Prostaglandin E_2 Induces the Expression of IL-1α in Colon Cancer Cells[1]

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PGE_2 has been shown to exert pro-oncogenic effects in colorectal neoplasia through producing autocrine or paracrine growth factors. In the present study, we demonstrate that PGE_2 induced the expression of IL-1α in colon cancer cells, which plays critical roles in tumor metastasis and neoangiogenesis in a variety of cancers. PGE_2 increased the levels of both IL-1α mRNA and protein, suggesting a positive feedback loop between the IL-1 pathway and PGE_2 signaling. Mechanistically, PGE_2 induced the expression of IL-1α at both transcriptional and posttranscriptional levels. PGE_2 stimulated the transcriptional activity of the IL-1α promoter and significantly stabilized IL-1α mRNA. Moreover, we show that IL-1α enhanced colorectal neoplasia, stimulating cell migration and neoangiogenesis. Knockdown of the expression of IL-1α by small-interfering RNA resulted in a reduction of vascular endothelial growth factor secretion in colon cancer cells and an inhibition of tube formation by HUVECs. Thus, our results suggest that PGE_2 induces the expression of proinflammatory cytokine IL-1α, which may potentially enhance the proneoplastic actions of the cyclooxygenase-2/PGE_2 signaling pathway. *The Journal of Immunology,* **2007, 178: 4097–4103.**

The IL-1 cytokine family consists of three members, IL-1α, IL-1β, and IL-1R antagonist. IL-1 is a crucial regulator of the innate immune system and inflammatory responses (1, 2). A variety of cell types, including lymphocytes, monocytes, fibroblasts, endothelial cells, and epithelial cells, can produce IL-1. The proinflammatory activities of IL-1 result largely from stimulating the expression of genes encoding inflammatory mediators. It is well-documented that IL-1 increases the expression of cyclooxygenase-2 (COX-2)[3] and the production of PGE_2 (3, 4). Many biological activities of IL-1 are actually due to increased PGE_2 production (5). Inhibition of COX-2 enzyme activity significantly reduces IL-1-induced inflammation, suggesting that the COX-2/PGE_2 signaling system plays critical roles in the proinflammatory activities of this cytokine.

Accumulative evidence suggests that IL-1 plays critical roles in the development of malignant lesions. The most compelling evidence was generated in IL-1 knockout (KO) mouse models. Voronov et al. (6) have demonstrated the critical roles of IL-1 in tumor invasiveness and angiogenesis. Mice solely deficient in IL-1α or IL-1β exhibit dramatically impaired tumor development and blood vessel growth. B16 melanoma cells do not metastasize to the lung of IL-1β KO mice; however, wild-type mice die from lung metastasis by day 20 after inoculation of B16 cells. DA/3 mammary cancer cells fail to grow tumors in the foot pad of IL-1α KO mice, whereas progressive tumor growth is observed in wild-type mice. In support of these findings, the expression of IL-1 is significantly increased in a variety of malignant lesions and particularly in metastatic human tumor specimens, including non-small cell lung cancer, colorectal carcinoma, and melanoma (7, 8). Furthermore, antagonism of IL-1R using IL-1R antagonist results in significant inhibition of angiogenesis and growth of xenografted human tumors that produce IL-1. These data suggest that the IL-1 signaling system plays critical roles in neoangiogenesis and tumor metastasis in a variety of cancers and inhibition of this pathway may be used for treatment of certain cancers.

A large body of studies indicates that COX-2 exerts pro-oncogenic effects on a variety of tumors (9). COX-2 enzyme catalyzes the conversion of arachidonic acid to PGG_2 and PGH_2. PGH_2 is subsequently converted to a variety of prostaglandins which include PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and thromboxaneA_2 by each respective PG synthase. Recent studies provide strong evidence that PGE_2 is a key mediator for proneoplastic actions of COX-2. PGE_2 promotes proliferation of human colorectal carcinoma cells. DNA synthesis is increased by PGE_2 treatment in several colon cancer cell lines (10). PGE_2 stimulates the growth of human colorectal cancer cells when grown in extracellular matrix (11–13). In addition, PGE_2 promotes colon cancer cell migration and increases their metastatic potential (12, 14–16). Further evidence demonstrates that PGE_2 promotes intestinal neoplasia through enhancing tumor angiogenesis (17–20). Knockout of the EP_2 receptor or inhibition of COX-2 enzyme results in a reduction of neoangiogenesis in $APC^{\Delta 716}$ mouse tumors (19, 21). The molecular mechanisms by which PGE_2 promotes such a wide range of malignant phenotypes are poorly understood. A potential mechanism is that PGE_2 stimulates the production of a variety of growth factors, so that provides a proneoplastic environment. In the present study, we attempted to elucidate the role of IL-1α in COX-2/PGE_2 proneoplastic actions in colorectal neoplasia. We found that PGE_2 induced the expression of IL-1α in colon cancer cells through both transcriptional and posttranscriptional mechanisms. IL-1α strongly stimulated colon cancer cell migration and induced the expression of VEGF. Our results suggest a positive loop between COX-2/PGE_2 and IL-1α that

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[3] Abbreviations used in this paper: COX-2, cyclooxygenase-2; KO, knockout; C_T, cycle threshold; VEGF, vascular endothelial growth factor; PKA, protein kinase A; ARE, adenosine uridine-rich element; UTR, untranslated region; siRNA, small-interfering RNA; DRB, 5,6,-dichlorobenzimidazole riboside; GPCR, G protein-coupled receptor; CRE, cAMP responsive element; EP, E-prostanoid.

may play critical roles in neoangiogenesis and metastasis of colon cancer.

Materials and Methods

Cell culture and reagents

LS-174T, T-84, and DLD-1 cells were purchased from American Type Culture Collection and maintained in McCoy's 5A medium containing 10% FBS. HCA-7 cells were a gift from Dr. S. Kirkland (University of London, London, U.K.). HUVECs were purchased from Cascade Biologics and grown in medium 200 supplemented with low serum growth supplement. PGE_2, 17-phenyl-trinor-PGE_2, butaprost, sulprostone, and PGE_1 alcohol were purchased from Cayman Chemical. H-89, LY-294002, PD-98059, and SB-203580 were purchased from Calbiochem. Human IL-1α was purchased from R&D Systems.

Human cytokine gene array

To determine the relative expression levels of cytokines, GEArray Q Series Human Growth Factor/Cytokine Gene Array (SuperArray Bioscience) was conducted according to the manufacturer's instructions. Biotin-labeled probe was synthesized from total RNA and hybridized with a nylon membrane printed with cDNAs of 96 growth factors and cytokines. The array image was captured with chemiluminescence detection and analyzed using the software of GEArray Expression Analysis Suite.

Cell migration assay

LS-174T cells suspended in 400 μl of serum-free McCoy's 5A medium were placed in uncoated Transwell chamber (8 μm; Corning Costar). The Transwell chambers were then inserted into a 24-well plate containing vehicle, PGE_2, or IL-1α. After an incubation period of 24 h at 37°C, cells on the upper surface of the filter of Transwell chambers were removed with a cotton swab. The filters were fixed and stained with 0.5% crystal violet solution. Three microscope fields (×200) from each Transwell chamber were randomly selected and cells adhering to the undersurface of the filter were counted.

HUVEC tube formation

HUVEC were suspended in 0.1 ml of indicated conditioned medium and placed on growth factor-reduced Matrigel (Collaborative Biomedical Products) in 96-well plates. Morphology of the cells was documented using a digital camera attached to an inverted microscope. Three photographs from random fields of each microtiter well (quadruplicate wells for each group) were analyzed. Tubes were defined as straight cellular extensions joining two cell masses (22). Tube formation was assessed by the numbers of tubular structures and the length of tubes.

RNA extraction and RT-PCR

Extraction of total cellular RNA was conducted as previously described (13). Expression of IL-1α in LS-174T cells was determined using RT-PCR as described previously (12). Human IL-1α primer pairs were purchased from R&D Systems. RT-PCR was conducted using ProStar RT-PCR system (Stratagene) according to the manufacturer's instructions.

Real-time RT-PCR

IL-1α expression was quantified using real-time quantitative PCR or TaqMan technique (Applied Biosystems). The sequence of the primer/probe set was based on IL-1α mRNA sequence (GenBank accession no. NM_000575) and includes the following: forward primer, CCTCTTCT GGGAAACTCACG; reverse primer, AAGTTTGGATGGGCAACTG ATGT. 18S rRNA TaqMan assay reagent was used for internal control. One-step RT-PCR was performed with 40 ng of RNA for both target gene and endogenous controls. Duplicate cycle threshold (C_T) values were analyzed in Microsoft Excel using the comparative C_T ($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta C_T}$) was obtained by normalized to 18s and relative to a calibrator.

ELISA

Levels of human VEGF and IL-1α proteins in cell culture medium and cell lysates were quantified using ELISA kits (R&D Systems). Cells were seeded in 60-mm plates and serum was deprived for 24 h before PGE_2 treatment. Culture medium were collected and cell lysates were prepared in radioimmunoprecipitation assay buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate) for ELISA.

Table I. *Expression of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, and IL-22 was analyzed*[a]

Cytokines	Fold Change					
	4 h		8 h		24 h	
Gene symbol	PGE_2: 1 μM	10 μM	1 μM	10 μM	1 μM	10 μM
IL-1α	5.8	6.7	4.6	5.8	2.7	2.3
IL-1β	1.5	1.6	1.6	1.6	1.2	1.4
IL-7	1.3	1.5	1.7	1.6	1.8	2.1
IL-8	0.7	0.9	0.7	0.8	0.9	0.8
IL-12α	1.0	1.1	0.9	0.8	1.0	1.9
IL-14	1.3	1.1	1.2	1.1	1.2	1.2
IL-15	0.9	1.3	1.1	1.1	0.7	0.8
IL-18	0.9	1.0	0.8	0.7	0.8	1.2
IL-19	1.0	1.1	1.0	1.0	0.8	0.9

[a] Listed are relative levels of cytokines expressed in LS-174T cells. Fold changes of differential expression are expressed as PGE_2 treated/vehicle treated.

Transient transfection and luciferase assay

Fragments of the human *IL-1α* promoter (GenBank accession no. X03833) were PCR amplified from human genomic DNA and inserted into pGL-3 vector (Promega). For construction of the IL-1α 3′ untranslated region (UTR) reporter plasmid, expression vector pCMV-Luc was used (23). The addition of the IL-1α 3′ UTR (1167 bp) was accomplished by PCR amplification of the IL-1α mRNA 3′ UTR (nt 1777–2943, GenBank accession no. NM_000575) and inserting them adjacent to the luciferase coding region to yield pCMV-Luc-IL-1α 3′ UTR plasmid. Assay to determine luciferase activity was described previously (13). Briefly, LS-174T cells were transfected with 0.5 μg of IL-1α reporter plasmid along with 0.1 μg of the pRL-CMV plasmid, containing the *Renilla* luciferase gene (Promega), using the FuGENE 6 procedure (Roche) as described in the manufacturer's protocol. Transfected cells were lysed at indicated times for luciferase assay. Firefly and *Renilla* luciferase activities were measured using a dual-luciferase reporter assay system (Promega) and a luminometer. Firefly luciferase values were standardized to *Renilla* values.

RNA interference

IL-1α-specific small-interfering RNA (siRNA) sequences were purchased from Ambion (GenBank accession no. NM_000575, siRNA ID 121265 and 121266). Transfection was accomplished using XtremGene transfection reagent (Roche) according to the supplier's instruction. Twenty-four hours after transfection, cells were placed in serum-free medium for 24 h and then treated with PGE_2 for 24 h. Levels of vascular endothelial growth factor (VEGF) in cell culture medium and levels of IL-1α in cell lysates were determined by ELISA.

Data analysis

All statistical analyses were performed on a personal computer with the StatView 5.0.1 software (SAS Institute). Analyses between two groups were determined using the unpaired Student t test. Differences with a p value of <0.05 were considered as statistically significant.

Results

PGE_2 induction of IL-1α expression

PGE_2 modulates a number of signaling pathways and, therefore, regulates the expression of an array of genes. To determine whether PGE_2 regulates the expression of inflammatory cytokines, we conducted targeted cDNA arrays, using GEArray Human Growth Factor/Cytokine Array. LS-174T cells were treated with PGE_2 at relatively low (0.5 μM) or high (10 μM) concentrations for 4, 8, and 24 h; levels of a panel of cytokines were analyzed. A number of cytokines, including IL-1α, IL-1β, IL-7, IL-8, IL-12α, IL-14, IL-15, IL-18, and IL-19, were expressed in LS-174T cells. IL-1α appeared to be the leading cytokine that was strongly induced by PGE_2. A 4-h treatment with either low or high concentrations of PGE_2 resulted in an ~6-fold increase in the expression

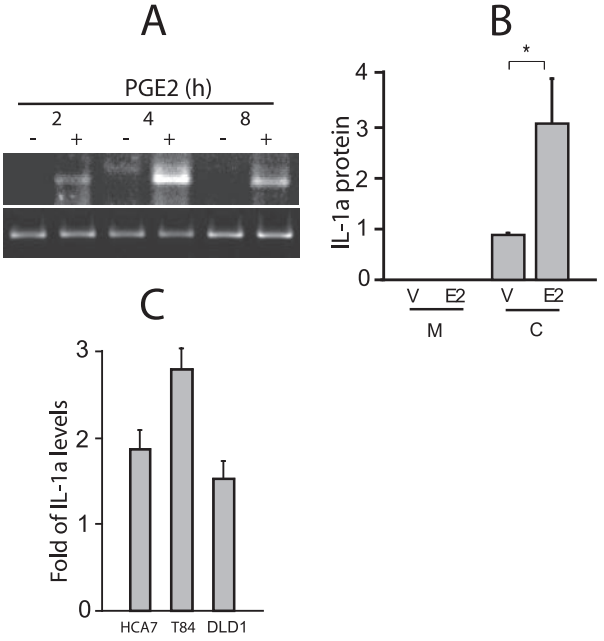

FIGURE 1. PGE_2 induction of IL-1α expression in colon cancer cells. *A*, LS-174T cells were serum-deprived for 24 h before PGE_2 treatment. Levels of IL-1α mRNA were analyzed by RT-PCR. *B*, LS-174T cells were serum-deprived for 24 h and treated with vehicle (V) or 0.5 μM PGE_2 (E2) for 24 h. Cell culture medium and cellular lysates were collected. Levels of IL-1α protein in cell culture medium (M) or cell lysates (*C*) were determined by ELISA. Plotted is the mean $\pm$ SD of IL-1α content performed in triplicate. $*$, $p < 0.05$. ELISA in all figures were performed at least three times independently. *C*, HCA-7, T-84, or DLD-1 cells were serum-deprived for 24 h and treated with vehicle or PGE_2 for 24 h. Cell lysates were collected and levels of IL-1α protein were determined by ELISA. Plotted is the fold of increase in IL-1α levels by PGE_2 engagement.

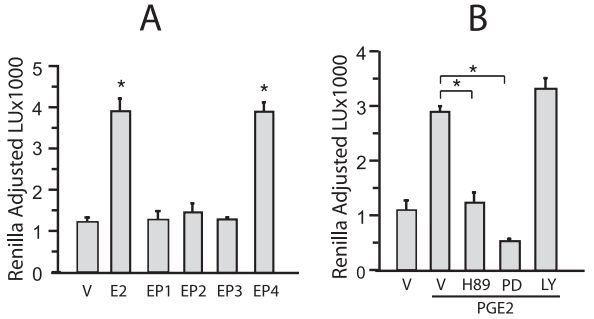

FIGURE 2. PGE_2 induction of IL-1α transcription. *A*, LS-174T cells were transiently transfected with IL-1α promoter driving reporter vector. Treatment was added 6 h before harvest (V, ETOH; E2, 0.5 μM PGE_2; EP1, 0.5 μM 17-phenyl-trinor-PGE_2; EP2, 0.5 μM butaprost; EP3, 0.5 μM sulprostone; and EP4, 0.5 μM PGE_1 alcohol). Firefly and *Renilla* luciferase activities were measured and standardized. Plotted is the mean $\pm$ SD of *Renilla* adjusted luciferase values performed in quadruplicate. $*$, $p < 0.05$. Luciferase assays in all figures were performed at least three times independently. *B*, LS-174T cells were transiently transfected with IL-1α promoter reporter vector and then treated with 0.5 μM PGE_2 in the presence of vehicle (V), 5 μM H-89 (H89), 25 μM PD-98059 (PD), or 10 μM LY-294002 (LY) for 6 h. Firefly and *Renilla* luciferase activities were measured and standardized. Plotted is the mean $\pm$ SD of *Renilla* adjusted luciferase values performed in quadruplicate. $*$, $p < 0.05$.

of IL-1α (Table I). In addition, IL-1β and IL-7 were modestly induced by PGE_2. RT-PCR analysis reproduced similar results; levels of IL-1α mRNA were strongly increased at 4 and 8 h after PGE_2 exposure (Fig. 1*A*). A 3-fold increase in IL-1α protein level was detected in LS-174T cell lysates by ELISA, whereas IL-1α protein was not found in cell culture medium (Fig. 1*B*), suggesting that PGE_2-induced IL-1α may function as an intracellular messenger in LS-174T cells (24). Furthermore, PGE_2 engagement increased levels of IL-1α production in additional colon cancer cell lines, including HCA-7, T-84, and DLD-1 (Fig. 1*C*). Based on these findings, we decided to further investigate the regulatory mechanism and functional roles of PGE_2-induced IL-1α.

PGE_2 stimulation of IL-1α transcription

PGE_2 regulates a number of genes at the transcriptional level. To elucidate the mechanism by which PGE_2 induced IL-1α expression, the 1.4-kb sequence immediately upstream of the 5′ end of the IL-1α mRNA start site was PCR amplified and subcloned into luciferase report vector pGL-3 (pGL-IL-1α 5′). PGE_2 treatment increased the activity of the IL-1α promoter ~2- to 3-fold in LS-174T cells, which were transiently transfected with pGL-IL-1α 5′ plasmid (Fig. 2*A*). The EP_4 signaling pathway was the predominant pathway which mediated PGE_2-induced IL-1α transcription. Agonists for EP_2 did not appear to stimulate the activity of the IL-1α promoter. Inhibition of PKA activity by H-89 significantly reduced PGE_2-induced IL-1α transcription (Fig. 2*B*). Interestingly, blocking of the MEK1/ERK signaling pathway by a selective MEK inhibitor, PD-98059, strongly inhibited both basal and PGE_2-induced activation of the IL-1α promoter. These results suggest that MEK/ERK activation is critical for basal activity of the IL-1α promoter, whereas PGE_2-stimulated IL-1α transcription requires the activation of the cAMP/protein kinase A (PKA) pathway.

PGE_2 stabilization of IL-1α mRNA

Many mRNA-encoding inflammatory gene products, including *TNF-α*, *IL-8*, *IL-6*, and *IL-1β*, undergo rapid degradation; their stability can be regulated in response to extracellular stimulus (25, 26). Because IL-1α transcription was increased by PGE_2 modestly, it was of interest to determine whether PGE_2 regulated the stability of IL-1α mRNA. Adenosine uridine-rich elements (AREs) located in the 3′ UTR of many inflammatory cytokines are determined to promote rapid degradation of mRNA (25, 27–30). The 3′ UTR of human IL-1α mRNA (GenBank accession no. NM_000575) contains 1167 nt (1777 to 2943) where a number of class I and II AREs can be mapped. A cluster of AREs is found in a 56-nt AU-rich region (2389–2445), which includes at least two class I and two class II AREs (Fig. 3*A*).

LS-174T cells were treated with PGE_2 and new transcription was blocked by addition of 5, 6,-dichlorobenzimidazole riboside (DRB). The decay rate of IL-1α mRNA was evaluated by real-time quantitative PCR. Although the half-life of IL-1α mRNA in vehicle-treated LS-174T cells was ~2 h, PGE_2 treatment significantly stabilized IL-1α mRNA, extending the half-life to ~4.5 h (Fig. 3*B*).

To determine the functional role of the 3′ UTR of IL-1α mRNA in PGE_2 stabilization of IL-1α mRNA, we constructed a luciferase reporter vector (pCMV-Luc-IL-1α 3′ UTR), in which luciferase cDNA was under the control of the CMV promoter and followed by the 3′ UTR of IL-1α mRNA. The pCMV-Luc-IL-1α 3′ UTR was transiently introduced into LS-174T cells and addition of PGE_2 increased the luciferase activity ~3-fold (Fig. 3*C*), suggesting that PGE_2 stabilized the chimerical luciferase-IL-1α 3′ UTR mRNA and, therefore, increased luciferase activity. To determine the roles of the AREs in IL-1α mRNA 3′ UTR, we constructed several deletion mutants of IL-1α 3′ UTR reporter vectors. PGE_2 did not induce the luciferase activity in LS-174T cells transfected

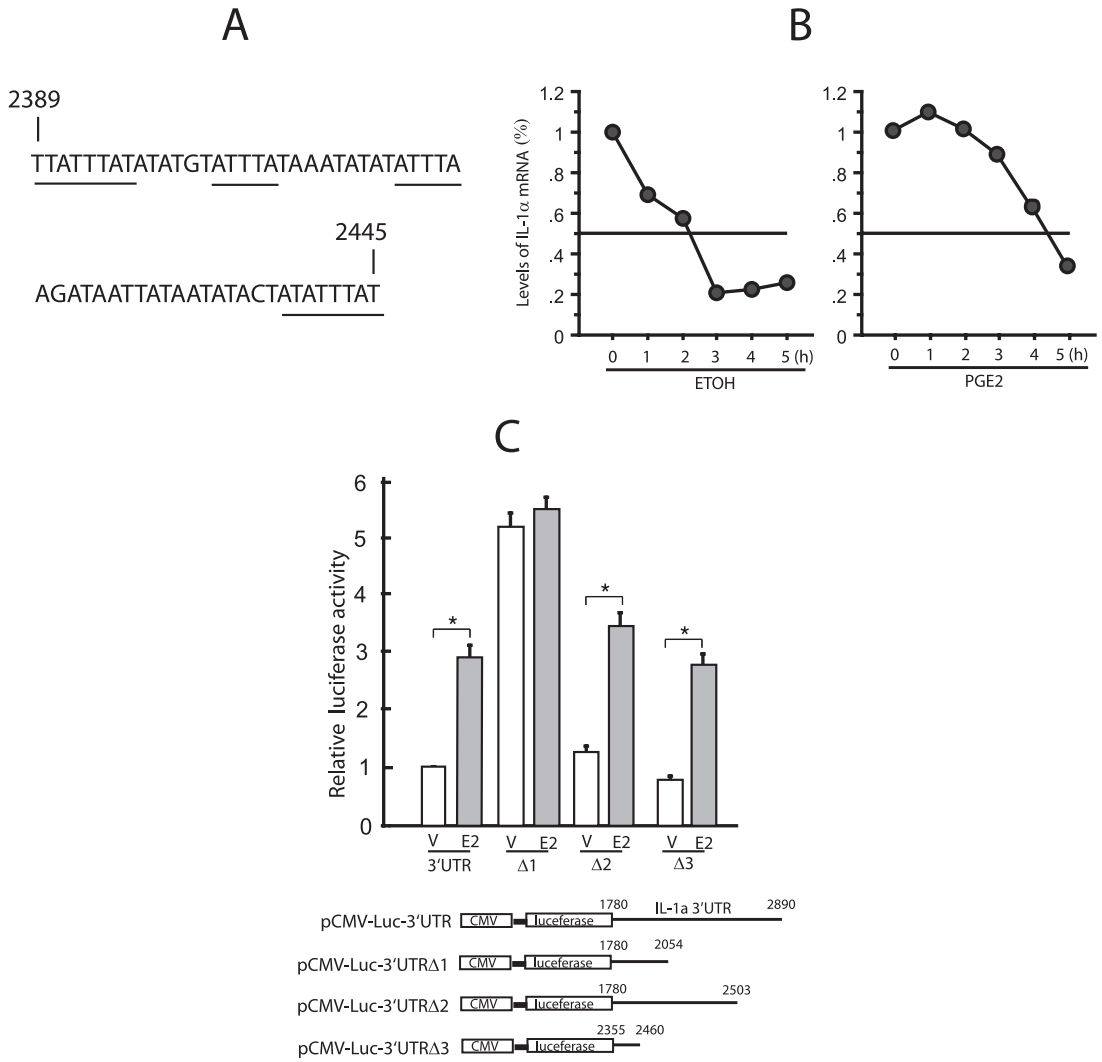

FIGURE 3. PGE$_2$ stabilization of IL-1α mRNA. *A*, The sequence of IL-1α mRNA 3′ UTR between nt 2389 and 2445 contains multiple AREs. Class I or II AREs are marked. *B*, Degradation curves of IL-1α mRNA. LS-174T cells were treated with ETOH (*left panel*) or 0.5 μM PGE$_2$ (*right panel*) for 4 h, and then 50 μM DRB was added. Total RNA was extracted at the indicated time points after the addition of DRB. Steady-state levels of IL-1α mRNA were determined by real-time PCR. Results are expressed as the percentage of the level at 0 h. *C*, pCMV-Luc-IL-1α 3′UTR reporter vector and its derived mutants were constructed by inserting the various sequences of IL-1α mRNA 3′ UTR into the pCMV-Luc vector as illustrated. LS-174T cells were transiently transfected with IL-1α 3′ UTR reporter plasmids. Cells were treated with 0.5 μM PGE$_2$ (E2) or vehicle (V) for 6 h. Firefly and *Renilla* luciferase activities were measured and standardized. Plotted is the mean $\pm$ SD of *Renilla* adjusted luciferase values performed in quadruplicate. $*$, $p < 0.05$.

with pCMV-Luc-IL-1α 3′ UTR (1780–2054), which did not include the ARE cluster between nt 2389 and 2445. PGE$_2$-regulated stabilization of IL-1α 3′ UTR was fully restored in cells that were transfected with pCMV-Luc-IL-1α 3′ UTR (1780–2503). Next, we inserted a sequence of 106 bp (nt 2355–2460), which includes the ARE clusters, into the pCMV-Luc reporter vector. This minimum sequence mimicked the role of the entire IL-1α 3′ UTR, facilitating the degradation of luciferase mRNA and appeared to be responsive to PGE$_2$-induced stabilization of IL-1α mRNA 3′ UTR.

Roles of EP/cAMP/PKA in PGE$_2$ stabilization of IL-1α 3′ UTR

PGE$_2$ acts via specific transmembrane G protein-coupled receptors (GPCR) (31). Previous studies suggest that PGE$_2$ exerts proneoplastic actions predominantly through the EP$_{2,4}$/cAMP/PKA pathway. To elucidate the signaling pathways involved in PGE$_2$ stabilization of IL-1α mRNA 3′ UTR, pCMV-Luc-IL-1α 3′ UTR reporter was stably transfected into LS-174T cells (LS-174T-Luc-IL-1α3′ UTR). Stimulation with PGE$_2$ increased luciferase activity ~3-fold (Fig. 4*A*). Both EP$_2$ and EP$_4$ agonists mimicked the effect of PGE$_2$ and significantly stabilized Luc-IL-1α 3′ UTR. Interestingly, addition of EP$_1$ agonist induced luciferase activity as well. It was not surprising that an increase in the level of cAMP by addition of dibutyryl cAMP reproduced the effects of PGE$_2$ and EP agonists in LS-174T-Luc-IL-1α 3′ UTR cells. The stimulatory action of PGE$_2$ on Luc-IL-1α 3′ UTR activity required activation of the PKA pathway because inhibition of PKA by H-89 completely attenuated the PGE$_2$-induced luciferase activity (Fig. 4*B*). In contrast, an MEK inhibitor, PD-98059, and a PI3K inhibitor, LY-294002, did not block PGE$_2$-induced Luc-IL-1α 3′ UTR expression. In support of these findings, ectopic expression of active PKA also reproduced the effect of PGE$_2$ and robustly increased the luciferase expression of pCMV-Luc-IL-1α 3′ UTR in LS-174T cells (Fig. 4*C*).

Functional roles of IL-1α in colon cancer cells

Both PGE$_2$ and IL-1 play critical roles in tumor invasiveness. Therefore, we next compared the roles of IL-1α and PGE$_2$ in colon

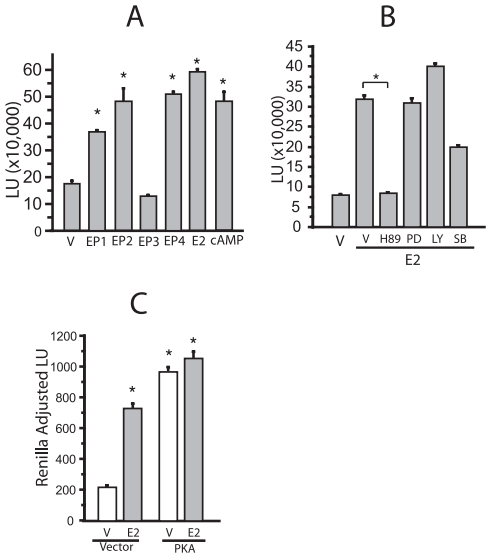

FIGURE 4. Signaling mechanisms of PGE_2 stabilization of IL-1α mRNA 3′ UTR. *A*, pCMV-Luc-IL-1α 3′ UTR reporter was stably transfected into LS-174T cells (LS-174T-Luc-IL-1α 3′ UTR). Cells were serum-deprived for 24 h before treatments (V, ETOH; E2, 0.5 μM PGE_2; EP1, 0.5 μM 17-phenyl-trinor-PGE_2; EP2, 0.5 μM butaprost; EP3, 0.5 μM sulprostone; EP4, 0.5 μM PGE_1 alcohol; cAMP, 5 mM dibutyryl cAMP). After a 6-h incubation, firefly luciferase activity was measured. Plotted is the mean ± SD of luciferase values performed in quadruplicate. *B*, LS-174T- Luc- IL-1α 3′ UTR cells were treated with vehicle (V), 5 μM H-89 (H), 25 μM PD-98059 (P), 10 μM LY-294002 (L), or 10 mM SB 203580 for 15 min before the addition of 0.5 μM PGE_2 (E2). After a 6-h incubation, firefly activity was measured. Plotted is the mean ± SD of luciferase values performed in quadruplicate. *C*, LS-174T cells were transiently transfected with pCMV-Luc-IL-1α 3′ UTR reporter along with empty vector, or active PKA expression construct (PKA). After a 24-h incubation, cells were treated with vehicle (V) or 0.5 μM PGE_2 (E2) for 6 h. Firefly and *Renilla* luciferase activities were measured and standardized. Plotted is the mean ± SD of *Renilla* adjusted luciferase values performed in quadruplicate. ∗, $p < 0.05$.

cancer cell migration. LS-174T cells were subjected to the modified Boyden chamber assay. Vehicle-treated LS-174T cells formed noninvasive clumps on the membrane after a 24-h incubation. Addition of either IL-1α or PGE_2 in the lower chambers significantly stimulated the migration of LS-174T cells (Fig. 5*A*). TGF-α-activated epidermal growth factor receptor signaling is critical for colon cancer cell migration (32). We have demonstrated that TGF-α and PGE_2 synergistically stimulate LS-174 cell migration (33) (Fig. 5*B*). Interestingly, TGF-α and IL-1α also promoted the migration of LS-174T cells in a synergistic fashion and robustly increased the number of migrating cells. These results indicate that IL-1α and PGE_2 exerted similar effects on LS-174T cell migration, suggesting that activation of the IL-1 pathway may contribute to PGE_2 promoted cell migration.

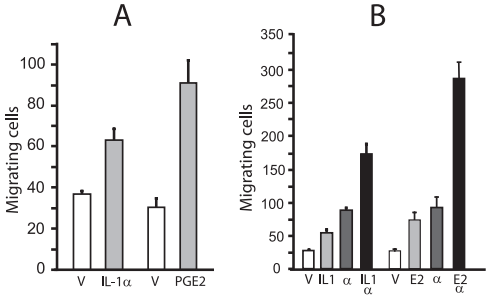

FIGURE 5. Stimulation of LS-174T cell migration by both IL-1α and PGE_2. *A* and *B*, A total of 5×10^4 LS-174 cells were seeded into the upper chamber and the assay was conducted for 24 h with indicated attractants in the bottom chamber (V, vehicle; IL-1, 5 ng/ml IL-1α; E2, 0.5 μM PGE_2; α, 100 ng/ml TGF-α). Cells that attached to the bottom side of the upper chamber were stained and counted per field (×200). Plotted is the mean ± SD of migrating cells of three experiments.

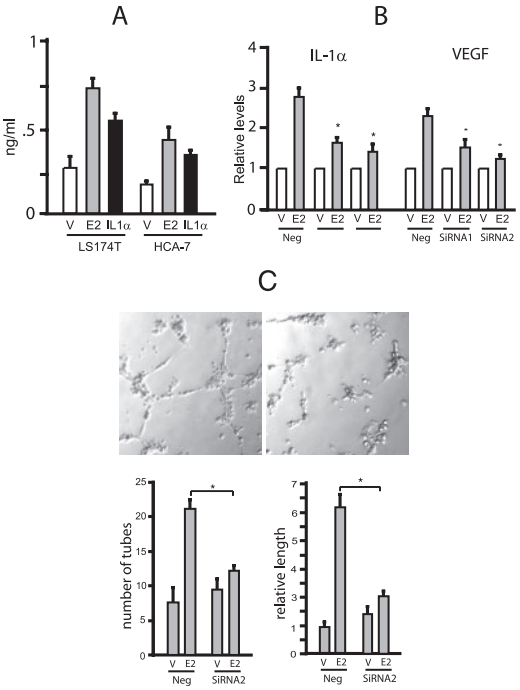

FIGURE 6. IL-1α promotion of angiogenesis. *A*, LS-174T and HCA-7 cells were serum-deprived for 24 h before vehicle (V), 0.5 μM PGE_2 (E2), or 5 ng/ml IL-1α treatments. Levels of VEGF protein in culture medium were determined by ELISA. Plotted is the mean ± SD of VEGF content performed in triplicate. *B*, HCA-7 cells were transfected with negative control siRNA (Neg), or IL-1α-specific siRNA1 and siRNA2 (siRNA ID 121265 and 121266). Transfection was accomplished using XtremGene transfection reagent. Twenty-four hours after transfection, cells were placed in serum-free medium for 24 h and then treated with vehicle (V) or 0.5 μM PGE_2 for 24 h. Levels of VEGF in cell culture medium and levels of IL-1α in cell lysates were determined by ELISA. Results were normalized by the value of respective negative controls. ∗, $p < 0.05$. *C*, HCA-7 cells were transfected with negative control siRNA (Neg) or IL-1α-specific siRNA2. Twenty-four hours after transfection, cells were placed in serum-free medium for 24 h and then treated with vehicle (V) or 0.5 μM PGE_2 for 24 h. Conditioned medium were collected for growing HUVEC. A total of 1×10^4 HUVEC suspended in 100 μl of HCA-7-conditioned medium were placed onto growth factor reduced Matrigel. After an 8-h incubation, cells were photographed (×100). *Upper panels*, *left*, HUVEC grown in negative control siRNA-transfected and PGE_2-treated HCA-7-conditioned medium; *right*, HUVEC grown in IL-1α siRNA2-transfected and PGE_2-treated HCA-7-conditioned medium. Numbers of tubes were counted and the relative length of the tubular structure was measured (*lower panels*). Plotted is the mean ± SD. ∗, $p < 0.05$.

IL-1 is known to stimulate tumor angiogenesis through increasing the secretion of angiogenic factors (34). Genetic disruption of the *IL-1* genes results in significantly reduced secretion of VEGF by tumor cells (6). Both LS-174T and HCA-7 cells constitutively released VEGF into cultural medium. Addition of either PGE_2 or IL-1α significantly increased the levels of VEGF in both LS-174T and HCA-7 cell culture medium, determined by ELISA (Fig. 6*A*). To determine the effects of endogenous IL-1α on VEGF production and secretion, we performed an acute knockdown of IL-1α

expression using siRNA. LS-174T and HCA-7 cells were transfected with IL-1α siRNA before PGE_2 treatment. Efficient down-regulation of IL-1α protein was achieved 72 h after siRNA transfection. As a result, PGE_2-induced IL-1α expression was significantly inhibited by two independent siRNA sequences in both LS-174T (data not shown) and HCA-7 cells (Fig. 6*B*). Interestingly, transfection of IL-1α siRNA also significantly reduced the levels of PGE_2-induced VEGF in both LS-174T and HCA-7 cell-conditioned medium, suggesting the involvement of IL-1α signaling in PGE_2 induction of VEGF. To determine the effects of colon cancer cell-released growth factors on tubular organization, HUVECs were placed on growth factor-reduced Matrigel. HUVEC spontaneously formed tubular structures on extracellular matrix. As expected, conditioned medium collected from PGE_2-stimulated HCA-7 cells robustly increased the number and length of HUVEC-formed tubes (Fig. 6*C*). Transfection with IL-1α siRNA before PGE_2 engagement, however, significantly attenuated the stimulatory effects of HCA-7-conditioned medium on tubular formation by HUVEC.

Discussion

PGE_2 is a key mediator for COX-2 proneoplastic actions and activates a number of oncogenic signaling pathways, including the β-catenin/T cell factor pathway (20, 35), the epidermal growth factor receptor signaling system (16), the Ras-signaling cascade (36), and the PI3K pathway (12). Accumulative evidence suggests that PGE_2 proneoplastic actions often involve the production of either autocrine or paracrine growth factors. PGE_2 induces the expression of a member of the epidermal growth factor family, amphiregulin, in colon cancer cells, which in turn stimulates cell proliferation and growth via an autocrine fashion (13, 33). Chemokine CXCL1 (growth-regulated oncogene-α) can be induced by PGE_2 in colon cancer cells, which then promotes neoangiogenesis in intestinal neoplasia via a paracrine pathway (37). Amphiregulin, hepatocyte growth factor, and VEGF are induced by PGE_2 in intestinal subepithelial myofibroblasts, which stimulate the growth of intestinal epithelial cells and promote angiogenesis through a paracrine mechanism (38). In the present study, we added the proinflammatory cytokine IL-1α to the list of growth factors/cytokines, which are induced by PGE_2 and mediate PGE_2 proneoplastic actions through autocrine or paracrine mechanisms. IL-1α is commonly cell associated and is not found in the circulation or body fluids, it regulates cell growth and differentiation through intracellular signaling as an autocrine growth factor (25). We found that PGE_2-induced IL-1α was cell associated and not detected in cell culture medium. Previous studies demonstrate that IL-1 increases the expression of COX-2 and the production of PGE_2, which mediates the proinflammatory effects of IL-1 (5). Thus, our data suggest a positive feedback between the COX-2/PGE_2 signaling system and the IL-1α pathway. Because both IL-1α and COX-2 are overexpressed in colon cancers, this positive loop may play critical roles in colorectal carcinogenesis.

Although IL-1 plays crucial roles in inflammatory responses, its proneoplastic actions have been well-documented. IL-1 may stimulate the growth of a number of cell types (39). IL-1 is particularly critical for the metastasis of a variety of tumors (6). In the present study, we show that IL-1α stimulated the migration of colon cancer cells. Interestingly, PGE_2 exerts similar prometastatic effects to colon cancer cells, stimulating cell migration (10, 12). Furthermore, IL-1α enhanced TGF-α-stimulated cell migration in a synergistic manner, mimicking the effect of PGE_2 (34). COX-2/PGE_2 signaling plays critical roles in neoangiogenesis; homozygous deletion of EP_2R significantly reduces the number and size of intestinal polyps in $APC^{\Delta 716}$ mice that is associated with a reduction of VEGF expression, suggesting that PGE_2/EP_2 signaling is critical for increased levels of VEGF in intestinal neoplasm (19). Fukuda et al. (40) have reported that PGE_2 induction of VEGF in HCT-116 colon cancer cells is mediated by the transcriptional activator hypoxia-inducible factor 1. We have demonstrated that PGE_2 induces the transcription of the *VEGF1* in LS-174T cells through activation of the β-catenin/T cell factor pathway (20). Our results from this study suggest that PGE_2 induction of VEGF expression involves a variety of mechanisms. Apparently, activation of the IL-1 pathway critically contributes to the induction of VEGF by PGE_2.

PGE_2 acts via specific transmembrane GPCR (31). EP_1R signals via generation of IP_3 and increased intracellular Ca^{2+}. EP_2 and EP_4 receptors are coupled to stimulatory G (G_s) proteins and signal through increased cAMP, whereas the EP_3R is coupled to inhibitory G (G_i) proteins which inhibit the generation of cAMP. PGE_2 stimulates the transcription of a number of genes through the cAMP/PKA pathway where the cAMP responsive element (CRE) within the promoter plays critical roles (13, 41). The CRE consists of an 8-bp palindrome (TGACGTCA) and is typically found within 100 nt of the TATA box. Although typical CRE/TATA structures are not found in the IL-1α promoter, PGE_2 modestly stimulated IL-1α transcription through activation of the cAMP/PKA pathway. Our results show that the MEK/ERK signaling pathway was essential for IL-1α transcription. Similar findings have been demonstrated in previous studies (42, 43). Additional experiments are required for understanding of the functional role of the MEK/ERK pathway in PGE_2 induction of IL-1α transcription, because PGE_2 has been shown to transactivate the MEK/ERK signaling pathway via different mechanisms (15, 20, 36).

An important finding of this study is that PGE_2 regulated the expression of IL-1α via a posttranscriptional mechanism. Because the steady-state level of mRNA is determined by both the rate of transcription and the rate of degradation, the regulation of mRNA decay is critical for the control of gene expression. Regulation of mRNA stability is achieved through fluctuations in half-lives in response to extracellular stimuli and deregulated mRNA stability can lead to the aberrant accumulation of mRNAs and the proteins they encode (44). Many mRNAs encoding inflammatory gene products are structured for rapid degradation. The 3′ UTR of the IL-1α transcript is AU rich (ARE), and contains at least six copies of the Shaw-Kamens sequence (ATTTA) (28, 45). This motif is present in many immediate early genes and is thought to be involved in regulating the rate of mRNA degradation (46). The IL-1α mRNA was relatively unstable with a half-life of 2 h. There was a significant reduction in the IL-1α mRNA decay rate in PGE_2-stimulated LS-174T cells as compared with vehicle-treated cells. The activation of the EP/cAMP/PKA pathway was required for the stabilization of IL-1α mRNA 3′ UTR, suggesting that this signal transduction pathway either induced factor(s) that stabilized or inhibited factors that destabilize IL-1α mRNA.

In summary, PGE_2 stimulates the production of a number of autocrine and paracrine growth factors and cytokines, which provide a proneoplastic environment for tumor growth and metastasis. IL-1α is a PGE_2-regulated proinflammatory and pro-oncogenic cytokine, which stimulated the migration of colon cancer cells. Moreover, IL-1α increased the expression of VEGF and stimulated neoangiogenesis. Thus, our results suggest that IL-1α is a mediator of PGE_2 proneoplastic effects and a positive loop between IL-1 and PGE_2 signaling may collaboratively promote tumor metastasis and neoangiogenesis in colorectal neoplasia.

Disclosures

The authors have no financial conflict of interest.

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