino *et al*., 1998). However, it is not well understood whether endogenous PGE_2 participates in the production of soluble factors by human synovial fibroblasts through PGE_2 receptors.

The physiological and pharmacological actions of PGE_2 on cell growth and function are mediated by a specific group of seven transmembrane receptors. PGE_2 receptors have been classified into four subtypes, designated the EP_1, EP_2, EP_3, and EP_4 receptors (Coleman *et al*., 1994; Negishi *et al*., 1995). EP_1 receptors associate phospholipase C and phosphoinositol turnover, and stimulate the release of intracellular calcium. EP_2 and EP_4 receptors increase cyclic AMP levels *via* activation of adenylate cyclase, whereas EP_3 receptor variants mediate multiple signal pathways such as inhibition or stimulation of cyclic AMP levels, activation of phospholipase C, and mobilization of intracellular calcium. Recently, the prostanoid derivative EP receptor agonists such as ONO-DI-004 for EP_1, ONO-AE1-259 for EP_2, ONO-AE-248 for EP_3, and ONO-AE1-329 for EP_4 (Figure 1) have been developed (Suzawa *et al*., 2000) and used to examine the role and function of PGE_2 receptor subtypes (Zacharowski *et al*., 1999; Suzawa *et al*., 2000; Yamane *et al*., 2000). We report here, for the first time, that endogenous PGE_2 regulates the production of IL-6, VEGF, and M-CSF by IL-1β in synovial fibroblasts obtained from patients with OA and RA through the activation of EP2 and EP4 receptors. Furthermore, the results from this study favour the hypothesis that PGE_2 plays an important role as a regulator in the production of chemical mediators driven inflammation.

Methods

Reagents

Recombinant human IL-1β was purchased from Becton Dickinson Labware (Bedford, MA, U.S.A.). PGE_2 and pentoxifylline were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 8-Bromo cyclicAMP and dibutyryl cyclic AMP were purchased from Biomol Research Laboratory (Plymouth Meeting, PA, U.S.A.), and NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulphonamide) was purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). ONO-DI-004 ((17S)-2,5-ethano-6-oxo-17,20-dimethylPGE_1), ONO-AE1-259((16S)-9-deoxy-9β-chloro-15-deoxy-16-hydroxy-17, 17-trimethylene-19, 20-didehydroPGE_2 sodium salt), ONO-AE-248 (11, 15-O-dimethylPGE_2), and ONO-AE1-329 (16-(3-methoxymethyl)phenyl-ω-tetranor-3, 7-dithiaPGE_1) were generous gifts from Ono Pharmaceutical Co. (Osaka, Japan).

Synovial fibroblast culture

Primary cultures of human synovial fibroblasts were obtained by enzymatic digestion of synovial tissue obtained from patients with OA and RA (Takayanagi *et al*., 1997). Fibroblasts were cultured in α-minimum essential medium (α-MEM; Gibco BRL, Gaithersbung, MD, U.S.A.) containing heat-inactivated 10% foetal calf serum (FCS) (Biowhittaker, Walkersville, MD, U.S.A.) and 60 μg ml^{-1} kanamycin

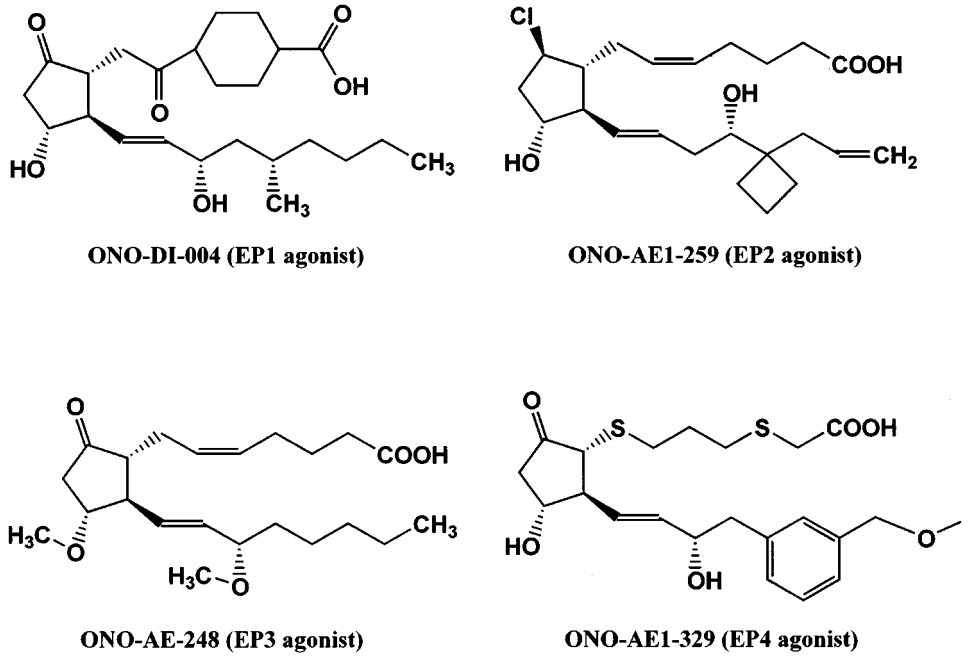

Figure 1 Chemical structures of ONO-DI-004, ONO-AE1-259, ONO-AE-248, and ONO-AE1-329.

Table 1 Gene-specific primer sequences used in PCR amplification

Gene		*Primer sequence*	*Product size, bp*
EP_1 receptor	Sense	5′-CTCGCCGCGCTGGTGTGCAACACGC-3′	519
	Antisense	5′-GGCCTCCCAGGCGCTCGGTGTTAGGCC-3′	
EP_2 receptor	Sense	5′-TTCATCCGGCACGGGCGGACCGC-3′	510
	Antisense	5′-GTCAGCCTGTTTACTGGCATCTG-3′	
EP_3 receptor	Sense	5′-CGTGTCGCGCAGCTACCGGCG-3′	398
	Antisense	5′-CGGGCCACTGGACGGTGTACT-3′	
EP_4 receptor	Sense	5′-CCTCCTGAGAAAGACAGTGCT-3′	366
	Antisense	5′-AAGACACTCTCTGAGTCCT-3′	

sulphate (Gibco BRL) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was replaced twice each week. The confluent cells were dispersed with trypsinization and then transferred to new plastic dishes in a split ratio of 1 : 2 or 1 : 4. The cells at more than six passages (eight population doubling levels, PDL) were used for subsequent experiments. These cells consisted of fibroblasts alone, with no dendritic or monocytic cells.

Synovial fibroblasts were plated onto 24-well plates (Becton Dickinson Labware) at 2×10^4 cells ml^{-1} to perform the experiments. When cells grew up to confluence, cells were kept in 0.5 ml α-MEM containing 0.5% FCS for 48 h and then exposed to a concentration of 1 ng ml^{-1} IL-1β (Inoue *et al*., 2001) in the presence or absence of different agents dissolved in DMSO (0.1%) for 24 h. Each study was repeated another one or two times.

Measurement of IL-6, IL-8, M-CSF, PGE_2, and VEGF levels in the culture medium

The culture media were collected at 24 h after IL-1β stimulation and kept at −80°C until used for assay. Concentrations of IL-6 (detection limit: 3 pg ml^{-1}), M-CSF (detection limit: 9 pg ml^{-1}), PGE_2 (detection limit roughly 12 pg ml^{-1}), and VEGF (detection limit: 5 pg ml^{-1}) in the culture supernatant were measured directly by the enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's directions. ELISA kits used were purchased from PerSeptive Biosystems (Framingham, MA, U.S.A.) for IL-6, R & D systems (Minneapolis, MN, U.S.A.) for M-CSF and VEGF, and Cayman Chemical for PGE_2. Sample and standard dilutions were made with experimental medium, and results were expressed as the mean ± s.e.m. The statistical significance was determined with the Tukey–Kramer multiple comparison test after one-way analysis of variance. Appropriate groups were compared by Student's *t*-test.

RT–PCR analysis of EP receptor subtypes

Total RNA was extracted from synovial fibroblasts (10^6 cells) by acid guanidine-phenol-chloroform extraction using ISOGEN® (Nippon Gene, Toyama, Japan) and treated with a DNA-free kit (Ambion, Austin, TX, U.S.A.) for elimination of contaminating DNA. cDNA was synthesized from isolated RNA with oligo-dT primer and AMV reverse transcriptase XL (TaKaRa RNA PCR kit (AMV) Ver. 2.1, Takara, Osaka, Japan), and used as templates for PCR. PCR for EP_2 and EP_4 receptors was carried out for 30–32 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, followed by a final of 72°C for 6 min. That for EP_1 and EP_3 receptors was performed for 42 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min, followed by a final of 72°C for 6 min. The synthetic primers for the human PGE_2 receptors were designed to according to Zeng *et al*. (1998) (Table 1). The constitutively expressed gene encoding GAPDH was used as internal control in RT–PCR to normalize the amounts of mRNA in each sample. The cDNA of the GAPDH gene was amplified from the same volume of cDNAs as for EP receptors, but only 10–12 cycles of PCR. The PCR products were analysed by electrophoresis in a 2% agarose gel visualized using ethidium bromide. No PCR product was amplified without reverse-transcription reaction.

Results

Effects of exogenous PGE_2 on the spontaneous production of IL-6, M-CSF, and VEGF by OA fibroblasts

We first examined whether PGE_2 alone affects the spontaneous release of IL-6, M-CSF, and VEGF from OA fibroblasts (Table 2). OA fibroblasts themselves released immunoreactive IL-6, M-CSF, and VEGF for 24 h culture without any stimulants. Among them, spontaneous level of M-CSF was higher than that of IL-6 and VEGF. PGE_2 dose-dependently increased the secretion of IL-6 and VEGF from OA fibroblasts. When cells were treated with 1 ng ml^{-1} PGE_2, significant ($P<0.05$) increases in the release of IL-6 and VEGF were observed. Also, levels of IL-6 and VEGF were further enhanced a 12 and 2 fold by 20 ng ml^{-1} PGE_2, respectively, compared with PGE_2-untreated cells. In contrast, PGE_2 at a concentration of 20 ng ml^{-1} significantly ($P<0.05$) decreased M-CSF level.

Effects of exogenous PGE_2 on IL-6, M-CSF, and VEGF production by OA fibroblasts stimulated with IL-1β in the presence of NS-398

We had recently reported that IL-1β induced the production of IL-6, M-CSF, PGE_2, and VEGF in synovial fibroblasts of patients with OA and RA (Inoue *et al*., 2001). In fact, IL-1β (1 ng ml^{-1}) markedly increased IL-6, M-CSF, and VEGF production by OA synovial fibroblasts (Figure 2). Basal levels of IL-6, M-CSF, and VEGF were 119 ± 4, 540 ± 7, and 120 ± 5 pg ml^{-1} ($n=5–6$), respectively. Increase in IL-6 production by IL-1β was greater than that in M-CSF and VEGF production. The release of PGE_2 was also enhanced significantly ($P<0.001$, Student's *t*-test) by IL-1β from the

Table 2 Effects of exogenous PGE_2 on the spontaneous production of IL-6, M-CSF, and VEGF by OA synovial fibroblasts

Dose (ng ml^{-1})		*IL-6* (pg ml^{-1})	*M-CSF* (pg ml^{-1})	*VEGF* (pg ml^{-1})
PGE_2	0	150±8	581±31	101±2
	1	230±8*	625±30	122±3*
	10	930±4**	523±27	175±5**
	20	1740±4**	481±8*	196±10**

Confluent fibroblasts were treated with PGE_2 at a concentration of 1, 10 or 20 ng ml^{-1} for 24 h. The result represents the mean±s.e.m. of 5–6 samples. *$P<0.05$ or **$P<0.01$ vs control (PGE_2:0) (Tukey–Kramer multiple test).

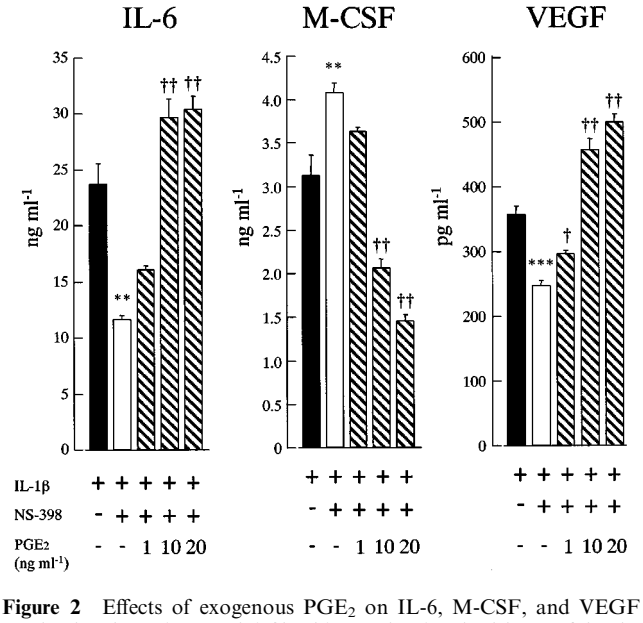

Figure 2 Effects of exogenous PGE_2 on IL-6, M-CSF, and VEGF production by OA synovial fibroblasts stimulated with IL-1β in the presence of NS-398. Confluent fibroblasts were treated with IL-1β (1 ng ml^{-1}) in the presence or absence of NS-398 (1 μM) and PGE_2 at various concentrations for 24 h. +: with and −: without. Values are the mean±s.e.m. of 5–6 samples. *$P<0.05$ or **$P<0.01$ vs IL-1β (Student's *t*-test). † $P<0.01$ vs IL-1β+NS-398 (Tukey–Kramer multiple test).

basal value 0.030±0.003 to 8.1±0.5 ng ml^{-1} ($n=6$). NS-398, a COX-2 inhibitor (Futaki *et al*., 1994), at a dose of 1 μM which completely blocks the production of PGE_2 in this model (Inoue *et al*., 2001), significantly ($P<0.01$ or $P<0.001$) inhibited IL-6 and VEGF production. In contrast, treatment with NS-398 resulted in approximately 30% increase in M-CSF production compared with IL-1β alone value. Furthermore, treatment of OA fibroblasts with PGE_2 completely abolished the effects of NS-398 on IL-1β-induced IL-6, M-CSF, and VEGF production. Simultaneous addition of 10 or 20 ng ml^{-1} PGE_2 to IL-1β resulted in further enhancement of IL-6 and VEGF production in the presence of NS-398. Contrary, PGE_2 dose-dependently inhibited the enhancement by NS-398 of IL-1β-induced M-CSF production. M-CSF level increased in response to IL-1β was significantly ($P<0.01$) reduced up to 64% by 20 ng ml^{-1} PGE_2.

Involvement of cyclic AMP in the production of IL-6, M-CSF, and VEGF by OA fibroblasts stimulated with IL-1β

Effects of cyclic AMP analogues were examined on IL-6, M-CSF, and VEGF production by IL-1β in OA fibroblasts. 8-Bromo-cyclic AMP and dibutyryl-cyclic AMP, cyclic AMP analogues, at 1 mM significantly ($P<0.01$, Student's *t*-test) increased the basal level of IL-6 from 84±3 to 2490±260 and 2364±54 pg ml^{-1} ($n=5$), and of VEGF from 115±3 to 289±14 and 340±5 pg ml^{-1} ($n=5$), respectively. In contrast, 8-bromo-cyclic AMP and dibutyryl-cyclic AMP markedly ($P<0.01$, Student's *t*-test) reduced the basal level of M-CSF from 509±8 to 288±11 and 344±12 pg ml^{-1} ($n=5$), respectively. Both compounds dose-dependently enhanced IL-1β-induced IL-6 and VEGF production in the presence of NS-398 (Figure 3). However, dibutyryl-cyclic AMP at a high dose (1 mM) failed to enhance IL-6 production. 8-Bromo-cyclic AMP and dibutyryl-cyclic AMP significantly ($P<0.01$) inhibited M-CSF production by IL-1β in a dose-dependent manner. Furthermore, pentoxifylline (0.5 mM), a non-selective phosphodiesterase inhibitor (Ward & Clissold, 1987), significantly ($P<0.05$, Student's *t*-test) enhanced VEGF production in response to IL-1β (IL-1β: 583±31 vs pentoxifylline: 695±29 pg ml^{-1}, $n=5$) whereas it markedly ($P<0.01$, Student's *t*-test) reduced IL-1β-induced M-CSF production (IL-1β: 1992±47 vs pentoxyfylline: 1647±36 pg ml^{-1}, $n=5$).

Effects of EP receptor agonists on IL-6, M-CSF, and VEGF production by IL-1β in OA and RA fibroblasts

We next determined whether PGE_2 regulates IL-1β-stimulated IL-6, M-CSF, and VEGF production *via* PGE_2 receptor subtypes by employing selective EP receptor agonists such as ONO-DI-004, ONO-AE1-259, ONO-AE-248, and ONO-AE1-329. The specificities of the agonists have been confirmed by the binding assay for the respective receptor subtypes expressed in CHO cells (Suzawa *et al*., 2000). In addition, ONO-AE1-259 and ONO-AE1-329 have no difference in binding affinity and agonist activity between mouse and human EP receptors (personal communications). Basal levels of IL-6, M-CSF, and VEGF in OA fibroblasts were 107±12, 527±13, and 120±7 pg ml^{-1} ($n=5-6$), respectively. Similar to the exogenous PGE_2, the EP_2 receptor agonist ONO-AE1-259 and the EP_4 receptor agonist ONO-AE1-329 at a dose of 2 or 20 nM not only enhanced the production of IL-6 and VEGF but also reduced M-CSF production by OA fibroblasts treated with IL-1β in the presence of NS-398 (Table 3). However, the EP_1 receptor agonist ONO-DI-004 and the EP_3 receptor agonist ONO-AE-248 had no effect on the production of each mediator until 1 μM. Both agonists for EP_2 and EP_4 receptors, as well as PGE_2, enhanced spontaneous levels of IL-6 and VEGF, and attenuated spontaneous M-CSF level in non-stimulated OA fibroblasts (data not shown).

Furthermore, we examined the effects of EP receptor agonists on IL-1β-induced IL-6, M-CSF, and VEGF production in RA synovial fibroblasts (Table 4). Basal levels of IL-6, M-CSF, and VEGF were 202±7, 357±9, and 69±4 pg ml^{-1} ($n=5-6$), respectively. Similar to OA fibroblasts, basal level of M-CSF was higher than that of IL-6 and VEGF in RA fibroblasts. In addition, IL-1β strongly induced

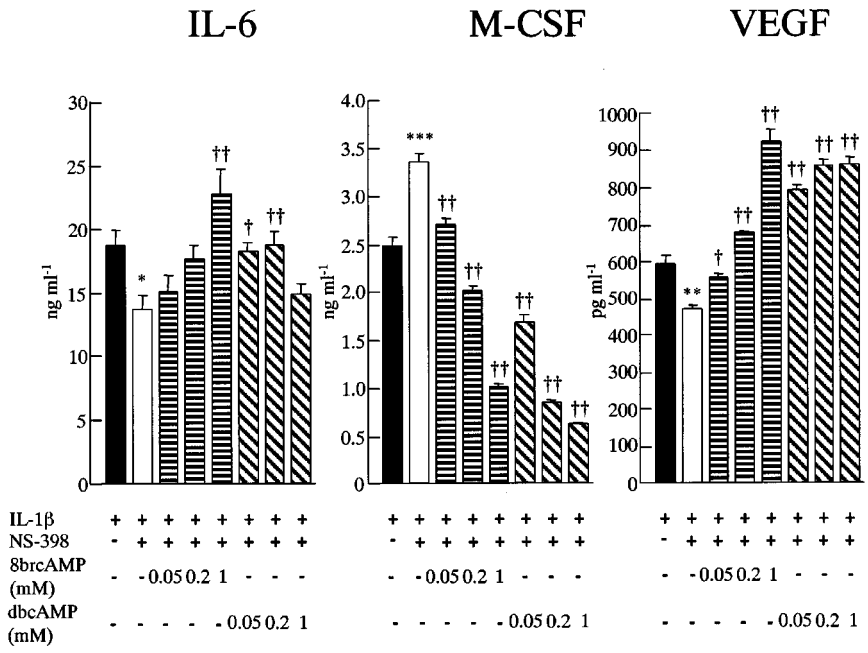

Figure 3 Effects of cyclic AMP analogues on IL-6, M-CSF, and VEGF production by OA synovial fibroblasts stimulated with IL-1β in the presence of NS-398. Confluent fibroblasts were treated with IL-1β (1 ng ml^{-1}) in the presence or absence of NS-398 (1 μM) and dibutyryl-cyclic AMP (dbcAMP) or 8-bromo-cyclic AMP (8brcAMP) at various concentrations for 24 h. +: with and −: without. Values are the mean ± s.e.m. of 5–6 samples. $^*P<0.05$ or $^{**}P<0.01$ vs IL-1β (Student's t-test). †$P<0.01$ vs IL-1β + NS-398 (Tukey–Kramer multiple test).

Table 3 Effects of EP receptor agonists on IL-1β-induced IL-6, M-CSF, and VEGF production in OA synovial fibroblasts

	IL-6 (ng ml^{-1})	*M-CSF* (pg ml^{-1})	*VEGF* (pg ml^{-1})
IL-1β	153 ± 8	2164 ± 86	457 ± 11
IL-1β + NS-398	93 ± 9**	3260 ± 50***	347 ± 34*
IL-1β + NS-398 ± EP_1 agonist ONO-DI-004 (μM)			
0.2	97 ± 5	3184 ± 61	318 ± 26
1.0	98 ± 8	3195 ± 98	352 ± 11
IL-1β + NS-398 ± EP_3 agonist ONO-AE-248 (μM)			
0.2	92 ± 1	3049 ± 29	350 ± 11
1.0	87 ± 2	3188 ± 69	339 ± 7
IL-1β	168 ± 5	2334 ± 127	457 ± 11
IL-1β + NS-398	116 ± 11**	3257 ± 149**	347 ± 34*
IL-1β + NS-398 ± EP_2 agonist ONO-AE1-259 (nM)			
0.02	116 ± 5	2905 ± 48	335 ± 32
2.0	166 ± 6††	2281 ± 76††	469 ± 15†
IL-1β + NS-398 ± EP_4 agonist ONO-AE1-329 (nM)			
0.02	108 ± 3	2714 ± 64††	319 ± 9
2.0	176 ± 20†	2783 ± 75†	429 ± 11
20.0	175 ± 7††	2267 ± 5††	472 ± 29††

Confluent fibroblasts were teated with IL-1β (1 ng ml^{-1}) in the presence or absence of NS-398 (1 μM) and EP receptor agonists at various concentrations for 24 h. The result represents the mean ± s.e.m. of 5–6 samples. $^*P<0.05$, $^{**}P<0.01$ or $^{***}P<0.001$ vs IL-1β (Student's t-text). †$P<0.05$ or ††$P<0.01$ vs IL-1β + NS-398 (Tukey–Kramer multiple test).

IL-6 production compared with M-CSF and VEGF production. IL-6 and VEGF production in response to IL-1β were inhibited, but M-CSF production was enhanced by treatment with NS-398. In OA and RA fibroblasts, inhibition by NS-398 of IL-1β-induced IL-6 and VEGF production was 35 ± 4% and 26 ± 2% (n = 4–5 experiments), respectively, whereas enhancement of M-CSF was 38 ± 4% (n = 5 experiments). As expected, the EP_2 receptor agonist ONO-AE1-259 and the EP_4 receptor agonist ONO-AE1-329 at a dose of 2 nM were effective in enhancing IL-6 and VEGF production,

Table 4 Effects of EP receptor agonists on IL-1β-induced IL-6, M-CSF, and VEGF production in RA synovial fibroblasts

	IL-6 (ng ml^{-1})	*M-CSF* (pg ml^{-1})	*VEGF* (pg ml^{-1})
IL-1β	134±4	2367±105	301±13
IL-1β+NS-398	96±4***	3123±187**	219±18**
IL-1β+NS-398+EP_1 agonist ONO-DI-004 (μM)			
0.2	108±3	2825±75	230±3
1.0	112±1	3012±143	229±7
IL-1β+NS-398+EP_3 agonist ONO-AE-248 (μM)			
0.2	103±6	3701±112†	199±7
1.0	119±2	2817±87	237±8
IL-1β+NS-398+EP_2 agonist ONO-AE1-259 (nM)			
0.02	99±1	2948±67	212±5
2.0	139±12††	1567±97††	300±8††
IL-1β+NS-398+EP_4 agonist ONO-AE1-329 (nM)			
0.02	93±8	3638±35†	206±5
2.0	136±11†	2058±129††	281±7††

Confluent fibroblasts were teated with IL-1β (1 ng ml^{-1}) in the presence or absence of NS-398 (1 μM) and EP receptor agonists at various concentrations for 24 h. The result represents the mean±s.e.m. of 5–6 samples. *$P<0.01$ or ***$P<0.001$ vs IL-1β (Student's *t*-text). †$P<0.05$ or ††$P<0.01$ vs IL-1β+NS-398 (Tukey–Kramer multiple test).

and in inhibiting M-CSF production by IL-1β-stimulated RA fibroblasts. In contrast to this, EP_1 and EP_3 receptor agonists at 1 μM did not change the production of IL-6, M-CSF, and VEGF by IL-1β in the presence of NS-398. EP_2 receptor agonist effectively attenuated M-CSF production compared with EP_4 receptor agonist in both OA and RA fibroblasts.

Enhancement by IL-1β of EP_2 and EP_4 receptor mRNAs in OA and RA fibroblasts

As EP_2 and EP_4 receptors were suggested to be involved in the production of IL-6, M-CSF, and VEGF by IL-1β, we examined expression of mRNA encoding EP receptor subtypes in OA and RA synovial fibroblasts by RT–PCR. Co-expression of EP_2, EP_3, and EP_4 receptor mRNAs was confirmed in total RNA isolated from OA fibroblasts (Figure 4a, lane 1). Expression of EP_2, EP_3, and EP_4 receptor mRNAs was up-regulated at up to 3 h after IL-1β stimulation (Figure 4a, lane 3). As shown in Figure 4b, mRNA encoding EP_2 and EP_4 receptors was confirmed in RA fibroblasts (Figure 4b, lane 1) and up regulated at 3 h after IL-1β stimulation (Figure 4b, lane 2). EP_3 receptor mRNA was not observed in either IL-1β-treated or -untreated RA fibroblasts. Nevertheless, RA fibroblasts derived from other donors expressed EP_3 receptor mRNA as well as EP_2 and EP_4 receptor mRNAs (data not shown). Expression of EP_1 receptor mRNA was not observed in both OA and RA fibroblasts, and induced even by treatment with IL-1β (data not shown).

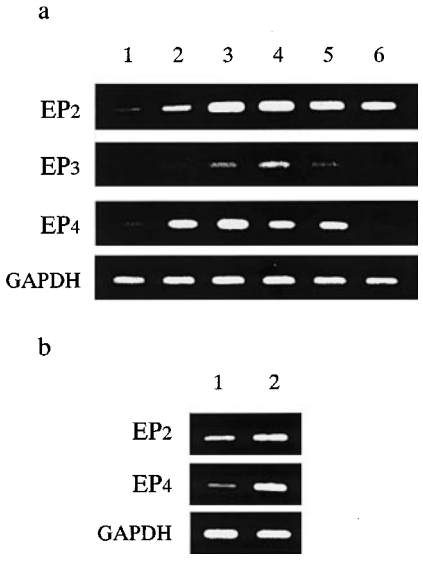

Figure 4 Time-course of mRNA encoding PGE_2 receptor subtypes in OA (**a**) and RA (**b**) synovial fibroblasts treated with IL-1β. Total RNA was isolated from confluent fibroblasts at 1, 3, 6, 12 or 24 h after IL-1β (1 ng ml^{-1}) treatment and subjected to RT–PCR with a specific primer for each EP receptor or GAPDH. After RT–PCR, amplified products were analysed by agarose gel electrophoresis with ethidium bromide staining. The cultures without IL-1β treatment served as the control. (**a**) Lane 1: control; lane 2 : 1 h; lane 3 : 3 h; lane 4 : 6 h; lane 5 : 12 h; lane 6 : 24 h. (**b**) Lane 1: control; lane 2 : 3 h. The figure was one representative of three independent experiments.

Discussion

In this study, we have presented evidence showing that endogenous PGE_2 regulates the production of IL-6, M-CSF, and VEGF in response to IL-1β through the activation of EP_2 and EP_4 receptors in human synivial fibroblasts. Synovial fluid of patients with RA and OA contains many soluble mediators, such as cytokines and growth factors, which contribute to the development of joint diseases (Feldmann *et al*., 1996; Westacott & Sharif, 1996). IL-6 increases B-cell proliferation, immunoglobulin production,

and acute phase protein production, and may stimulate rheumatoid factor secretion in the synovium (Isomaki & Punnonen, 1997). M-CSF, as well as granulocyte macrophage-colony stimulating factor (GM-CSF), activates monocytes/macrophages to produce cytokines and increases the number of mature macrophages in the inflamed synovium (Moss & Hamilton, 2000). VEGF plays an important role of angiogenesis and endothelial migration during development of RA synovitis (Folkman, 1995; Nagashima *et al*., 1995). In addition, inflammatory mediators including cytokines and PGs are also involved in the induction of angiogenensis (Fidler & Ellis, 1994). In our study, the COX-2 inhibitor NS-398 inhibited IL-6 and VEGF production as well as PGE_2 production in response to IL-1β whereas it enhanced M-CSF production in synovial fibroblasts, suggesting that autocrine stimulation by PGs is involved in the production of them. In fact, exogenous PGE_2 abolished the effect of NS-398 in response to IL-1β. PGE_2 also potentiated the spontaneous production of IL-6 and VEGF, and attenuated M-CSF production by non-stimulated synovial fibroblasts. However, the ability of PGE_2 to produce IL-6 and VEGF was low compared with IL-1β. Thus, endogenous PGE_2 seems to play as a promoter in the production of cytokine and growth factor by synovial fibroblasts stimulated with IL-1β. Others have found that PGE_2 enhances IL-6 production but inhibits GM-CSF production by IL-1α-stimulated human synovial fibroblasts (Agro *et al*., 1996). IL-11, a functionally pleiotropic cytokine classified as an IL-6-type cytokine, produced by RA fibroblasts treated with IL-1α and TNF-α is inhibited by indomethacin, and its inhibition is prevented by PGE_2 treatment (Mino *et al*., 1998). Furthermore, PGE_2 participates in the upregulation of VEGF by cobalt chloride-stimulated hypoxia in human prostate cancer cells (Liu *et al*., 1999). Taken together, our results indicate that endogenous PGE_2 regulates the production of IL-6, M-CSF, and VEGF by IL-1β in synovial fibroblasts derived from arthritis. In addition to PGE_2, other prostanoids also might modulate the production of cytokine and growth factor in response to IL-1β, as suggested by others that prostacyclin regulates the release of GM-CSF and granulocyte-colony stimulating factor from human vascular smooth muscle cells stimulated with IL-1β (Stanford *et al*., 2000).

It has been reported that human synovial fibroblasts express mRNA encoding the EP_1 and EP_2 subtypes of PGE_2 receptors (Ben-Av *et al*., 1995). We confirmed that both OA and RA fibroblasts express EP_2, EP_3, and EP_4, but not EP_1, receptor mRNAs, although there was difference in expression of EP_3 receptor mRNA between RA donors. A recent report has shown that RA fibroblasts express EP_3 receptor mRNA as well as EP_2 and EP_4 receptor mRNAs (Yoshida *et al*., 2001). Thus, it is conceivable that the expression of EP receptor mRNAs in synovial fibroblasts may be affected by the condition of diseases or the medical application for donors. We also found that the expression of mRNA encoding EP_2 and EP_4 mRNAs was up-regulated by IL-1β treatment in both OA and RA fibroblasts. IL-1β is known to augment EP_1 receptor level, as well as PGE_2 production, in amnion cells (Spaziani *et al*., 1997). However, in the present study, the induction of mRNA encoding EP_1 receptor by IL-1β was not observed in synovial fibroblasts. In addition, specific agonists for EP_2 and EP_4 receptors excluding EP_1 and EP_3 receptors enhanced IL-6 and VEGF production, and attenuated M-CSF production by fibroblasts stimulated with IL-1β in the presence of NS-398. Moreover, there was no difference in the effects of EP receptor agonists between OA and RA fibroblasts. These findings suggest that both EP_2 and EP_4 receptors may participate in the regulation of IL-1β-induced IL-6, M-CSF, and VEGF production in human synovial fibroblasts. Additionally, it seems likely that an increase in expression of mRNA encoding EP_2 and EP_4 receptors contributes to the PGE_2 effects in this model. Recently, PGE_2 has been found to induce parathyroid hormone-related peptide *via* EP_2 and EP_4 receptors in RA fibroblasts treated with IL-1α (Yoshida *et al*., 2001). Others have reported that while EP_3 receptors mediate the production of matrix metalloproteinase-9 in response to PGE_2 (Zeng *et al*., 1996), EP_2 and EP_4 receptors regulate the production of IL-6 by PGE_2 in human leukemic T cells (Zeng *et al*., 1998) and mediate the modulation of TNF-α-induced M-CSF synthesis by PGE_2 in human bone marrow stromal cells (Besse *et al*., 1999). Furthermore, PGE_2 is shown to down-regulate adhesion molecule-1 expression by interferon-γ *via* EP_2 receptor in human gingival fibroblasts (Noguchi *et al*., 1999). Expression of VEGF mRNA by PGE_2 in synovial fibroblasts has been suggested to be related to the activation of EP_2 receptor (Ben-Av *et al*., 1995). It has also been shown that the augmentation of IL-6 production is mediated mainly by EP_2 receptor whereas the suppression of TNF-α production is predominantly regulated by EP_4 receptor in lipopolysaccharide-stimulated mouse neutrophils (Yamane *et al*., 2000). These reports indicate that EP_2 and EP_4 receptors mediate the production of soluble factors in cells through cyclic AMP dependent-mechanism. Accordingly, we examined whether cyclic AMP contributes to the regulation by PGE_2 of the production of IL-6, M-CSF, and VEGF in synovial fibroblasts.

PGE_2 is known to stimulate cyclic AMP accumulation in Swiss 3T3 fibroblasts (Burch *et al*., 1989). In the present study, the cyclic AMP analogues, 8-bromo-cyclic AMP and dibutyryl-cyclic AMP, themselves increased spontaneous levels of IL-6 and VEGF but reduced M-CSF level in OA fibroblasts. Furthermore, the cyclic AMP analogues mimicked the effects of PGE_2 on the production of soluble factors by IL-1β, indicating that cyclic AMP is involved as a second messenger in the PGE_2 effects. This is further supported by the finding that pentoxifylline, a non-selective phosphodiesterase inhibitor, also enhanced VEGF production and attenuated M-CSF production by fibroblasts stimulated with IL-1β. EP_2 and EP_4 receptors generate cyclic AMP production through stimulation of adenyl cyclase (Coleman *et al*., 1994; Negishi *et al*., 1995). In fact, it has been reported that the EP_2 receptor agonist ONO-AE1-259 and the EP_4 receptor agonist ONO-AE1-329, as well as PGE_2, increase cyclic AMP formation (Yamane *et al*., 2000). It is therefore, well possible that cyclic AMP accumulation facilitates the process of IL-6 and VEGF production and interferes with that of M-CSF production in synovial fibroblasts, although the potentiation by PGE_2 of IgE-induced IL-6 and GM-CSF production in rodent mast cells is mediated *via* EP_1 and/or EP_3 receptors with cyclic AMP-independent mechanisms (Gomi *et al*., 2000). Furthermore, the activation of cyclic AMP-dependent protein kinase A by EP_2 and EP_4 receptors may mediate the signals for the PGE_2

effects (Ben-Av *et al*., 1995; Zeng *et al*., 1998; Suzawa *et al*., 2000). However, the signalling pathways after cyclic AMP accumulation leading to the PGE_2 regulation on the production of IL-6, M-CSF, and VEGF in response to IL-1β remain to be examined.

In conclusion, we have demonstrated for the first time that endogenous PGE_2 regulates, at least in part, the production of IL-6, M-CSF, and VEGF by IL-1β through the activation of EP_2 and EP_4 receptors in synovial fibroblasts from patients with OA and RA. This implies that in addition to the function of inflammatory mediator, PGE_2 plays an important role as a regulator in the production of various soluble factors at inflammatory sites.

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