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Differential Effects of Anti-IL-1R Accessory Protein Antibodies on IL-1α or IL-1β-induced Production of PGE_2 and IL-6 from 3T3-L1 Cells

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Soluble or cell-bound IL-1 receptor accessory protein (IL-1RAcP) does not bind IL-1 but rather forms a complex with IL-1 and IL-1 receptor type I (IL-1RI) resulting in signal transduction. Synthetic peptides to various regions in the Ig-like domains of IL-1RAcP were used to produce antibodies and these antibodies were affinity-purified using the respective antigens. An anti-peptide-4 antibody which targets domain III inhibited 70% of IL-1β-induced productions of IL-6 and PGE_2 from 3T3-L1 cells. Anti-peptide-2 or 3 also inhibited IL-1-induced IL-6 production by 30%. However, anti-peptide-1 which is directed against domain I had no effect. The antibody was more effective against IL-1β compared to IL-1α. IL-1-induced IL-6 production was augmented by co-incubation with PGE_2. The COX inhibitor ibuprofen blocked IL-1-induced IL-6 and PGE_2 production. These results confirm that IL-1RAcP is essential for IL-1 signaling and that increased production of IL-6 by IL-1 needs the co-induction of PGE_2. However, the effect of PGE_2 is independent of expressions of IL-1RI and IL-1RAcP. Our data suggest that domain III of IL-1RAcP may be involved in the formation or stabilization of the IL-1RI/IL-1 complex by binding to epitopes on domain III of the IL-1RI created following IL-1 binding to the IL-1RI.

Keywords: Cyclooxygenase, Cytokines, IL-1, IL-1RAcP, Ig-like domain, Inflammation

Abbreviations: IL-1, interleukin-1; hIL-1, human IL-1; mIL-1, murine IL-1; IL-1R, IL-1 receptor; IL-1RI, type I IL-1R; IL-1RAcP, IL-1R accessory protein; IL-1Ra, IL-1R antagonist; TNF, tumor necrosis factor; PGE_2, prostaglandin E_2; COX, cyclooxygenase; IBP, ibuprofen; RT, reverse transcriptase; IRAK, IL-1R associated kinase

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Introduction

Microbes and microbial toxins induce interleukin-1 (IL-1), tumor necrosis factor and IL-6 via cellular activation pathways including the synthesis of small molecules such as prostaglandin E_2 (PGE_2) (Araki *et al.*, 1994). In turn, IL-1 stimulates PGE_2 synthesis in various cells, particularly fibroblasts and vascular smooth muscle cell (Balavoine *et al.*, 1986; Libby *et al.*, 1988; Chernoff *et al.*, 1995). In fact, IL-1 is a potent inducer of PGE_2 and PGE_2 production accounts for several biological effects of IL-1 and in most cells capable of expressing cyclooxygenase (COX) (Dinarello *et al.*, 1996). For example, IL-1-induced COX products mediate fever, lower pain thresholds and augment vascular permeability and the formation of edema (Schweizer *et al.*, 1988). Most attention on IL-1-induced PGE_2 has focused on COX-2. COX-2 is normally undetected in many tissues, but is rapidly induced by IL-1 (Cao *et al.*, 1996; Kagiwada *et al.*, 2004; Fukushima *et al.*, 2005). COX-2 plays an essential role in inflammation, cellular differentiation, mitogenesis and appears to be critical for normal kidney growth, differentiation, and function (Morham *et al.*, 1995). Human orbital fibroblasts are more susceptible to some actions of proinflammatory cytokines than are fibroblasts from other anatomic regions. These cells produce high levels of PGE_2 when activated by cytokines (Cao *et al.*, 2003).

Following IL-1 binding to its extracellular receptor complex, a rapid succession of protein phosphorylations take place, particularly the p38 mitogen-activated protein (MAP) kinase (Freshney *et al.*, 1994; Karnitz *et al.*, 1995; Banda *et al.*, 2005). Recent studies have demonstrated that IL-1 activation of p38 MAP kinase activates MAP kinase-activated protein kinase-2 which results in up-regulation of PGE_2 biosynthesis (Guan *et al.*, 1997; Lin *et al.*, 2004). There is an important role for IL-1-induced PGE_2 in regulation of IL-1 receptors. The IL-1 receptor type I (IL-1RI) is expressed on nearly all

cells and is necessary for IL-1 signal transduction (Sims *et al*., 1993). IL-1 up-regulates gene and cell surface expression of IL-1RI in human fibroblasts via induction of PGE_2 (Akahoshi *et al*., 1988; Takii *et al*., 1992). In the absence of PGE_2 synthesis, however, IL-1 down-regulates IL-1RI mRNA in human fibroblast cell lines (Takii *et al*., 1994; Greenfeder *et al*., 1995b). Therefore, since IL-1 is a potent inducer of PGE_2 synthesis, the regulation of the IL-1RI by PGE_2 is of considerable significance since it provides a mechanism for self-augmentation of responses to IL-1. Until recently, surface expression of the IL-1RI was thought to explain the biological response to IL-1. However, the second chain of the IL-1R complex, called the IL-1R accessory protein (IL-1RAcP) (Greenfeder *et al*., 1995a), must now be considered since in the absence of this chain, no biological responses to IL-1 take place (Martin *et al*., 1997; Martin *et al*., 2002). For example, cell lines expressing IL-1RI but are unresponsive to IL-1 do not express the IL-1RAcP (Wesche *et al*., 1997) and transfection with IL-1RAcP into IL-1 non-responders restores IL-1 responsiveness (Korherr *et al*., 1997). These results support the concept that IL-1RAcP plays a pivotal role in IL-1 signaling. Therefore, understanding the regulation of IL-1 responsiveness clearly requires the effect of PGE_2 on both IL-1RI as well as on IL-1RAcP expression. In the present study, we used affinity-purified antibodies to four regions of hydrophilic amino acid sequences of the extracellular portion of the murine IL-1RAcP. We examined whether blocking IL-1RAcP would inhibit IL-1-induced PGE_2 and IL-6 synthesis in the mouse fibroblast 3T3-L1 cell line. In addition, we elucidated the regulatory effect of PGE_2 on IL-1 signaling in 3T3-L1 cells. In the previously study, we checked the effects of 4 kinds of anti-IL-1RAcP peptide antibodies on IL-1 induced proliferative response of D10S cells which do not secrete PGE_2 (Yoon and Dinarello, 1998). In this study, we used 3T3-L1 cells which secrete spontaneously PGE_2 and investigated whether IL-1-induced PGE_2 would be required for IL-1-stimulated IL-6 production from 3T3-L1 cells and effects of PGE_2 on target cells would be receptor-mediated.

Materials and Methods

Reagents. The following were purchased: RPMI 1640, penicillin and streptomycin (Mediatech, Inc.), CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals), ibuprofen, prostaglandin E_2 (PGE_2), bovine serum albumin, rabbit IgG and keyhole lympet hemocyanin (Sigma Chemical Co.), fetal bovine serum (FBS) and glutamine (Gibco BRL Gaithersburg). Human recombinant IL-1β was kindly provided by Dr. Aldo Tagliabue (Sclavo) and human IL-1α was from Glaxo Inc. (Research Triangle). Murine IL-1α and β were from Hoffmann-La Roche Inc. (Nutley). Human IL-1 receptor antagonist (IL-1Ra) was obtained from Dr. Daniel Tracey (Upjohn Co.).

Synthetic peptides. Four synthetic peptides **YWTRQDRDLEEPI, VSNNGNYT, WTIDG KKPDDV,** and **YSSTEDETRTQ** were identified as hydrophilic regions (amino acids 71-83, 204-211, 282-292, and 304-314, respectively) of the extracellular portion of the murine IL-1RAcP using methods for displaying the hydropathic character of proteins (Chou *et al*., 1974; Kyte *et al*., 1982). There were the amino acid similarities between the mouse and human IL-1RAcP (Table 1). These peptides were without biological effect on murine and human cell responses to IL-1 (Yoon and Dinarello, 1998). The peptides were synthesized as previously described (Yamada *et al*., 1981) and were cross-linked with keyhole limpet hemocyanin for antibody production as described previously (Yoon and Dinarello, 1998).

Table 1. Comparison of the selected internal peptide sequences of mouse IL-1RAcP, human IL-1RAcP, and mouse IL-1RI

	IL-1RI (61-73)	YKNDSKTPISADR
Peptide-1	mIL-1RAcP (71-83)	YWTRQDRDLEEPI
	hIL-1RAcP (71-83)	YWTRQDRDLEEPI
	IL-1RI (189-198)	VAEEHRGDYT
Peptide-2	mIL-1RAcP (204-211)	VSNNGNYT
	hIL-1RAcP (204-211)	ISNNGNYT
	IL-1RI (263-275)	WKWNGSEIEWNDP
Peptide-3	mIL-1RAcP (282-292)	WTIDGKKPDDV
	hIL-1RAcP (282-292)	WTIDGKKPDDI
	IL-1RI (287-297)	PSTKRKYTLIT
Peptide-4	mIL-1RAcP (304-314)	YSSTEDETRTQ
	hIL-1RAcP (304-314)	HSRTEDETRTQ

Affinity-purified antibodies to synthetic peptides. Details concerning the production, affinity purification and characterization of rabbit anti-murine IL-1RAcP have been published (Yoon and Dinarello, 1998). These antibodies immunoprecipitate native, cell surface as well as recombinant IL-1RAcP; each anti-IL-1RAcP peptide antibody does not cross-react with the other peptides. Control rabbit IgG for these experiments was purchased from Sigma.

3T3-L1 cell cultures. Mouse embryo 3T3-L1 fibroblasts cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in RPMI/10% FBS culture medium (RPMI 1640 containing 10% heat-inactivated FBS, 2 mM glutamine, 100 units/ml of penicillin, 100 μg/ml streptomycin, and 5 μM of 2-mercaptoethanol) at 37°C in a 5% CO_2 incubator. For measuring IL-6 and PGE_2 release, fibroblasts were washed twice in PBS, trypsinized (0.05% trypsin, 0.1% EDTA) for 5 min at 37°C, diluted in RPMI/10% FBS, centrifuged at $250 \times g$ and cell pellets were resuspended in RPMI/10% FBS. Cells were seeded at 2.5×10^4/ml in 24 well plates. After 30 h at 37°C, the culture medium was replaced with 1 ml of fresh RPMI/1% FBS and used for experimental studies. Antibodies, IL-1Ra or ibuprofen were added 1 or 2 h before stimulation with IL-1.

Expression of IL-1RAcP using reverse transcriptase polymerase chain reaction. 3T3-L1 cells were grown for 30 h in 6 well plates at 1×10^5/ml and then replaced with fresh RPMI/1% FBS medium containing PGE_2 (10 ng/ml) for 24 h. Total RNA from confluent 3T3-L1 cells in 6 well plates was extracted using Tri Reagent

(Molecular Research Center, Inc., Cincinnati, OH). RT-PCR was performed as described (Wesche *et al*., 1996) with slight modifications using RNA PCR Core Kit (Roche Molecular Systems, Inc., Branchburg, NJ). Briefly, cDNA was made from RNA samples using random oligonucleotide primers and reverse transcriptase in a thermocycler with a program of 30 min at 42°C, 5 min at 90°C, 5 min at 5°C. As negative control, reverse transcriptase was replaced with DEPC-treated water. cDNA was amplified with appropriate primers and Taq DNA polymerase in a thermocycler with a program of 5 min annealing at 90°C and 5 min at 60°C, followed by 28 cycles with 1 min at 72°C, 1 min at 90°C, 1 min at 60°C, followed by 7 min at 72°C. The following primers were used: IL-1RI (product length: 363 bp), primer 1: 5'-CTG GAG ATT GAC GTA TGT ACA GAA TAT CCA AAT-3'; primer 2: 5'-ATC CCC GGC AAT GTG GAG CCG CTG TGG GAA GGT GGC CTG TGT-3'. IL-1RAcP (product length: 677 bp), primer 1: 5'-AAC CAT CGG TCA CTT GGT ATA AGG G-3'; primer 2: 5'-TTC ATC TGT TCC AAA GTG AGC TCG G-3'. Glyceraldehyde phosphate dehydrogenase (GAPDH) (product length: 452 bp) was used as an internal control, primer 1: 5'-ACC ACA GTC CAT GCC ATC AC-3'; primer 2: 5'-TCC ACC ACC CTG TTG CTG TA-3'. The PCR products were analyzed on a 1.5% agarose containing ethidium bromide, visualized with UV light and photographed with Polaroid 55 film. The photograph was scanned with a densitometer (Molecular Dynamics, Sunnyvail, CA).

IL-6 assay. Murine IL-6 was measured in culture supernatants of 3T3-L1 cells using an ELISA (Endogen, Inc.). The level of quantification was 10 - 1,200 pg/ml.

Quantification of prostaglandin PGE_2. Immunoreactive PGE_2 was measured by ELISA (Cayman Chemical Inc.) based on the competition between free PGE_2 and a PGE_2 tracer (PGE_2 linked to an acetylcholine esterase molecule) for a limited number of PGE_2-specific antibody binding sites. Wavelength absorption was at 405 nm and the range for PGE_2 was 15 to 2,000 pg/ml.

Statistical analysis. Analysis of variance (ANOVA) using Fisher's least significant difference was used. Data were expressed as the mean ± SEM. Comparisons of antibody- or ibuprofen-treated samples to IL-1-treated controls were statistically evaluated by a paired *t*-test.

Results

Downregulation of IL-1-induced IL-6 production by ibuprofen in 3T3-L1 cells. As shown in Fig. 1, the biological activity of four isoforms of IL-1 were tested. Various isoforms of human and murine IL-1 were approximately equipotent in inducing IL-6, reaching statistical significance between 0.1 and 1 ng/ml. In each case, ibuprofen pretreatment of 3T3-L1 cells completely inhibited IL-1-stimulated IL-6 production, demonstrating that IL-1-induced IL-6 is dependent on a

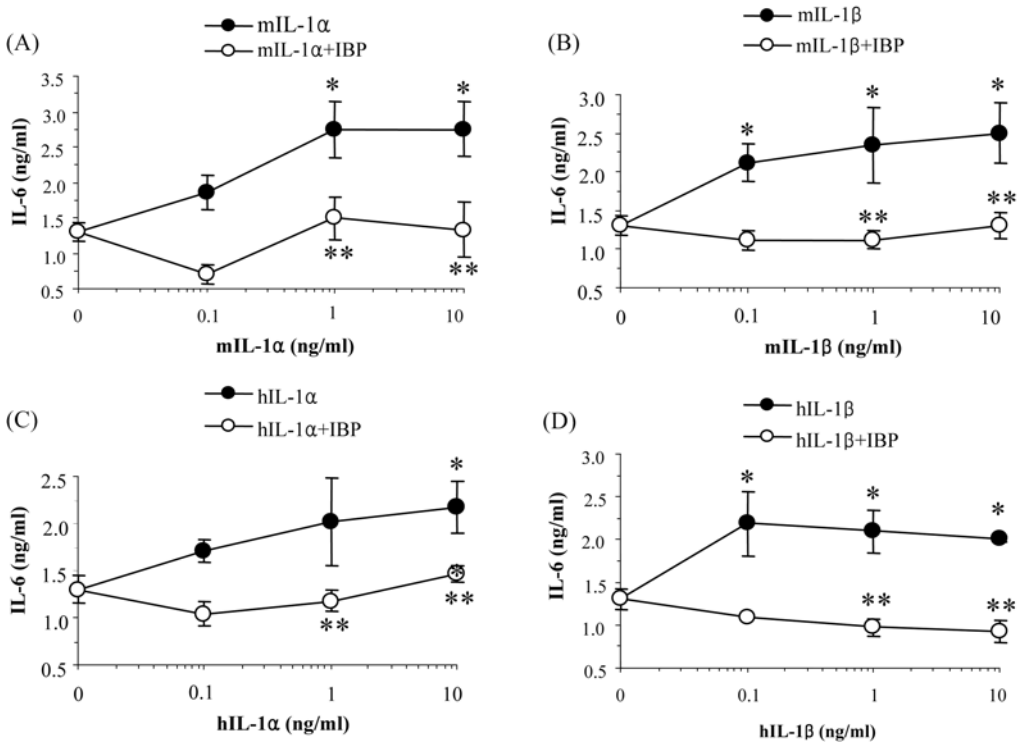

Fig. 1. The effect of ibuprofen on IL-1-induced IL-6 production in 3T3-L1 fibroblast cells. 3T3-L1 cells were pretreated for 2 h at 37°C with ibuprofen (IBP) at 1 μg/ml or vehicle. Then 3T3-L1 cells were stimulated with each of the different forms of IL-1. After 24 h, IL-6 was measured in the supernatants. *$P < 0.05$ compared control cells not stimulated with IL-1. Comparisons of IBP-treated samples to IL-1-treated controls were statistically evaluated by a paired *t*-test. **$P < 0.01$ compared to IL-1 treated controls (n = 4).

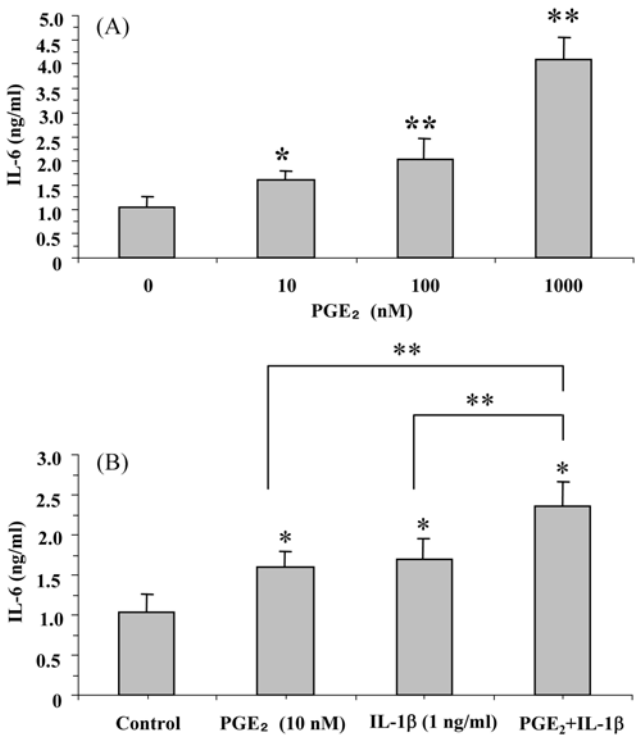

Fig. 2. Effect of PGE_2 on IL-6 production in 3T3-L1 cells. A. 3T3-L1 cells were stimulated with increasing concentrations of PGE_2 as indicated. After 24 h, the supernatants were measured for IL-6. $^*P < 0.05$, $^{**}P < 0.01$ compared to cells not treated with PGE_2. B. PGE_2 (10 nM) and/or hIL-1β (1 ng/ml) were added to 3T3-L1 cells and then IL-6 was measured after 24 h. $^*P < 0.05$ compared to control cells not treated with IL-1. $^{**}P < 0.01$ compared to PGE_2 or hIL-1β-treated cells (n = 3).

cyclooxygenase product. As expected, IL-1α or IL-1β activities were reduced to untreated control levels by the presence of saturating concentrations (5 μg/ml) of IL-1Ra (data not shown, n = 6).

Augmentation of IL-1-induced IL-6 production by PGE_2. The effect of exogenous PGE_2 on IL-6 production was then evaluated. PGE_2 alone induced a dose-dependent, statistically significant rise in IL-6 production (Fig. 2A). As shown in Figs 2A and 2B, PGE_2-induced IL-6 was significant at the lowest concentration tested (10 nM). In cells stimulated with IL-1ß, a significant further increased IL-6 production was observed when PGE_2 was present in 3T3-L1 cell cultures (Fig. 2B). Compared to either PGE_2 or IL-1ß stimulated IL-6 production, the combination of both agents appeared to be additive and not synergistic.

Suppression of IL-1β-induced IL-6 and PGE_2 production by anti-IL-1RAcP peptide antibody. In order to assess the role of IL-1RAcP in IL-1-induced IL-6 and PGE_2, we used blocking antibodies to the IL-1RAcP (Yoon and Dinarello, 1998). 3T3-L1 cells were pretreated with anti-IL-1RAcP peptide-4 antibody for 1 h and then stimulated with mIL-1β for 24 h. As shown in Fig. 3, IL-1β-induced IL-6 and PGE_2 were inhibited to the same degree (Figs 3A and 3B). The degree of suppression of PGE_2 and IL-6 by the anti-IL-1RAcP was comparable. However, there was no effect when the same concentrations of normal rabbit IgG were tested on IL-1β-induced PGE_2 or IL-6 production (Figs 3A and 3B). 3T3-L1 cells spontaneously secrete PGE_2 and hIL-1β (1 ng/ml) increased PGE_2 production by 39% compared to non-treated control cells (Fig. 3B) (n = 4, $p < 0.05$). Similar to data stated above, ibuprofen (μg/ml) suppressed IL-1β-induced PGE_2 production in these cells below spontaneous levels from control cells not stimulated with IL-1 (Fig. 3B). IL-1Ra (5 μg/ml) suppressed IL-1-induced PGE_2 in all cases (data not shown).

Inhibition of the biological activity of hIL-1β by anti-IL-1RAcP peptide 4. In order to assess the role of IL-1RAcP in this model of IL-1-induced IL-6 and PGE_2, we studied the effects of antibodies targeted to the different domains of IL-1RAcP in 3T3-L1 cells. 3T3-L1 cells were incubated with anti-peptide antibodies for 1 h at RT and then stimulated with IL-1β for 24 h. As shown in Fig. 4, anti-IL-1RAcP peptide-4 reduced the biological activity of IL-1β on 3T3-L1 cells by 70% whereas anti-IL-1RAcP peptide-2 or 3 inhibited by 30%. However, anti-IL-1RAcP peptide-1, which is directed against the domain I of IL-1RAcP, had no effect.

Effective inhibition of IL-1β activity by anti-IL-1RAcP antibody. Since anti-IL-1RAcP peptide-4 possessed the greatest IL-1 blocking activity, we compared the effect of this antibody on mouse and human IL-1α and IL-1β. 3T3-L1 cells were pretreated with anti-IL-1RAcP peptide-4 antibody for 1 h, stimulated with different forms of IL-1 for 24 h, and IL-6 was measured. As shown in Fig. 5, there was no effect of anti-IL-1RAcP peptide-4 on human IL-1α-induced IL-6 production. Similarly, murine IL-1α responsiveness was only modestly reduced (30% and 23% reduction, respectively, $P < 0.05$ compared to control cells without antibody pretreatment). On the other hand, both human and mouse IL-1β responses were greatly reduced at 4 μg/ml by 70% and 65%, respectively ($P < 0.05$ compared to control cells) and 67% and 47% ($P < 0.05$ compared to respective IL-1α).

Expression of IL-1RAcP and IL-1RI genes by PGE_2. In order to ascertain whether the enhancing effect of PGE_2 on IL-1-induced IL-6 production was receptor mediated, the effect of PGE_2 on steady state mRNA levels for IL-1RI and IL-1RAcP was assessed. 3T3-L1 cells were incubated with PGE_2 for 24 h. Steady state levels of IL-1RI and IL-1RAcP mRNA were determined by RT-PCR after total RNA was obtained. Expressed mRNAs for both IL-1RI (363 bp) and IL-1RAcP (677 bp) were detected in 3T3-L1 cells as previously described (Yoon and Dinarello, 1998). As a negative control, no PCR product was detected in the absence of reverse transcriptase (data not shown). There is no significant visible increase in

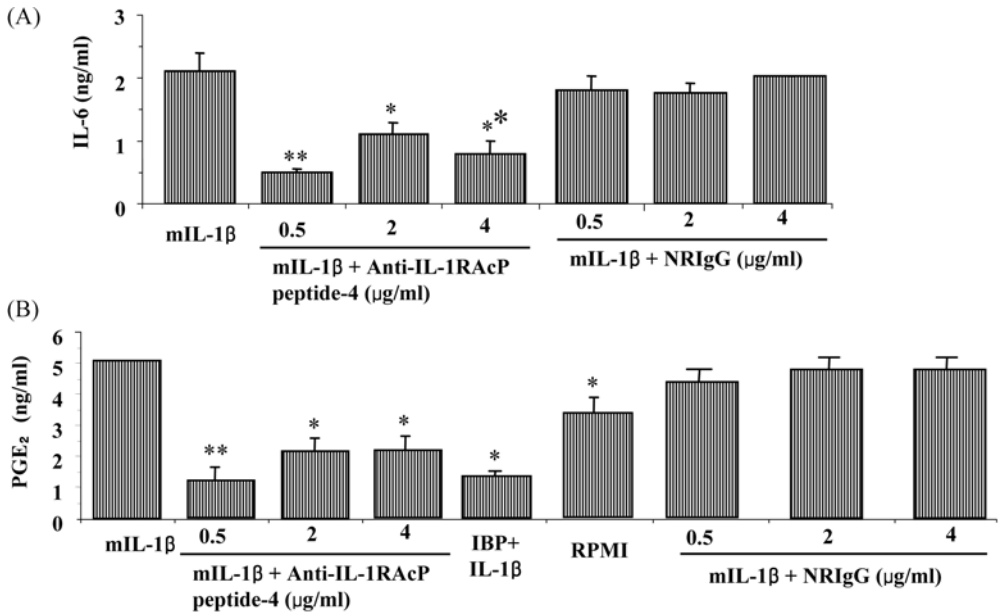

Fig. 3. Effect of anti-IL-1RAcP peptide-4 on productions of IL-6 and PGE_2 induced by IL-1β in 3T3-L1 cells. Cells were pretreated with increasing concentrations of the antibody for 1 h at 37°C. 3T3-L1 cells were then stimulated with murine IL-1β (1 ng/ml) for 24 h. IL-6 and PGE_2 were measured in the supernatants. A. IL-6 from Ab-treated or control rabbit IgG-treated cells (n = 4). B. PGE_2 from one of the experiments shown in A. $^*P < 0.05$ and $^{**}P < 0.01$ compared to IL-1 treated controls.

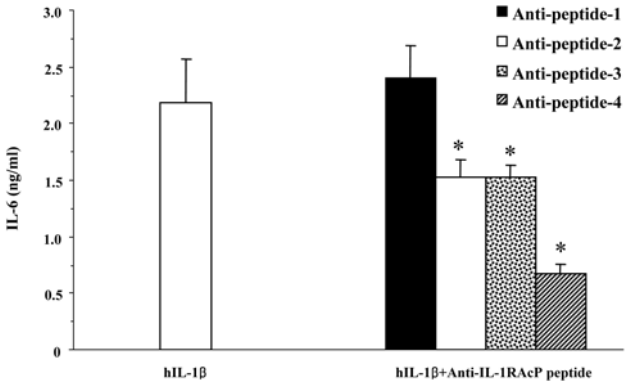

Fig. 4. Effects of different anti-IL-1RAcP peptide antibodies on IL-1β-induced IL-6 production in 3T3-L1 cells. 3T3-L1 cells were pretreated for 1 h with various anti-IL-1RAcP peptides (0.1 μg/ml) or control rabbit IgG and then stimulated for 24 h with hIL-1β (1 ng/ml). IL-6 was measured in the supernatants. $^*P < 0.05$ compared to IL-1 treated control (n = 4).

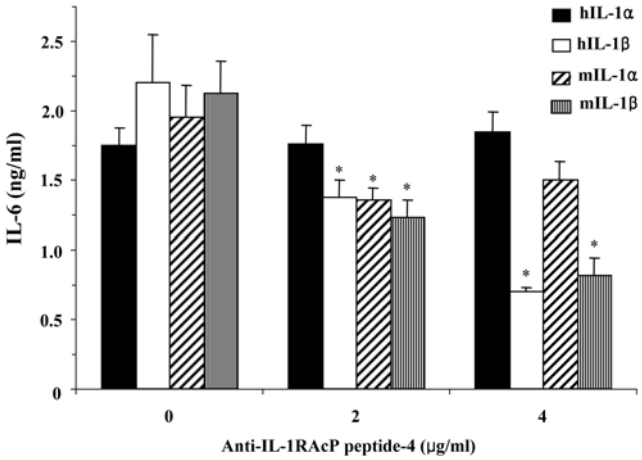

Fig. 5. Effects of anti-IL-1RAcP peptide-4 on the IL-6 production from 3T3-L1 cells stimulated by various isoforms of IL-1. 3T3-L1 cells were incubated with 2 or 4 μg/ml of anti-peptide-4 for 1 h at room temperature and then stimulated with hIL-1α, hIL-1β, mIL-1α, or mIL-1β at the final concentration of 1 ng/ml. IL-6 was measured after 24 h at 37°C. Comparisons of Ab-treated samples to IL-1 controls (no antibody) were statistically evaluated by a paired *t*-test. $^*P < 0.05$ compared to IL-1 treated controls.

the IL-1RI PCR product in cells exposed to PGE_2 which is not consistent with previous studies (Akahoshi *et al.*, 1988). Because this difference is not easily visible, this experiment was repeated on three additional occasions and changes were assessed by densitometer. Steady state levels of mRNA of IL-1RI and IL-1RAcP were not significantly affected by PGE_2 compared to control cells (Fig. 6). It has been reported that IL-1 up-regulates gene and cell surface expression of IL-1RI in human fibroblasts via induction of PGE_2 (Akahoshi *et al.*, 1988; Takii *et al.*, 1992). However, in the absence of PGE_2 synthesis, IL-1 down-regulates IL-1RI mRNA in human fibroblast cell lines and IL-1 specific Th1 D10S cells (Takii *et al.*, 1994; Greenfeder *et al.*, 1995b; Yoon and Dinarello, 1998). As shown in Fig. 3, IL-1 induced a little amount of PGE_2 production (5 ng/ml) and this concentration might not

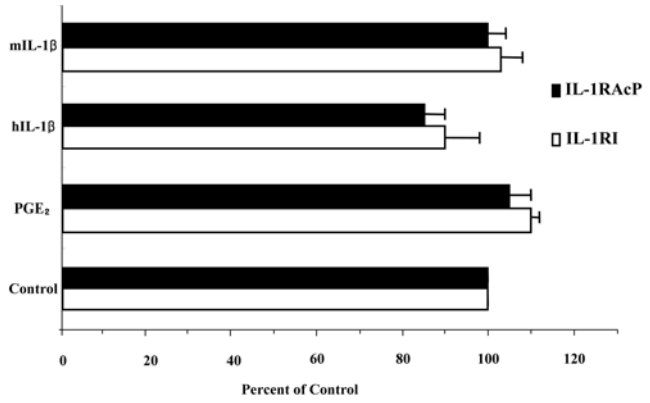

Fig. 6. Regulation of IL-1RAcP and IL-1RI mRNA expression in 3T3-L1 cells by IL-1 and PGE_2. 3T3-L1 cells were incubated with RPMI/1% FBS containing 10^{-6} M PGE_2 or IL-1β (1 ng/ml) for 24 h. Total RNA was isolated, and IL-1RI, IL-1RAcP, and GAPDH mRNA steady state levels were determined by RT-PCR. The RT-PCR products were scanned using a densitometer (Molecular Dynamics, Sunnyvail, CA). Values were normalized based on the density of GAPDH. The ratio of mRNA levels of IL-1Rs to GAPDH of cells in medium alone was used as the controls. Results represent the mean SEM of four experiments. Differences were analyzed for significance by ANOVA.

be enough to induce IL-1R mRNA expression in this fibroblast cells.

Discussion

In these studies, we have shown that IL-1-induced PGE_2 is required for IL-1-stimulated IL-6 production from 3T3-L1 cells. Blocking endogenous PGE_2 synthesis by using a cyclooxygenase inhibitor, ibuprofen, reduced IL-1-induced IL-6 and PGE_2 production from 3T3-L1 cells. The combination effect of IL-1β and PGE_2 caused an additive effect on IL-6 production (Fig. 2B), suggesting IL-1β and PGE_2 stimulate IL-6 production by different pathways. Effects of PGE_2 on target cells are receptor mediated, triggering G protein coupling, adenylcyclase activation, and rises in intracellular cAMP. Increased cAMP serves as a second messenger and may be essential for the expression of certain genes (Roesler *et al*., 1988) such as IL-1ß, IL-6, TNF-α, and class I and II MHC genes (Lenard *et al*., 1989). It has been reported that IL-1 and TNF-α increase intracellular levels of cAMP and IL-6 gene expression in FS-4 fibroblast cells (Zhang *et al*., 1998). This suggests that certain cytokines (IL-1 and TNF-α) coupled with endogenous PGE_2 production and increased intracellular cAMP may regulate the secretory function of fibroblasts in inflammatory reactions. It has been well known that during cytokine-mediated activation of macrophagic cells, the co-induction of PGE_2 takes place; however, unlike the situation with lipopolysaccharide, the co-induction of PGE_2 actually increases the production of these cytokines. This has been demonstrated in human blood monocytes, where induction of IL-1 or IL-6 is enhanced by the addition of PGE_2 or by mechanisms of elevating cAMP (Vannier *et al*., 1993; Vannier *et al*., 1994).

An anti-peptide-4, which targets the terminal segment in domain III of the IL-1RAcP, inhibited IL-1-induced IL-6 and PGE_2 production from 3T3-L1 cells. Anti-IL-1RAcP peptide-2 or 3, which is against peptides in domains II and III, also inhibited IL-1-induced IL-6 production by about 30%. Anti-IL-1RAcP-peptide-4 inhibited 70% of IL-1β-induced IL-6 and PGE_2 production from 3T3-L1 cells, and was more effective against IL-1β compared to IL-1α (Fig. 5), suggesting that IL-1α and IL-1β have different roles as modulators to regulate IL-6 and PGE_2 production from 3T3-L1 cells. However, anti-IL-1RAcP peptide-1 which is directed against domain I had no effect. It has been recently reported that stimulation with IL-1α and IL-1β in bovine corpora lutea cells decreased the PGE_2: $PGF_{2\alpha}$ ratio in the developing stage ($P < 0.05$), whereas it increased the ratio in the mid stage ($P < 0.05$) (Nishimura *et al*., 2004). In the late stage, the ratio of IL-1β-treated cells was greater than that of IL-1α-treated cells ($P < 0.05$).

Anti-IL-1RAcP peptide 4 antibody inhibited IL-1-induced productions of IL-1 and PGE_2 and a COX inhibitor ibuprofen inhibited IL-6 production in 3T3-L1 cells stimulated with IL-1. These results confirm that IL-1RAcP is essential for IL-1 signaling and that IL-6 production by IL-1 requires the co-induction of PGE_2. Also, our data suggest that domain III of IL-1RAcP may be involved in the formation or stabilization of the IL-1RI/IL-1 complex by binding to epitopes on domain III of the IL-1RI which may be created following IL-1 binding to the IL-1RI. The IL-1RAcP is a 570- amino acid transmembrane glycoprotein, and like the IL-1RI, is a member of the Ig superfamily. Unlike the IL-1RI, IL-1RAcP does not bind IL-1 but rather binds to the complex formed by IL-1 and IL-1RI (Greenfeder *et al*., 1995a). The importance of the IL-1RAcP to IL-1 responses has been demonstrated when a blocking antibody to this chain prevented IL-1 responses (Greenfeder *et al*., 1995a). However, it is likely that neo-epitopes are created following the binding of IL-1 to IL-1RI so that the IL-1RAcP can dock to the complex (Schreuder *et al*., 1997; Vigers *et al*.,1997). Following formation of the IL-1/IL-1RI/IL-1RAcP complex, the heterodimer IL-1RI/IL-1RAcP triggers the activation of the IL-1 receptor associated kinase (IRAK) (Croston *et al*., 1995; Boch *et al*., 2003), which has been shown to bind to the IL-1RacP (Volpe *et al*., 1997).

In the previously studies, the inability of anti-IL-1RAcP to block IL-1 binding to D10S Th2 cell lines suggests that none of the three domains of the IL-1RAcP bind to IL-1 itself, but rather to the complex of IL-1RI/IL-1, perhaps to stabilize the complex (Yoon and Dinarello, 1998). This would facilitate dimerization of the cytosolic segments of IL-1RI and IL-1RAcP. Several ligand signaling complexes are stabilized by binding to receptor molecules that are structurally separate

from those involved in ligand binding (Heldin *et al*., 1996). An antibody directed against the fourth Ig-domain of the stem cell factor receptor apparently interferes with signaling by directly blocking dimerization rather than by blocking ligand binding (Blechman *et al*., 1995). It has been recently reported that two kinases, 63- and 83-kD kinase, as well as a protein similar to IRAK, associate with cytosolic domain of the IL-1R following IL-1 binding to the cell surface receptor (Singh *et al*., 1997; Singh *et al*., 1999). Since anti-IL-1RAcP peptide-4 was most effective in blocking the IL-1 bioactivity on 3T3-L1 cells, we suggest that domain III of IL-1RAcP is involved in dimerization of the cytosolic segments of IL-1RI and IL-1RAcP, which would recruit signaling molecules to activate proinflammatory genes.

Though IL-1α and IL-1β bind to the IL-1RI and trigger identical responses, some recent evidence suggests that IL-1α and IL-1β may have differential actions. For example, in the previous report, we demonstrated that IL-1β-induced D10S cell proliferation was more effectively inhibited by anti-IL-1RAcP antibody than that of IL-1α (Yoon and Dinarello, 1998). The cross-linking pattern produced with IL-1β is more intense than that of IL-1α (Greenfeder *et al*., 1995a). In addition, low concentration of human IL-1β down-regulated IL-1RI of D10S cells whereas human IL-1α failed to reduce the receptor surface expression, despite inducing a full proliferative response (Ye *et al*., 1992). IL-1α but not IL-1β was required during skin sensitization to a chemical antigen (Nakae *et al*., 2001). In this case, transfer of antigen-conjugated IL-1α deficient epidermal cells is unable to prime T-lympocytes for sensitization. Using IL-1α (-/-) and IL-1β (-/-) mice, it has been demonstrated that both IL-1α and IL-1β are involved in ovalbumin induced airway hypersensitivity response (AHR). Both IgG_1 and IgE levels were reduced in IL-1β (-/-) mice, while only IgE levels were affected in IL-1α (-/-) mice, indicating a functional difference between IL-1α and IL-1β (Nakae *et al*., 2003).

We here show that affinity purified anti-peptide-4 antibody inhibition of IL-1-induced IL-6 from 3T3-L1 cells was more effective against IL-1β than against either human or mouse IL-1α (Fig. 5). This suggests that IL-1β binding can more easily cause dimerization of IL-1RI and IL-1RAcP than IL-1α. IL-1β is the secreted form of IL-1 and these levels are usually in the low picogram/ml even in severe diseases such as sepsis. In contrast, the IL-1α precursor is not cleaved by caspase-1, IL-1α is not secreted from the cells, and only in severe disease can detect serum IL-1α, which may result from its release from dying cells. IL-1α remains intracellularly where it can function as a DNA-binding transcription factor and perhaps as an oncogene (Werman *et al*., 2004; Buryskova *et al*., 2004). Anti-peptide-4, in the Ig-like domain III (304-314), is of particular importance because the region shares little sequence homologies to IL-1RI and there are only two different amino acid residues between human and mouse IL-1RAcP (Table 1) which would allow its inhibition on IL-1 responses in human cell lines.

IL-1 upregulates its own receptor expression on human fibroblasts through the endogeneous production of PGE_2 (Akahoshi *et al*., 1988; Takii *et al*., 1992). However, in the absence of PGE_2 synthesis, IL-1 down-regulated its own receptor in a human fibroblast cell line TIG-1 (Takii *et al*., 1994). It seems that IL-1 negatively regulates IL-1RI mRNA in cells that do not produce PGE_2 in response to IL-1. For example, IL-1 downregulates IL-1RI mRNA expression and its cell surface receptor in Th2 cell line, D10S cells (Ye *et al*., 1992; Yoon and Dinarello, 1998). T lymphocytes or T helper cells do not secrete PGE_2 after activation with IL-1, IL-2, a combination of both, or concanavalin A (Radeke *et al*., 1992). In the present studies, however, there were no changes in mRNA levels of IL-1RI and IL-1RAcP after stimulation of PGE_2 (Fig. 6). Although IL-1 produced PGE_2 (Fig. 3), the amount of IL-1-induced PGE_2 (5 ng/ml) might be not enough to increase the IL-1RI mRNA. Anti-peptide 4 antibody inhibited IL-1 induced productions of IL-6 and PGE_2 in the 3T3-L1 cells. These observations lead us to conclude that IL-1 signaling requires IL-1RAcP, and IL-1ß binding to IL-1RI can more easily cause dimerization of IL-1RI and IL-1RAcP, thus the 3rd domain of IL-1RAcP is critical for the dimerization between IL-1RI and IL-1RAcP.

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