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Pro-migratory actions of the prostacyclin receptor in human breast cancer cells that over-express cyclooxygenase-2

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ABSTRACT

Metastasis is the major cause of death in cancer patients. Elevated expression of cyclooxygenase-2 (COX-2) is observed in many human cancers and over-production of downstream prostaglandins (PGs) has been shown to stimulate metastasis. A role for increased PGE_2 production has been proposed, but whether other PGs contribute is currently unclear. In this study the pro-migratory actions of individual PGs were evaluated in MDA-MB-468 breast cancer cells that stably over-expressed COX-2 (MDA-COX-2 cells); cell migration was quantified using 3D-matrigel droplet assays. Inhibition of the prostacyclin and PGE synthases, but not alternate prostanoid synthases, prevented the increase in MDA-COX-2 cell migration produced by arachidonic acid (AA); direct treatment of cells with the stable prostacyclin analogue cicaprost also promoted migration. Pharmacological antagonism and knockdown of the IP receptor decreased cell migration, while antagonists of the alternate DP, EP2, FP, and TP prostanoid receptors were inactive. In support of these findings, activation of the IP receptor also enhanced migration in the MDA-MB-468, MDA-MB-231 and A549 cell lines, and IP receptor knock-down in MDA-COX-2 cells decreased the expression of a number of pro-migratory genes. In further studies, the prostacyclin/IP receptor and PGE_2/EP4 receptor pathways were found to be functionally independent and the inhibition of phosphatidylinositol 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK) selectively impaired the IP-receptor-dependent migration in MDA-COX-2 cells. Taken together, the prostacyclin/IP/PI3K-p38 MAPK axis has emerged as a novel pro-migratory pathway in breast cancer cells that over-express COX-2. This information could be utilized in novel treatment strategies to minimize tumor metastasis.

1. Introduction

In metastasis, tumor cells migrate through surrounding tissue, undergo transportation in the lymphatic system or bloodstream and then re-invade and colonize distant sites, to promote the formation of new tumors [1]. The survival, proliferative, and migratory characteristics of metastatic cancer cells undermine the effectiveness of drug therapy, and new treatments for advanced cancers are urgently needed. To develop anti-metastatic treatments it is essential that pro-migratory pathways in tumor cells are clearly defined.

Cyclooxygenase (COX-2) is over-expressed in a wide range of cancers [2,3], and is implicated in tumor metastasis [4,5]. COX-2 converts the ω-6 polyunsaturated fatty acid (PUFA) arachidonic acid (AA) to prostaglandin (PG) G_2/H_2, which is in turn converted to a series of prostanoids, including PGI_2 (prostacyclin), PGE_2, PGD_2, $PGF_{2\alpha}$, and thromboxane A_2 through the action of different synthases [6]. COX-2-specific inhibitors have been found to decrease migratory activity in tumor cells and experimental animals by preventing the formation of pro-migratory prostanoids [4], but the long-term clinical use of COX-2-selective inhibitors is not viable due to toxicity [7].

Several studies have established that PGE_2 is a major pro-migratory prostanoid that is formed in tumor cells [4,8–11]. PGE_2 promotes migration by activating the EP4 receptor [3,8,12–14]. However, whether other AA-derived prostanoids contribute to tumor metastasis is unclear. PGD_2 has been reported to inhibit metastasis in tumor cells [15], while $PGF_{2\alpha}$ promoted

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tumorigenesis in endometrial adenocarcinoma cells [16]. On the other hand, azoxymethane-induced carcinogenesis produced similar numbers of pre-neoplastic colonic lesions in control mice and in mice that lacked the FP prostanoid receptor for $PGF_{2\alpha}$ [17]. Prostacyclin reportedly activates migration in endothelial cells [18], but was anti-migratory in melanoma and certain rodent tumor cells [19,20] and in some non-tumor cell types, including myocytes, where it activates cAMP-dependent pathways [21,22]. Prostacyclin has also been shown to activate angiogenesis, which promotes the development of vascular structures that facilitate tumorigenesis [18,23,24]. Whether prostacyclin also activates metastatic behavior in breast cancer-derived cells has not been evaluated directly.

The present study was undertaken to delineate the pro-migratory actions of multiple prostanoids in the metastatic behavior of breast cancer cells that were engineered to over-express COX-2. The principal finding to emerge was that activation of the prostacyclin/IP receptor pathway promotes the migratory behavior of tumor cells. Downstream activation of phosphatidylinositol 3-kinase (PI3K)/p38 mitogen-activated protein kinase (MAPK) pathways by the IP receptor was required for pro-migratory activity. These findings implicate the prostacyclin/IP receptor/PI3K/p38 MAPK axis as a novel pro-migratory pathway in breast cancer cells that over-express COX-2.

2. Materials and methods

2.1. Materials

AA, PGE_2, PGD_2, $PGF_{2\alpha}$, and the stable prostacyclin analogue cicaprost were purchased from Cayman Chemical (Ann Arbor, MI, USA). Selective inhibitors of COX-1 (FR122047), COX-2 (CAY10404), PGE synthase (CAY10526), PGD synthase (AT-56), and prostacyclin synthase (U-51605), the receptor antagonists AH 6809 (EP2 receptor), L-161,982 (EP4 receptor), CAY10441 (IP receptor), BW A868C (DP1/2 receptors), $PGF_{2\alpha}$-dimethylamine (PGF-DMA; FP receptor) and SQ 29,548 (TP receptor), and the PKA inhibitor KT 5720 were also from Cayman Chemical. The extracellular signal-regulated protein kinase kinase (MEK1/2) inhibitor PD98059, the p38 MAPK inhibitor SB203580 and the PI3K inhibitor (LY294002) were purchased from Life Research (Scoresby, Vic, Australia). The thromboxane synthase inhibitor dazmegrel was generously provided by Prof G Dusting (CERA, University of Melbourne). All modulators of enzymes and receptors were used at concentrations used routinely in the literature.

The murine COX-1 monoclonal antibody (cat no 160140), rabbit polyclonal IgGs directed against IP (10005518), EP2 (101750), EP4 (101775), and TP (10004452) receptors, PGE synthase (160140), thromboxane synthase (160715), and prostacyclin synthase (160640) were from Cayman Chemical. The anti-β-actin IgG and murine COX-2 monoclonal antibodies (sc-47778 and sc-19999, respectively) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the anti-rabbit p38 MAPK and phospho-p38 MAPK antibodies (9212 and 9211, respectively) were from Cell Signaling Technology (Arundel, QLD, Australia). Goat anti-rabbit (LCR 926-32211) and anti-mouse (LCR 926-68020) secondary antibodies were purchased from Millennium Science (Mulgrave, Vic, Australia). Dimethylsulfoxide (DMSO) was from Sigma–Aldrich (Castle Hill, NSW, Australia), Dulbecco's modified eagle medium (DMEM, containing 1 g glucose/L) and fetal bovine serum were purchased from Thermo Scientific (Scoresby, Vic, Australia). Trypan blue, 0.5% trypsin–EDTA and penicillin–streptomycin (5000 U/mL) were purchased from Life Technologies™ (Mulgrave, Vic, Australia), and phosphate buffered saline (PBS) was from Amresco (Solon, OH, USA).

2.2. Cell lines

Human MDA-MB-468 and MDA-MB-231 breast cancer, A549 lung cancer and other cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). MDA-MB-468 cells were stably transfected with COX-2 and green fluorescent protein vectors under the control of the CMV promoter (MDA-COX-2 cells) or green fluorescent protein vector alone (MDA-CTL cells), as previously described [25]. MDA-MB-468 cells were selected because basal COX-2 expression is low, which enables the effects of COX-2 over-expression to be determined clearly. All cell lines were maintained in DMEM medium containing 10% fetal calf serum and 100 U/mL penicillin–streptomycin and grown in an atmosphere of 5% CO_2 at 37 °C.

2.3. 3D Matrigel migration assay

MDA-CTL, MDA-COX-2, MDA-MB-468, MDA-MB-231, and A549 cells were grown to a confluence of 50–60%, trypsinized briefly (trypsin–EDTA, 0.05%) and mixed with matrigel (basement membrane extract 3444-005-02 and 3430-005-01, ~17 mg/mL; Cultrex, Gaithersburg, MD, USA), at 4 °C in a 1:1 ratio, to a final concentration of 1.75×10^6 (MDA-MB-468 derived), 1.00×10^6 (MDA-MB-231) or 1.5×10^6 (A549) cells/mL. Matrigel droplets (20 μL; 3 per well) were applied to six-well plates (Nunc, Roskilde, Denmark), semi-solidified at 37 °C for 5 min and overlaid with 2.5 mL of DMEM supplemented with 10% fetal calf serum and 10% endothelial cell growth medium-2 (EGM™-2, SingleQuots, CC-4176; Lonza, Walkersville, MD). Plates were then incubated for 24 or 40 h and cells that had migrated out of matrigel droplets were visualized and counted using an inverted phase contrast microscope (Olympus CKX41; Olympus Australia, North Ryde, NSW, Australia). Cell number and viability were assessed after each assay using a Countess® Automated Cell Counter (Life Technologies).

2.4. Western blotting

Cells were seeded on six-well plates (2×10^5 cells/well). After 24 h minimal DMEM media was replaced with DMEM that was supplemented with 10% fetal bovine serum, 10% EGM-2 and AA (20 μM). After 40 h whole cell lysates were prepared in Laemmli sample buffer containing 50 mM dithiothreitol, sonicated for 5 s (Branson sonifier 250; Branson Ultrasonics Co., Danbury, CT, USA) and incubated at 95 °C for 5 min. Lysates were electrophoresed on 7.5% sodium dodecyl sulfate-polyacrylamide gels (except in the cases of PGE synthase and COX-2 where electrophoresis was conducted on 12% polyacrylamide gels), essentially as described previously [26].

Proteins were transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany) using a Trans-Blot® Turbo™ transfer system (Bio-Rad Laboratories; Gladesville, NSW, Australia), washed with 5% milk in Tris-buffered saline (TBS; pH 8.8) and then incubated overnight at 4 °C with a primary antibody, as described previously [27]. Antibody dilutions were 1:1000 (PGE synthase, EP4 and IP receptors), 1:500 (prostacyclin and thromboxane synthases, EP2 and TP receptors) or 1:200 (COX-1 and COX-2). Signals were detected and quantified on an Odyssey IP imaging system (LI-COR Biosciences, Lincoln, NE, USA) and expressed relative to immunoreactive β-actin protein.

2.5. RNA extraction and real-time PCR

Cells were seeded on six-well plates (2×10^5 cells/well) and incubated in minimal DMEM medium for 24 h prior to treatment with either DMSO (0.1%) or AA (20 μM) for 40 h. Cells were washed with PBS and total RNA was extracted using the Purelink RNA mini kit (Life Technologies) and quantified spectrophotometrically