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Prostaglandin E_2 regulates cellular migration via induction of vascular endothelial growth factor receptor-1 in HCA-7 human colon cancer cells

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ABSTRACT

An important event in the development of tumors is angiogenesis, or the formation of new blood vessels. Angiogenesis is also known to be involved in tumor cell metastasis and is dependent upon the activity of the vascular endothelial growth factor (VEGF) signaling pathway. Studies of mice in which the EP3 prostanoid receptors have been genetically deleted have shown a role for these receptors in cancer growth and angiogenesis. In the present study, human colon cancer HCA-7 cells were used as a model system to understand the potential role of EP3 receptors in tumor cell migration. We now show that stimulation of HCA-7 cells with PGE_2 enhanced the up-regulation of VEGF receptor-1 (VEGFR-1) expression by a mechanism involving EP3 receptor-mediated activation of phosphatidylinositol 3-kinase and the extracellular signal-regulated kinases. Moreover, the PGE_2 stimulated increase in VEGFR-1 expression was accompanied by an increase in the cellular migration of HCA-7 cells. Given the known involvement of VEGFR-1 in cellular migration, our results suggest that EP3 receptors may contribute to tumor cell metastasis by increasing cellular migration through the up-regulation of VEGFR-1 signaling.

1. Introduction

1.1. EP3 receptors and their involvement in cancer

Prostaglandin E_2 (PGE_2) can bind to and stimulate four major G protein-coupled EP receptor subtypes that have been named EP1 to EP4 [1,2]. Among the four subtypes, the EP3 receptors are the least well understood, particularly as it concerns their potential role in tumor development and progression. Knockout studies in mice have suggested possible roles of EP3 receptors in cancer, although there are some puzzling discrepancies. For instance, tumor growth and tumor-associated angiogenesis were reduced significantly in EP3 knockout mice [3], but on the other hand, genetic deletion of the EP3 receptor in $APC^{\Delta 716}$ mice, an animal model for colorectal cancer, had no effect on the formation of tumors [4,5]. This discrepancy could possibly be attributed to differences in the individual expression of isoforms of EP3 receptors. Thus, EP3 receptors are subdivided into eight isoforms in humans [6], which are generated by alternative splicing of mRNA at their carboxyl terminal tails [7].

1.2. Vascular endothelial growth factors and their receptors

Angiogenesis is important in the development and metastasis of tumors, providing nutrients to rapidly growing cancer cells. As a key regulator of angiogenesis, vascular endothelial growth factor (VEGF) plays an important role in endothelial proliferation under physiological conditions, especially in developing cancer [8–10]. VEGF-A is simply referred to as VEGF and is divided into five major isoforms based upon the number of amino acids in their sequence; *i.e.*, 121, 145, 165, 189, and 206. Among them, VEGF-A_{165} is considered as the most abundant and biologically active form [11]. VEGFs exert their angiogenic effects primarily via two receptors known as VEGF receptor (VEGFR)-1 and VEGFR-2 [9,10]. Because the tyrosine kinase activity of VEGFR-1 is approximately 10-fold weaker than that of VEGFR-2, VEGFR-2 has been considered to play a key role in angiogenesis [9]. However, the affinity for VEGF-A of VEGFR-1 is at least one order of magnitude higher than that of VEGFR-2 [12]. Therefore, increased expression of VEGFR-1 may trap VEGF-A due to its high affinity, rendering the factor less available to VEGFR-2 resulting in inhibition of the VEGF-A-mediated angiogenic process [9,10]. Thus, VEGFR-1 was consid-

Abbreviations: PGE_2, prostaglandin E_2; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; DMEM, Dulbecco's modified Eagle's medium; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; ERKs, extracellular signal-regulated kinases; MEK, mitogen-activated protein kinase/ERK kinase; PTX, pertussis toxin; BSA, bovine serum albumin; PI3 kinase, phosphatidylinositol 3-kinase; FGFR-1, fibroblast growth factor receptor-1; EGFR, epidermal growth factor receptor.

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ered to have only a relatively minor or even a negative role in angiogenesis, functioning as a decoy receptor [9]. However, more recent studies have revealed VEGFR-1 to be a critical mediator of physiologic and developmental angiogenesis, cellular migration, and tumor-mediated metastasis [9,10,13].

1.3. EP3 receptors and angiogenesis

As noted above, tumor-associated angiogenesis was reduced significantly in EP3 knockout mice and was accompanied by a reduction in VEGF mRNA expression [3]. Likewise, the topical injection of an EP3 agonist up-regulated VEGF mRNA expression in the granulation tissues surrounding sponge implants in wild-type mice [14]. These results suggest that PGE_2-stimulated VEGF secretion followed by angiogenesis are likely to be mediated through EP3 prostanoid receptors. Using HEK cells stably expressing recombinant human $EP3_I$ receptors as a model, we have recently shown that these $G_{\alpha i}$-coupled receptors can induce the expression of VEGF-A_{165} mRNA as well as VEGFR-1 mRNA [15]. In the present studies, we have now used human colon cancer cells, HCA-7 cells, as a model to examine the potential regulation of the VEGF signaling pathway by the endogenous EP3 receptors expressed natively in these cells. We have found that stimulation with PGE_2 enhanced cellular migration via up-regulation of VEGFR-1 expression following EP3 receptor activation. This finding provides a potential mechanism to explain the involvement of EP3 receptors in tumor metastasis.

2. Materials and methods

2.1. Cell culture and materials

HCA-7 human colon cancer cells were kindly provided by Dr. Mark Nelson (Arizona Cancer Center, The University of Arizona). HCA-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, MO) containing 10% fetal bovine serum (Thermo Scientific, Waltham, MA), 100 IU/ml penicillin (Meiji Seika, Tokyo, Japan), and 100 μg/ml streptomycin (Meiji Seika, Tokyo Japan). Prior to the experiments, the medium was replaced with fresh Opti-MEM (Invitrogen, Carlsbad, CA) containing 100 IU/ml penicillin and 100 μg/ml streptomycin. The amounts of PGE_2 produced by HCA-7 cells in the Opti-MEM were measured using a PGE_2 EIA kit (Cayman, Ann Arbor, MI) and were less than 10 nM within 48 h after the change of medium. All materials were obtained from Wako Pure Chemical (Osaka, Japan) unless otherwise stated.

2.2. RT-PCR

Cells were cultured in 6-well plates and the medium was replaced with fresh Opti-MEM containing 100 IU/ml penicillin and 100 μg/ml streptomycin. RNA was prepared using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription (RT) was carried out using the AMV reverse transcriptase (Promega, Madison, WI) and approximately 0.2 μg of RNA/sample (EP receptors) or 7–10 μg of RNA/sample (VEGF, VEGFR-1) that had been pretreated with DNase I (Promega, Madison, WI). This was followed by a polymerase chain reaction (PCR) with initial incubation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 20 s, 60 °C for 30 s and 72 °C for 60 s as shown previously [15]. The primers for the human EP receptors and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously [16]. Product sizes were 317 base pairs (bp) for EP1, 216 bp for EP2, 300 bp for EP3, 433 bp for EP4 and 737 bp for GAPDH. In the case of VEGFR-1 and VEGF, cells were treated with 1 μM PGE_2 (Cayman, Ann Arbor, MI) for the periods indicated in the figures at 37 °C. In the experiments using the inhibitors, cells were pretreated with vehicle (0.1% Me_2SO or water) or 10 μM U0126 (Promega, Madison, WI), a mitogen-activated protein kinase/extracellular signal-regulated kinases (ERKs) kinase (MEK) inhibitor, for 10 min, or with 100 ng/ml pertussis toxin (PTX, List Biological Laboratories, Campbell, CA) for 16 h at 37 °C, then treated with either vehicle (0.1% Me_2SO) or 1 μM PGE_2 for 2 h (VEGFR-1). PCR was performed following RT, with initial incubation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min for the VEGF primer set and 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min for the VEGFR-1 primer set. The human VEGF forward primer was 5′-CCCTGATGAGATCGAGTACATCTT-3, and backward primer was 5′-AGCAAGGCCCACAGGGATTT-3′ [17]. The human VEGFR-1 forward primer was 5′-AGGAGAGGACCTGAAACTGTCTT-3′ and backward primer is 5′-ATTCCTGGCTCTGCAGGCATAG-3′ [18]. Product sizes were 248 bp for VEGF-A_{165}, 214 bp for VEGFR-1. The products were resolved by electrophoresis on 2.0% agarose gels. Preliminary experiments were performed to find optimal conditions for the quantitative amplification of VEGF, VEGFR-1 and GAPDH mRNAs.

2.3. Western blotting

Cells were cultured in 6-well plates and the medium was replaced with fresh Opti-MEM containing 100 IU/ml penicillin and 100 μg/ml streptomycin prior to the immunoblotting experiments. Cells were then treated with 1 μM PGE_2 for the periods indicated in the figures at 37 °C, or else were treated with 5 μM butaprost (Cayman, Ann Arbor, MI) or 3 μM sulprostone (Cayman, Ann Arbor, MI) for 15 min. In the experiments using the inhibitors, cells were pretreated with either vehicle (water) or 100 ng/ml PTX for 16 h, or with vehicle (0.1% Me_2SO) or 100 nM wortmannin (Sigma, St Louis, MO) for 15 min, or 15 μM AH6809 (Cayman, Ann Arbor, MI) or 5 μM GW627368X (Cayman, Ann Arbor, MI) for 15 min at 37 °C followed by treatment with either vehicle (0.1% Me_2SO) or 1 μM PGE_2 for 15 min. In the case of VEGFR-1, cells were pretreated with either vehicle (water) or 100 ng/ml PTX for 16 h, or with vehicle (0.1% Me_2SO) or 10 μM U0126 (Sigma, St Louis, MO) for 15 min at 37 °C, then treated with either vehicle (0.1% Me_2SO) or 1 μM PGE_2 for 8 h. Cells were then scraped into a lysis buffer consisting of 150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 5 mM EDTA (pH 8.0), 1% Igepal CA-630 (MP Biomedicals, Aurora, OH), 0.5% sodium deoxycholate, 10 mM sodium fluoride, 10 mM disodium pyrophosphate, 0.1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin (Sigma, St Louis, MO), and 10 μg/ml aprotinin and transferred to microcentrifuge tubes. The samples were rotated for 30 min at 4 °C and centrifuged at 16,000 × *g* for 15 min. The supernatants of aliquots containing approximately 50 μg of protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes as described previously [15]. The membranes were incubated for 16 h at 4 °C in 5% bovine serum albumin (BSA, Sigma, St Louis, MO) for the detection of phosho-ERKs or in 5% non-fat milk for the detection of total ERKs. Incubations were done for 1–2 h at room temperature in 5% BSA containing a 1:1000 dilution of anti-phospho-ERK1/2 antibody (# 9106, Cell Signaling Technology, Danvers, MA); or a mixture of a 1:500 dilution of anti-ERK1 antibody and a 1:20,000 dilution of anti-ERK2 antibody (sc-93 and sc-154, Santa Cruz Biotechnology, Santa Cruz, CA) in 5% non-fat milk. In the case of VEGFR-1, the membranes were incubated for 1 h at room temperature in 5% non-fat milk, then for 16 h at 4 °C in 5% BSA containing a 1:1000 dilution of anti-VEGFR-1 antibody (sc-316, Santa Cruz Biotechnology, Santa Cruz, CA). After incubating with primary antibodies, membranes were washed twice and incubated for 1 h at room temperature with a 1:4000 dilution of the appropriate secondary antibodies conjugated with horseradish

peroxidase as described previously [15]. After washing twice, immunoreactivity was detected and visualized with a chemiluminescence imaging system, LAS-1000 (Fuji Film, Tokyo, Japan).

2.4. Human VEGF ELISA

Cells were cultured in 6-well plates and the medium was replaced with fresh Opti-MEM containing 100 IU/ml penicillin and 100 μg/ml streptomycin prior to the immunoblotting experiments. Cells were then treated with 1 μM PGE_2 for the periods indicated in the figures at 37 °C. In the experiments using the inhibitors, cells were pretreated with either vehicle (water) or 100 ng/ml PTX for 16 h, or with vehicle (0.1% Me_2SO), 10 μM U0126, or 10 μM VEGFR tyrosine kinase inhibitor II (Calbiochem/MERCK, Gibbstown, NJ), a potent cell-permeable inhibitor of the kinase activities of VEGFRs, for 15 min at 37 °C, and then treated with either vehicle (0.1% Me_2SO) or 1 μM PGE_2 for 16 h. The quantitative determination of human VEGF concentrations in the cell culture supernatants was assayed by ELISA according to the manufacturer's instructions (Quantikine, R&D Systems, Minneapolis, MN). Briefly, 200 μl of cell culture supernatant was incubated with an antibody raised against the recombinant human VEGF-A_{165}. The optical density was determined using a microplate reader with SUNRISE rainbowfilter (TECAN, Mannedorf, Switzerland) at 450 nm with a correction wavelength of 540 nm.

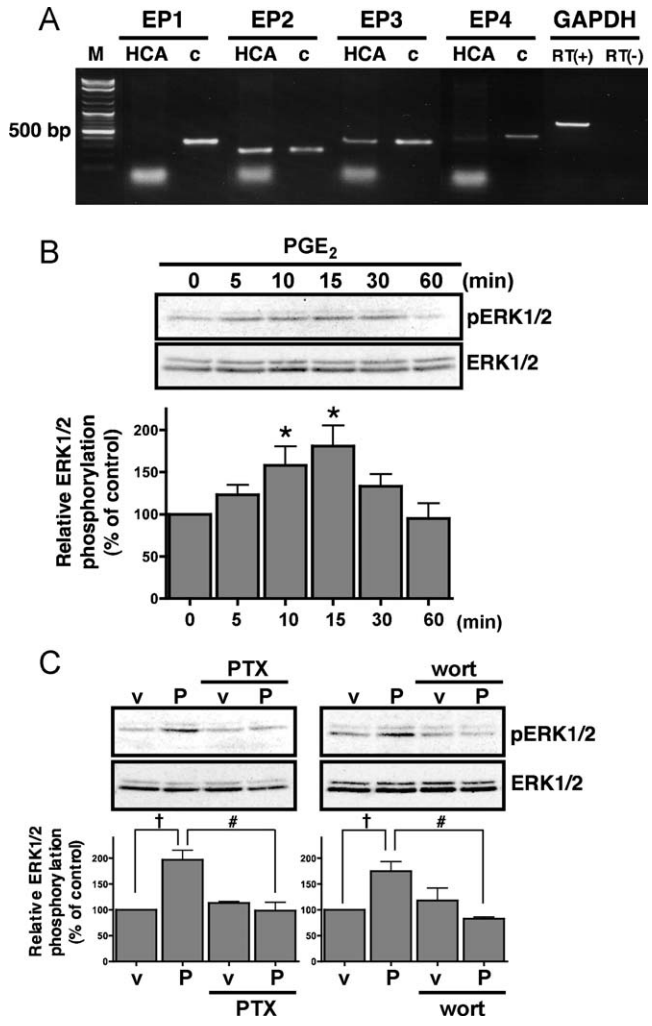

Fig. 1. Determination of mRNA encoding the human EP1, EP2, EP3 and EP4 prostanoid receptor subtypes (A), and immunoblots of time course (B), and effects of pertussis toxin (PTX) and wortmannin (wort) (C) on PGE_2-stimulated phosphorylation of ERKs in HCA-7 cells. (A) Total RNA from HCA-7 cells (HCA) and positive control RNA (c) from HEK cells stably expressing the human EP1, EP2, EP3 and EP4 receptors was isolated and subjected to RT-PCR as described in Section 2.2 using primer pairs that were specific for each of the human EP receptor subtypes. Primers for GAPDH were used in reactions in which reverse transcriptase (RT) was either present (+) or absent (−). Shown is a representative photograph from one of three independent experiments of the PCR products obtained following agarose gel electrophoresis and staining with ethidium bromide. Molecular size markers (M) are in the first lane. (B and C) Cells were incubated with 1 μM PGE_2 for the periods indicated at 37 °C (B), or cells were pretreated with either vehicle or 100 ng/ml PTX for 16 h or 100 nM wortmannin for 15 min, then treated with either vehicle (v) or 1 μM PGE_2 (P) for 15 min at 37 °C (C) and were subjected to immunoblot analysis. (Upper panels) Immunoblotting with antibodies against phospho-ERKs 1 and 2 (pERK1/2). (Lower panels) The blots shown in upper panels were stripped and re-probed with antibodies against ERKs 1 and 2 (ERK1/2). The histograms represent the ratio of pERK1/2 to total ERK1/2 as assessed with pooled densitometric data (mean ± S.D.) from three independent experiments. Data were normalized to the ratio of pERK1/2 to total ERK1/2 at 0 time point (B) or to the ratio of pERK1/2 to total ERKs of vehicle-treated control (C). $^{*}p < 0.05$, analysis of variance as compared with 0 time point. $^{\dagger}p < 0.05$, *t*-test, as compared with vehicle-treated control. $^{\#}p < 0.05$, *t*-test, as compared with PGE_2-treated HCA-7 cells.

2.5. Wound-healing assay

Cells were grown in 6-well plates to confluence and formed a monolayer covering the surface of the entire plate. Wounds were created with a pipette tip as similar to previous report [19], and washed extensively with serum-free Opti-MEM containing 100 IU/ml penicillin and 100 μg/ml streptomycin to remove cell debris. Cells were then pretreated with either vehicle (0.1% Me_2SO) or 10 μM U0126 for 15 min or with 10 μM VEGFR tyrosine kinase inhibitor II for 15 min at 37 °C before being treated with either vehicle (0.1% Me_2SO) or 1 μM PGE_2 for approximately 24 h. The wounds were assessed after 24 h by comparing the 0 and 24 h micrographs at several marked points along the wounded area in each well. Plates were examined by phase contrast microscopy using a Nikon Diaphot microscope (Nikon, Tokyo, Japan) and images were obtained using a Nikon D70 digital camera (Nikon, Tokyo Japan) and processed using Nikon Capture 4 (Nikon, Tokyo, Japan). The percentage of non-recovered wound area (open wound) was calculated by dividing the non-recovered area after 24 h by the initial wound area at the 0 time point.

2.6. Cell migration assay

Cells (3.5×10^3) were seeded on Costar Transwell (#3422, Corning, New York, NY) 24-well (6.5 mm in diameter) inserts with 8-μm pores in 100 μl of DMEM in their upper chambers and 600 μl of DMEM in the lower chambers. Cells were grown for 4 days and the medium was replaced with fresh Opti-MEM containing 100 IU/ml penicillin and 100 μg/ml streptomycin at 100 μl in upper chambers and 600 μl in lower chambers. Cells were then pretreated with either vehicle (0.1% Me_2SO) or 10 μM U0126 for 15 min or 10 μM VEGFR tyrosine kinase inhibitor II for 15 min at 37 °C and treated with either vehicle (0.1% Me_2SO_4) or 1 μM PGE_2 for approximately 48 h. Cells that had migrated through the filter membrane to the lower chamber were trypsinized and counted under a Nikon eclipse TS100 microscope (Nikon, Tokyo, Japan).

3. Results

3.1. HCA-7 cells express endogenous EP receptors

The expression of mRNA encoding the EP prostanoid receptor subtypes was examined by RT-PCR in total mRNA prepared from HCA-7 human colon cancer cells. Total RNA obtained from HEK cells stably expressing either the human EP1, EP2, EP3 or EP4 prostanoid receptors was used for the positive control as shown previously [20]. Fig. 1A shows that mRNAs encoding the human

EP2 and EP3 receptor subtypes were present in total RNA from HCA-7 cells. Although very faint, a band corresponding to the human EP4 receptor subtype was also present, however, no detectable expression of the human EP1 receptor subtype could be identified in HCA-7 cells. Consequently, based on the whole-cell radioligand binding of [^{3}H]PGE_2 to HCA-7 cells, maximal specific binding was approximately 30 fmol/mg protein. Although the EP2 and EP4 receptor subtypes are known to stimulate cAMP formation by coupling with $G_{\alpha s}$, while the EP3 receptor inhibits cAMP formation by coupling with $G_{\alpha i}$ [1,2]; treatment of cells with 1 μM PGE_2 either alone or following inhibition of $G_{\alpha i}$ with PTX only weakly stimulated cAMP formation suggesting that the regulation of cAMP formation by these receptors in HCA-7 cells is a minor signaling pathway (data not shown).

3.2. PGE_2-stimulated phosphorylation of ERKs in HCA-7 cells

The potential of $G_{\alpha i}$-coupled receptors to activate mitogen-activated protein kinase signaling is generally recognized. In fact, we have previously shown that HEK cells stably expressing $G_{\alpha i}$-coupled human EP3 receptors phosphorylated and activated ERK1/2 on stimulation with 1 μM PGE_2 [15]. We therefore examined the ability of PGE_2 to stimulate the phosphorylation of ERK1/2 in HCA-7 cells. Although a slight constitutive basal phosphorylation was detected, treatment with 1 μM PGE_2 resulted in a time-dependent further phosphorylation of ERK1/2 with a maximal effect at 10–15 min as shown in Fig. 1B. To ensure the equal loading of proteins, the blot shown in the upper panel was stripped and re-probed with non-phosphorylated forms of ERK1/2, and nearly identical amounts of ERK1/2 were present throughout the time course. We then examined if the PGE_2-stimulated phosphorylation of ERK1/2 involves the activation of $G_{\alpha i}$. HCA-7 cells were pretreated either with vehicle or PTX for 16 h and then incubated with either vehicle or PGE_2 for 15 min. Fig. 1C (left panels) shows that the pretreatment with PTX abolished the PGE_2-stimulated ERK1/2 phosphorylation in HCA-7 cells. We have previously shown that the activation of human EP3 receptors by PGE_2 can induce phosphorylation of ERK1/2 through the sequential activation of $G_{\alpha i}$ and the phosphatidylinositol 3-kinase (PI3 kinase) signaling pathway [15]. Thus, we next examined whether PGE_2-mediated ERK1/2 phosphorylation in HCA-7 cells involves signaling through PI3 kinase. As shown in Fig. 1C (right panels), pretreatment of the cells with PI3 kinase inhibitor, wortmannin, blocked the PGE_2-stimulated phosphorylation of ERK1/2. These results indicate that the further phosphorylation and activation of ERK1/2 by PGE_2 in HCA-7 cells is mediated by coupling to $G_{\alpha i}$ and activation of the PI3 kinase signaling pathway.

3.3. PGE_2-stimulated ERK1/2 phosphorylation is likely to be mediated by EP3 receptors

As shown in Fig. 1A, HCA-7 cells expressed mRNAs encoding the human EP2, EP3 and possibly EP4 receptor subtypes. We next used butaprost, an EP2 selective agonist, and sulprostone, an EP3 selective agonist, to address which receptor subtypes were responsible for promoting the PGE_2-stimulated ERK1/2 phosphorylation in HCA-7 cells. Fig. 2A shows that 15 min treatment of the cells with 3 μM sulprostone, increased the phosphorylation of ERKs to similar levels as that obtained with 15 min of treatment with 1 μM PGE_2. Furthermore, similarly as shown in Fig. 1C (left panels), sulprostone-stimulated ERKs phosphorylation was significantly inhibited by pretreatment with PTX. However, stimulation of the cells with 10 μM butaprost had no significant effect on ERK1/2 phosphorylation and was not affected with PTX pretreatment. In addition, the sulprostone-stimulated ERK1/2 phosphorylation was similarly inhibited when the cells were pretreated with

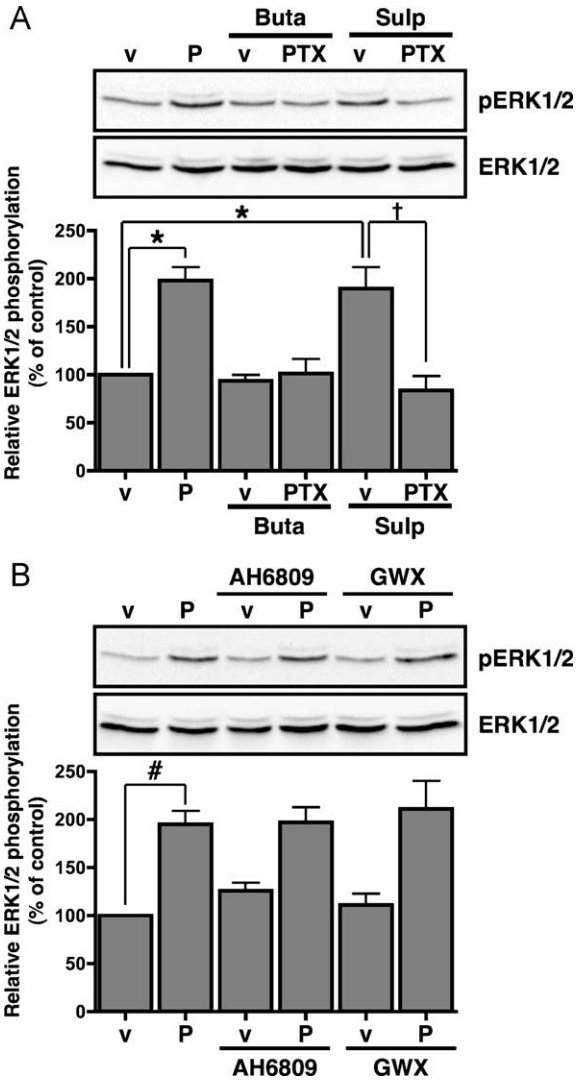

Fig. 2. Effects of butaprost (Buta) or sulprostone (Sulp) on phosphorylation of ERKs (A) and effects of AH6809 and GW627368X (GWX) on PGE_2-stimulated phosphorylation of ERKs (B) in HCA-7 cells. Cells were pretreated with either vehicle or 100 ng/ml PTX for 16 h, then treated with either vehicle (v) or 1 μM PGE_2, 5 μM butaprost or 3 μM sulprostone for 15 min at 37 °C (A), or cells were pretreated with either vehicle or 15 μM AH6809 or 5 μM GW627368X for 15 min, then treated with either vehicle (v) or 1 μM PGE_2 for 15 min at 37 °C (B) and were subjected to immunoblot analysis as described in Section 2.3. (Upper panels) Immunoblotting with antibodies against phospho-ERKs 1 and 2 (pERK1/2). (Lower panels) The blots shown in upper panels were stripped and re-probed with antibodies against ERKs 1 and 2 (ERK1/2). The histograms represent the ratio of pERK1/2 to total ERK1/2 as assessed with pooled densitometric data (mean ± S.D.) from three independent experiments. Data were normalized to the ratio of pERK1/2 to total ERKs of vehicle-treated control. $^*p < 0.05$, *t*-test, as compared with vehicle-treated control. $^\dagger p < 0.05$, *t*-test, as compared with sulprostone-treated HCA-7 cells.

100 nM wortmannin for 15 min (data not shown). These results indicate that the effect of PGE_2 on ERK1/2 phosphorylation in HCA-7 cells is mediated by EP3 receptors rather than EP2 receptors. We have previously reported that in addition to coupling with $G_{\alpha s}$, human EP4 receptors can also couple with $G_{\alpha i}$ and induce ERK1/2 phosphorylation though the activation of PI3 kinase [21]. Thus, we used AH6809 for antagonizing the EP2 receptors, and the EP4 selective antagonist, GW627368X, to examine the relative role of these receptors in the PGE_2-stimulated ERK1/2 phosphorylation in HCA-7 cells. As shown in Fig. 2B, pretreatment of cells for 15 min with either 20 μM AH6809 or 5 μM GW627368X failed to attenuate the PGE_2-stimulated phosphorylation of ERK1/2. These findings further support that the activation ERKs signaling by PGE_2

in HCA-7 cells is mediated by $G_{\alpha i}$-coupled EP3 receptors via activation of the PI3 kinase signaling pathway.

3.4. PGE_2-stimulated induction of VEGFR-1 mRNA and protein in HCA-7 cells

As described above, we have previously shown that HEK cells stably expressing $G_{\alpha i}$-coupled human $EP3_I$ receptor isoform can induce the expression of VEGFR-1 mRNA through a signaling pathway involving the sequential activation of PI3 kinase and ERK1/2. Therefore, we examined whether HCA-7 human colon cancer cells also express mRNA as well as protein expressions of VEGFR-1 in response to PGE_2. For these experiments HCA-7 cells were treated with PGE_2 for various periods ranging from 1 to 12 h and then mRNA was collected and examined by RT-PCR. As shown in Fig. 3A, there was a detectable basal level of VEGFR-1 mRNA present at the 0 time point. However, treatment with PGE_2 induced a time-dependent increase in VEGFR-1 mRNA expression that was maximal at 2 h and which slowly declined to baseline levels by 12 h. The levels of GAPDH mRNA were the same at each time point and were not affected by PGE_2. We next used immunoblot analysis to confirm if increased protein expression of VEGFR-1 followed the increased expression of mRNA. HCA-7 cells were treated with PGE_2 for 1 to 16 h and cell lysates were prepared for immunoblot analysis. Like the mRNA expression, Fig. 3B shows that there was a basal level of VEGFR-1 protein that increased significantly following 6 h of treatment with PGE_2 and remained elevated for 16 h. To ensure equal loading of proteins, the blot shown in the upper panel of Fig. 3B was stripped and re-probed with antibodies to β-tubulin. As shown in the lower panel, nearly identical amounts of β-tubulin were present throughout the time course of treatment. Interestingly, it has been reported that colon cancer cell lines express only VEGFR-1 but not VEGFR-2 or VEGFR-3 [22]. Indeed, we could not detect any VEGFR-2 expression either under basal conditions or when the cells were treated with 1 μM PGE_2 (data not shown).

We next examined the possible role of PGE_2-stimulated activation of ERK1/2 in the induction of VEGFR-1 mRNA and protein expression in HCA-7 cells. Cells were pretreated with either vehicle or 10 μM of the MEK/ERK inhibitor, U0126, for 15 min followed by treatment with either vehicle or 1 μM PGE_2 for either 2 h for RT-PCR experiments, or 8 h for immunoblot analysis. As shown in Fig. 4A, pretreatment with U0126 blocked both the PGE_2-stimulated increase in VEGFR-1 mRNA and protein expressions. To further confirm if the increase in VEGFR-1 mRNA and protein levels by PGE_2 was mediated by activation of $G_{\alpha i}$-coupled EP3 receptors, HCA-7 cells were pretreated with either vehicle or 100 ng/ml PTX for 16 h and then stimulated with PGE_2. As shown in Fig. 4B, pretreatment with PTX resulted in a significant inhibition of the PGE_2-stimulated increase in VEGFR-1 expression at both the mRNA and protein levels. These results further support a mechanism in which PGE_2-induced VEGFR-1 mRNA and protein expression is mediated by the coupling of human EP3 receptors to $G_{\alpha i}$ followed by activation of the ERK1/2 signaling pathway.

3.5. VEGF-A_{165} mRNA and protein expression were not altered by PGE_2 stimulation in HCA-7 cells

In HEK cells stably expressing $EP3_I$ receptors, treatment with PGE_2 resulted in a time-dependent induction of not only VEGFR-1 mRNA but also VEGF-A_{165} mRNA expression [15]. Therefore, the effect of PGE_2 on the expression of VEGF-A_{165} mRNA as well as protein was examined in HCA-7 cells. For these experiments, cells were treated with PGE_2 for the same periods as shown in Fig. 3 and then mRNA as well as protein was collected. As shown, respectively, in Fig. 5A and B the expression of VEGF-A_{165} mRNA and secreted VEGF-A_{165} protein expression in the cell culture supernatant was elevated under basal conditions (time 0) and remained unchanged following treatment with PGE_2. These results indicate that unlike the HEK cell model, in HCA-7 human colon cancer cells, mRNA and protein levels of VEGF are not affected by treatment with PGE_2. As we have previously shown, the expression of VEGF-A_{165} mRNA in HEK cells was regulated by activation of human $EP3_I$ receptors through a signaling pathway involving the ERKs [15]. We, therefore, examined if the constitutive expression of VEGF-A_{165} in HCA-7 cells is mediated through constitutive basal activation of an ERKs signaling pathway, possibly involving a $G_{\alpha i}$ coupled receptor or VEGFR. As shown in Fig. 5C, pretreatment of cells with 10 μM U0126 significantly reduced VEGF protein expression in the cell culture supernatant; however, pretreatment with 100 ng/ml PTX or with 10 μM VEGFR kinase inhibitor did not

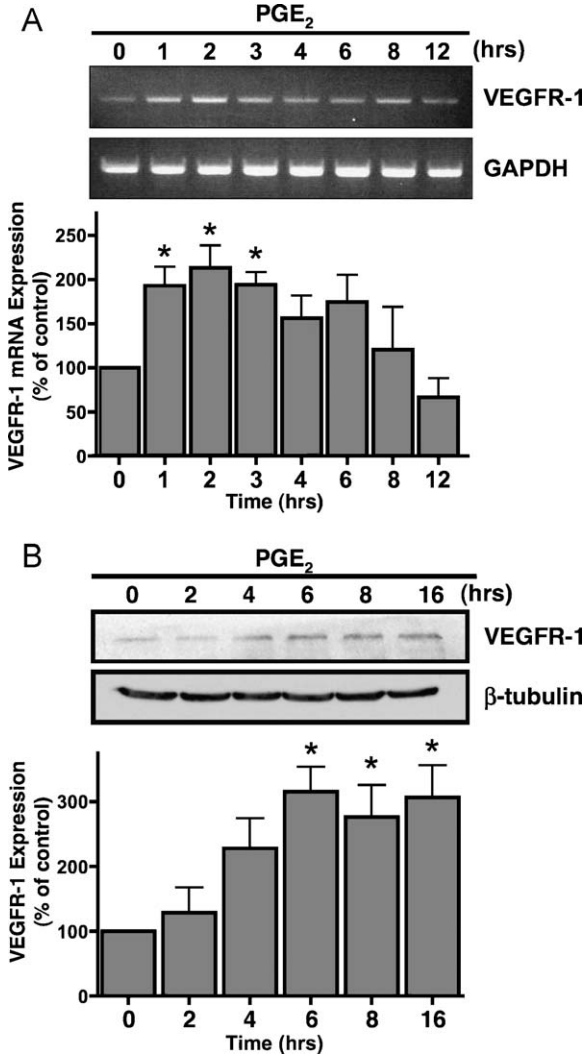

Fig. 3. The time course of PGE_2-stimulated VEGFR-1 mRNA (A) and protein (B) expression in HCA-7 cells. Cells were incubated with 1 μM PGE_2 at 37 °C for the periods indicated and were subjected to RT-PCR (A) or immunoblot analysis (B) as described in Sections 2.2 and 2.3. (A) Photographs from a representative experiment showing the PCR products obtained using primers specific for either human VEGFR-1 (upper panel) or for GAPDH (lower panel). (B) Immunoblotting results from a representative experiment first using antibodies against VEGFR-1 (upper panel) and then after the blot was stripped and re-probed with antibodies against β-tubulin (lower panel). The histograms represent ratios of the products obtained using the upper panels to the lower panels as assessed with pooled densitometric data (mean ± S.D.) from three independent experiments. Data were normalized to the ratio of VEGFR-1 products to GAPDH at 0 time point (A), or ratio of VEGFR-1 to β-tubulin at 0 time point (B). $^*p < 0.05$, analysis of variance as compared with 0 time point.

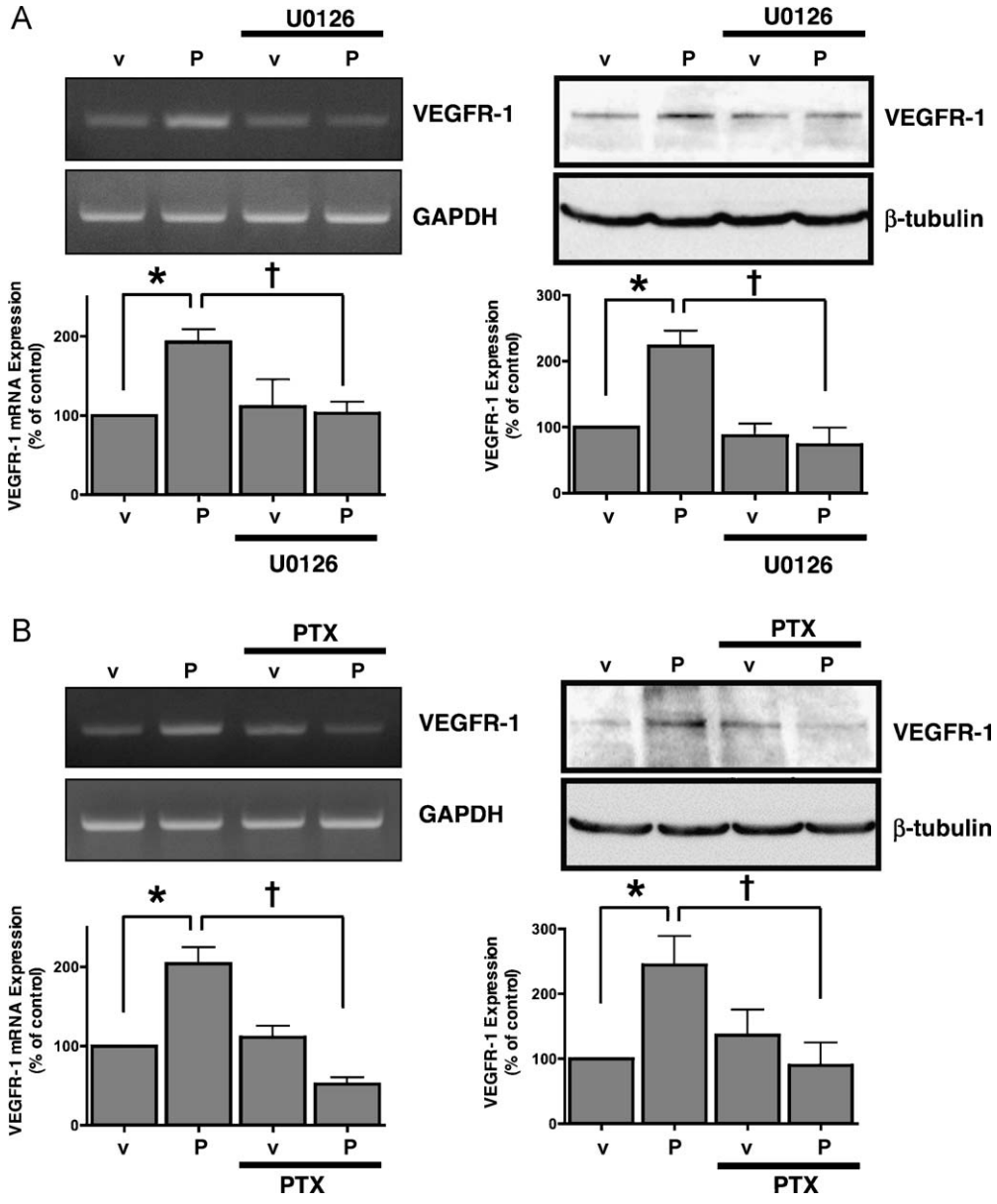

Fig. 4. The effects of the MEK/ERK inhibitor, U0126 (A), and pertussis toxin (PTX) (B) on PGE_2-stimulated VEGFR-1 mRNA (left panels) and protein (right panels) expression in HCA-7 cells. Cells were pretreated with either vehicle (v) or 10 μM U0126 for 15 min (A) or 100 ng/ml PTX for 16 h (B) at 37 °C then treated with either vehicle or 1 μM PGE_2 for 2 h (mRNA) or 8 h (protein) and subjected to RT-PCR (left panels) or immunoblot analysis (right panels) as described in Sections 2.2 and 2.3. (Upper panels) Photograph of PCR products obtained using primers specific for human VEGFR-1 subtype (left panels) or immunoblotting with antibodies against VEGFR-1 (right panels) in representative experiments. (Lower panels) Photograph of PCR products obtained using primers specific for GAPDH (left panels) or the blot shown in the upper panel was stripped and re-probed with antibodies against β-tubulin (right panels). The histograms represent ratios of the products obtained using the upper panels to the lower panels as assessed with pooled densitometric data (mean ± S.D.) from three independent experiments. Data were normalized to the ratio of VEGFR-1 products to GAPDH of vehicle-treated control (A), or ratio of VEGFR-1 to β-tubulin of vehicle-treated control (B). $^{*}p < 0.05$, *t*-test, as compared with vehicle-treated control. $^{\dagger}p < 0.05$, *t*-test, as compared with PGE_2-treated HCA-7 cells.

alter the expression of VEGF. It appears, therefore, that the constitutive expression of VEGF protein in HCA-7 cells involves the basal activation of an ERKs signaling pathway, but does not involve constitutive activation of either VEGFR or a $G_{\alpha i}$ coupled receptor.

3.6. PGE_2-stimulated migration of HCA-7 cells

As described in the introduction, activation of VEGFR-1 signaling is reported to be involved in cellular migration [13]. Thus, the potential for cells to migrate after PGE_2 stimulation was examined using a wound-healing assay. Confluent HCA-7 cells were scratched using a pipette tip to create wounds and wound closure was evaluated following PGE_2 stimulation for approximately 24 h. As shown in Fig. 6A and B, compared with the vehicle-treated control HCA-7 cells, in which the wound closed by approximately 25% after 24 h (Day 1, Fig. 6A (panels a and b)), wound closure was approximately 60% in PGE_2-treated HCA-7 cells (Fig. 6A (panels c and d)). We next examined the possible role of PGE_2-stimulated activation of ERK1/2 and induction of VEGFR-1 in HCA-7 cells. HCA-7 cells were pretreated with either vehicle or 10 μM U0126 for 15 min or 10 μM VEGFR kinase inhibitor for 15 min followed by treatment with either vehicle or 1 μM PGE_2 for 24 h. As shown in Fig. 6A (panels e–h) and (panels i–l), pretreatment with U0126, as well as VEGFR kinase inhibitor, decreased PGE_2-induced migration in HCA-7 cells (Fig. 6A (panels h for U0126, l for VEGFR kinase inhibitor)). In addition, the PGE_2-mediated wound closure was also inhibited by pretreatment with PTX and/or wortmannin (data not shown) and PGE_2 treatment for

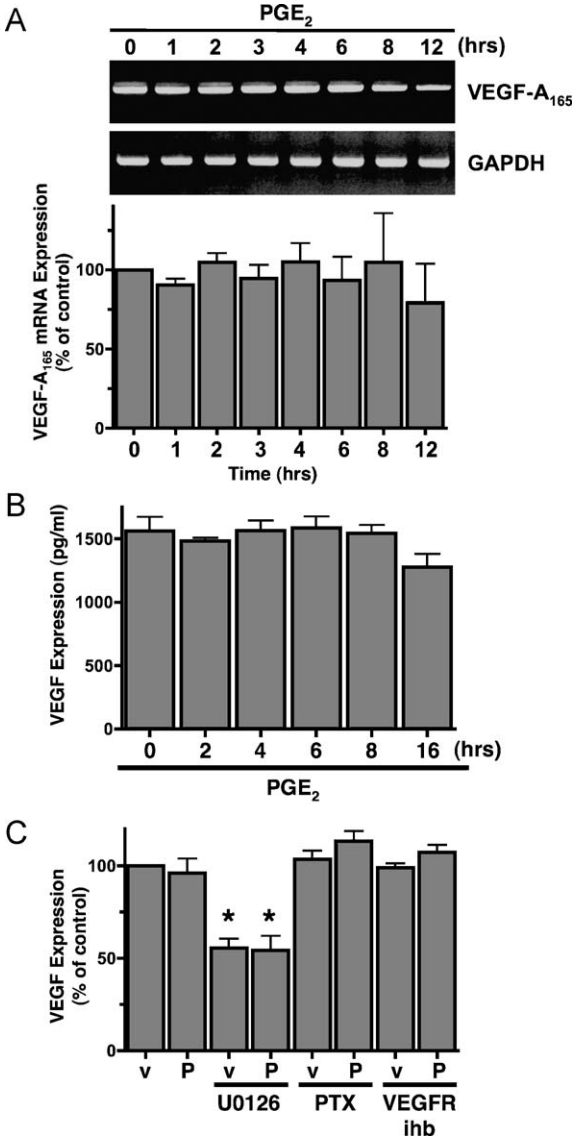

Fig. 5. The time course of PGE_2-stimulated VEGF-A_{165} mRNA (A) and protein (B) expression and the effects of the MEK/ERK inhibitor, U0126, pertussis toxin (PTX) and VEGFR inhibitor on PGE_2-stimulated VEGF protein expression (C) in HCA-7 cells. Cells were incubated with 1 μM PGE_2 at 37 °C for the periods indicated and were subjected to RT-PCR (A) or ELISA (B and C) as described in Sections 2.2 and 2.4. (A, upper panel) Photograph of PCR products obtained using primers specific for human VEGF-A_{165} isoform in representative experiments. (Lower panel) Photograph of PCR products obtained using primers specific for GAPDH. The histograms represent ratios of the products obtained using the upper panels to the lower panels as assessed with pooled densitometric data (mean ± S.D.) from three independent experiments. Data were normalized to the ratio of VEGF-A_{165} products to GAPDH at 0 time point. (B) The PGE_2-stimulated secreted VEGF protein in media were determined by ELISA and normalized to the numbers of cells. (C) Cells were pretreated with either 100 ng/ml PTX for 16 h, 10 μM U0126, or 10 μM VEGFR tyrosine kinase inhibitor II (VEGFR ihb) for 15 min at 37 °C followed by treatment with either vehicle (v) or 1 μM PGE_2 (P) for 16 h. The secreted VEGF protein in media was determined by ELISA. Data were normalized to the vehicle-treated control as 100%. $^*p < 0.05$, analysis of variance as compared with vehicle-treated control.

24 h did not alter the growth rates of HCA-7 cells compared to the vehicle-treated control (data not shown). Additionally, the levels of VEGF protein at 24 h were not altered by the treatment or pretreatment with either PGE_2, VEGFR kinase inhibitor or PTX, whereas U0126 pretreatment, decreased by approximately 20%, VEGF protein expression compared to the vehicle-treated control (data not shown).

We then further examined the ability of HCA-7 cells to migrate by using the Transwell filters. For these experiments, HCA-7 cells were seeded onto Transwell filters and treated with 1 μM PGE_2 for approximately 48 h. As shown in Fig. 6C, there was an ~2.5 fold increase in the migration of cells from the upper to lower chambers following treatment with PGE_2 as compared with vehicle-treated cells. However, when cells were pretreated with either 10 μM U0126 or 10 μM VEGFR kinase inhibitor for 15 min the PGE_2-induced migration was almost completely attenuated to the levels of vehicle-treated control cells. Again, PGE_2 treatment for 48 h did not alter the growth rates of HCA-7 cells as compared with the vehicle-treated control (data not shown). Therefore, PGE_2-induced HCA-7 cellular migration was mediated via the activation of ERK1/2 and VEGFR-1. In addition, the levels of VEGF protein at 48 h were not altered by the treatment or pretreatment with either PGE_2, VEGFR kinase inhibitor or PTX; whereas, pretreatment with U0126 slightly decreased (by ~5%) VEGF protein expression as compared with vehicle-treated control cells (data not shown). In summary the stimulation of HCA-7 cell migration by PGE_2 is consistent with an EP3 receptor-mediated activation of PI3 kinase and ERKs signaling, followed by up-regulation of VEGFR-1 and its subsequent activation by endogenously expressed VEGF-A_{165}.

4. Discussion

4.1. EP3 receptors and the up-regulation of VEGFR-1 expression

Our finding of a PGE_2 mediated up-regulation of VEGFR-1 expression and stimulation of cell migration could help explain some of the discrepancies noted in Section 1.1 concerning the role of EP3 receptors in tumorigenesis. For example, while VEGFR-2 mediates the stimulatory effects of VEGF on angiogenesis, VEGFR-1 actually attenuates the actions of VEGF by binding VEGF without activating angiogenesis; *i.e.*, by acting as a decoy receptor [11]. However, it has been reported that colon cancer cells express VEGFR-1, but not VEGFR-2 [22] and, indeed, we could not detect any expression of VEGFR-2 in HCA-7 cells either under basal conditions or following treatment with PGE_2 (data not shown). In contrast to angiogenesis, VEGFR-1 has been reported to stimulate endothelial cell migration, but not proliferation, in response to VEGF-A [23]. Similarly in the present studies we have found that PGE_2 stimulates the migration of HCA-7 cells (Fig. 6), but not their proliferation, most likely via EP3 receptor activation. Thus EP3 receptor signaling, through the up-regulation of VEGFR-1, could simultaneously inhibit cancer related angiogenesis while stimulating cellular migration and possibly metastasis. However, under normal physiological conditions (*i.e.*, in the absence of cancer) the stimulation of cell migration mediated by the EP3 receptor could be important in normal wound healing.

4.2. EP receptors and the constitutive expression of VEGF-A_{165}

As shown in Fig. 5, VEGF-A_{165} mRNA and protein were constitutively expressed in HCA-7 cells even though the levels of this protein were not affected by treatment with PGE_2. This constitutive expression of VEGF-A_{165} was probably not due to constitutive activity of the EP3 receptor because it was not blocked by pretreatment of the cells with PTX (data not shown). Another possibility is that EP2 and/or EP4 receptors, which have been previously implicated in the regulation of VEGF expression [24,25] and which we have presently shown to be expressed in HCA-7 cells, may be involved in the constitutive expression of VEGF-A_{165}. However, several lines of evidence suggest this is unlikely. First, and most obviously, treatment with PGE_2 did not further increase the expression of VEGF-A_{165}. We have determined that the concentration of endogenous PGE_2 is less than 10 nM (see Section

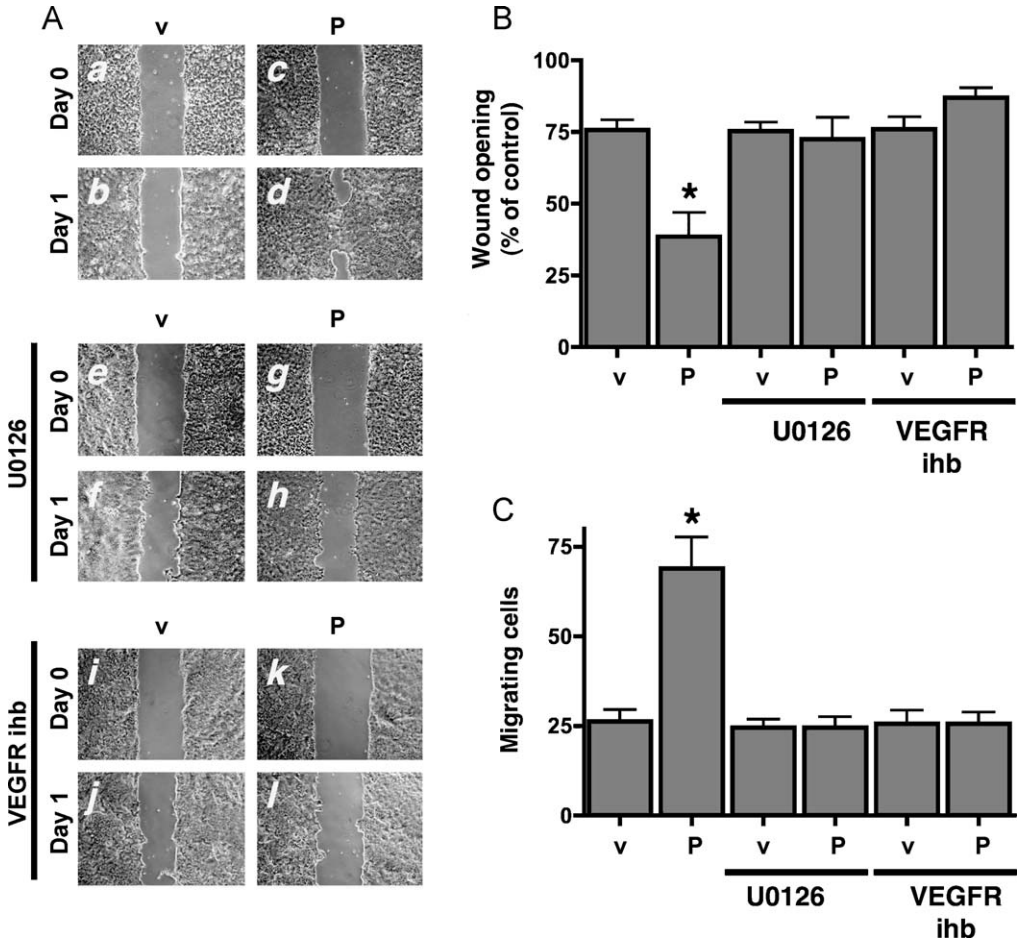

Fig. 6. The effects of the MEK/ERK inhibitor, U0126, and VEGFR tyrosine kinase inhibitor (VEGFR inh) on cell migration in a wound-healing assay (A and B) and on cell migration through Transwell filters (C). (A) Wound healing was assessed as described in Section 2.5. Cells were pretreated with either vehicle (panels a–d) or 10 μM U0126 (panels e–h) or 10 μM VEGFR inh (panels i–l) for 15 min and treated with either vehicle (v; panels a, b, e, f, i and j) or 1 μM PGE_2 (P; panels c, d, g, h, k and l) for 24 h at 37 °C. These results are all from a representative experiment that was repeated three times. (B) Histogram of the percentage of open wound remaining after 24 h (means ± S.D. from three independent experiments). Data were normalized to the vehicle-treated control in HCA-7 cells from day 0. (C) Cell migration through Transwell filters was determined as described in Section 2.6. Cells in upper chambers were pretreated with either vehicle or 10 μM U0126 or 10 μM VEGFR inh for 15 min then treated with either vehicle or 1 μM PGE_2 for 48 h at 37 °C. The histogram represents the cells that migrated to the lower chamber following treatment (mean ± S.D. from three independent experiments). $^*p < 0.05$, analysis of variance as compared with vehicle-treated controls.

2.1), which under most circumstances does not fully stimulate EP receptors. Second, constitutive expression of VEGF-A_{165} was decreased significantly by treatment of the cells with U0126 showing the requirement for activation of MEK/ERKs signaling (Fig. 5C). Nevertheless, stimulation with a selective EP2 receptor agonist did not lead to the phosphorylation of ERK1/2 (Fig. 2A) and treatment of cells with the selective EP4 receptor antagonist did not prevent the phosphorylation of ERK1/2 elicited by PGE_2 (Fig. 2B). Therefore, the constitutive expression of VEGF-A_{165} most likely results from the activity of other growth factors, such as epidermal growth factor, or platelet-derived growth factor, etc., [9] and is at a level that prevents further stimulation by PGE_2.

4.3. Possible involvement of transactivation with the EP3 receptor mediated induction of cell migration

Recently it has been shown that the activation of EP3 receptors by PGE_2 in human microvascular endothelial cells can induce angiogenesis and cell migration [26]. The mechanism of this induction, which was PTX sensitive, involved the transactivation of the fibroblast growth factor receptor-1 (FGFR-1) following proteolytic release of membrane-anchored fibroblast growth factor-2. Activation of ERKs signaling was also observed, but it appeared to be downstream of FGFR-1 activation; whereas, in the present study using HCA-7 cells the activation of ERK1/2 was upstream of the induction of VEGFR-1 expression. Additionally, the time course for FGFR-1 activation and the induction of migration was rapid, with phosphorylation of FGFR-1 occurring within 10 min and cellular migration occurring within 4 h [26]. It is interesting that the endogenous EP3 receptors in these two cell types appear to have co-opted similar, but different, signaling mechanisms to influence cell migration.

PGE_2 mediated transactivation of the epidermal growth factor receptor (EGFR) has also been implicated in the stimulation of cell migration in LS174T human colon cancer cells [27]. Although the specific EP receptor subtype(s) mediating this response were not identified, the stimulation of cell migration by PGE_2 was shown to be PKA and PKC independent. However, more recently PGE_2 has been to transactivate the EGFR in both epidermoid carcinoma cells [28] and mouse skin papillomas [29] via an intracellular signaling mechanism involving a G-protein independent activation of Src by the EP2 receptor. The activation of Src, in turn, leads to direct phosphorylation and activation of EGFR. Although in the present studies we have documented the expression of EP2 receptors in HCA-7 cells, such a mechanism involving EP2 receptors is unlikely to explain the PGE_2 mediated up-regulation of VEGFR-1 based

upon the results of our pharmacological studies. Thus, ERK1/2 phosphorylation was not stimulated by the EP2 selective agonist, butaprost, and PGE_2-induced ERK1/2 phosphorylation was not blocked by the EP2 selective antagonist, AH6809.

4.4. EP3 receptors as possible therapeutic targets for the treatment of cancer

Our finding that the VEGFR kinase inhibitor blocked the PGE_2 stimulated migration of HCA-7 cells suggests that the up-regulation of VEGFR-1 expression by the EP3 receptors may underlie the induction of cell migration. As we have shown, there is basal constitutive expression of both VEGFR-1 and its ligand, VEGF-A_{165}. Thus, a basal level of VEGFR-1 activation would be expected, but presumably not enough to stimulate cellular migration. However, we hypothesize that following PGE_2 stimulation of the EP3 receptor, and up-regulation of VEGFR-1, the additional activation of VEGFR-1 by endogenous VEGF-A_{165} is sufficient to exceed some threshold for the induction of cellular migration. Obviously this remains to be tested. In addition we cannot completely exclude the possibility of transactivation of VEGFR-1 by the EP3 receptor, or perhaps by another EP receptor, although this seems unlikely given the involvement of ERKs signaling in both the VEGFR-1 up-regulation and the cellular migration.

In summary, our findings indicate that in human colon cancer cells, the EP3 receptor can regulate cell migration and possibly metastasis through modulation of the VEGF signaling pathway. Therefore, in principle, a small molecule antagonist of the EP3 receptor would have the potential to decrease the activity of the VEGF pathway and could be used in the same manner as current anti-VEGF agents for the treatment of cancer. The potential advantage of such a small molecule inhibitor of the EP3 receptor is that it represents a fundamentally different therapeutic mechanism since the current anti-VEGF agents are all monoclonal antibodies directed against the VEGFR. Thus such a small molecule inhibitor of the EP3 receptor would have the potential to be used either to complement present therapies, or be used in patients who develop resistance or adverse reactions to currently approved anti-VEGF agents.

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