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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