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Iloprost, a prostacyclin analog, inhibits the invasion of ovarian cancer cells by downregulating matrix metallopeptidase-2 (MMP-2) through the IP-dependent pathway

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ABSTRACT

Recent studies have shown that a bioactive lipid prostacyclin (PGI_2) plays a role in various cancers, including lung cancer. However, the specific function of PGI_2 in ovarian cancer progression has not been determined. This study investigated the effects of PGI_2 on cell growth, migration, and invasion in ovarian cancer cells using iloprost, a stable PGI_2 analog. Iloprost significantly inhibited migration and invasion, but not cell growth, in a dose-dependent manner in human ovarian cancer cells (A2780 and SKOV3). Interestingly, the cell surface Gs protein-coupled PGI_2 receptor IP was enhanced in human ovarian cancer cells. The inhibitory effect of iloprost on migration and invasion was entirely reversed by an IP antagonist (CAY10449) and IP siRNA, whereas the knockdown of peroxisome proliferator-activated receptor δ (PPARδ), a nuclear receptor of PGI_2, did not rescue the effect of iloprost. Additionally, iloprost markedly decreased the expression of matrix metallopeptidase-2 and -9 (MMP-2 and MMP-9), which may be induced in the process of ovarian cancer metastasis. IP siRNA inhibited iloprost-reduced MMP-2 expression but not MMP-9 expression. Moreover, inhibition of protein kinase A (PKA) and overexpression of Akt and p38 rescued the inhibition of invasion and the reduction of MMP-2 expression by iloprost. Furthermore, iloprost-induced activation of PKA was associated with PKA-mediated Akt and p38 inactivation in ovarian cancer cells. Taken together, these results demonstrate that iloprost inhibits ovarian cancer cell invasion by downregulating MMP-2 expression via the IP-mediated PKA pathway. This study is the first to reveal a novel role for iloprost and to clarify its underlying mechanism in human ovarian cancer cells.

1. Introduction

Ovarian cancer is the leading cause of gynecological cancer-related death in developed countries. Researchers have estimated that approximately 22,280 cases of ovarian cancer are diagnosed per year worldwide, with approximately 14,240 deaths in 2016 [1]. Due to the absence of specific symptoms, 60–70% of women with ovarian cancer are diagnosed at a late stage (stage III and IV) in which cancer cells have spread to the entire body [2]. Additionally, patients presenting at stages III and IV have a 5-yr survival of around 21.9% and 5.6%, respectively. Thus, understanding the pathogenesis of ovarian cancer and the mechanism of metastasis will be helpful in developing efficient treatments for this deadly and highly metastatic disease.

Prostacyclin (prostaglandin I_2; PGI_2), a dienoic eicosanoid derived from the metabolism of arachidonic acid, is endogenously released in vascular endothelial cells [3]. Iloprost, which is a stable analog of PGI_2, is primarily used to treat pulmonary hypertension [4,5] and has known anti-inflammatory properties [6]. In addition to these effects, the antitumoral effect of iloprost has been reported in lung carcinoma [7–11]. However, the efficacy of iloprost as a chemotherapeutic agent in other cancers, including ovarian cancer, has not been evaluated. The biological effects of prostacyclin and iloprost are mediated by binding to two types of receptors: a cell surface Gs protein-coupled receptor IP and a nuclear hormone receptor peroxisome proliferator-activated receptor δ (PPARδ) [12]. It has been suggested that iloprost acts mainly on IP, which activates adenylate cyclase and leads to induction of cyclic AMP (cAMP) [13].

Matrix metallopeptidases (MMPs) are important enzymes in the degradation of the extracellular matrix (ECM) and are associated with tumorigenesis and metastasis [14]. In particular, matrix metallopeptidase-2 (MMP-2; 72 kDa type IV collagenase or gelatinase A) and matrix metallopeptidase-9 (MMP-9; 92 kDa type IV collagenase or gelatinase

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B) play important roles in ovarian cancer [15–18]. MMP-2 and MMP-9 are upregulated in ascitic fluid and in serum or plasma from patients with ovarian cancer, leading to the fast disaggregation of spheroids on adhesion to the surface mesothelial cell layer by cleavage of collagen types I and IV, fibronectin, and vitronectin [19,20]. Interestingly, several studies have demonstrated that iloprost regulates MMPs. However, the regulatory effect of iloprost on MMPs in cancer is not well studied.

In the present study, we investigated the potential effects of iloprost on ovarian cancer cell growth, migration, and invasion and explored the mechanisms underlying these processes. This study is the first to provide evidence for the clinical implications of iloprost in the management of the progression of ovarian cancer.

2. Materials and methods

2.1. Materials

Iloprost, prostacyclin analog, and a selective prostacyclin receptor (IP) antagonist (CAY10449) were purchased from Cayman Chemical Company (Cayman Chemical, Ann Arbor, MI, USA). N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89; protein kinase A (PKA) inhibitor) was obtained from Sigma–Aldrich Co. (St Louis, MO, USA). Polyethylenimine (PEI) and crystal violet were purchased from BD Biosciences (San Joes, CA, USA). Anti-p-ERK1/2, anti-ERK1/2, anti-Akt, anti-p38, anti-CREB, and anti-ß-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-IP, anti-MMP-2, anti-MMP-9, anti-p-CREB, anti-p-Akt, and anti-p-p38 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The p-ERK1/2 antibody detects ERK1 phosphorylated at Tyr 204 and correspondingly phosphorylated ERK2. The anti-Akt antibody detects Akt1, Akt2, and Akt3 and anti-p-Akt antibody detects Akt1 phosphorylated at Ser473 and Akt2/3 phosphorylated at the corresponding residues. Anti-PPARδ antibody was purchased from Abcam (Cambridge, MA, USA). 24-well transwell plate and BD BioCoat™ Matrigel™ were purchased from BD Bioscience.

2.2. Cell culture and iloprost treatment

Human ovarian cancer cells (MPSC1, A2780, and SKOV3 cells) were provided by Dr. IM Shih (Johns Hopkins School of Medicine, Baltimore, MD, USA). IOSE80PC (immortalized ovarian surface epithelial cell line) was kindly provided by Dr. Auersperg (University of British Columbia, Vancouver, BC, Canada) and Dr. A. Godwin (Fox Chase Cancer Center, Philadelphia, PA, USA). Cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin sulfate (100 μg/mL) (Life Technologies, Inc., Grand Island, NY, USA) in a humidified atmosphere of 5% CO_2–95% air at 37 °C. To investigate the regulation of MMP-2 and MMP-9, the cells were plated, cultured in above-mentioned culture conditions, and then treated with iloprost (1 and 10 nM).

2.3. Transfection

The pcDNA3-Akt-myr and pMT3 p38 were obtained from Addgene (Cambridge, MA, USA). Akt-myr is a constitutively active mutant of Akt1. Small interfering RNA against human IP and PPARδ, and control siRNA were from Bioneer technology (Daejon, Korea). After cells were cultured to 50–60% confluence, the cells were transfected with plasmid DNA constructs (2 μg/ml) or siRNAs (50 nM) by polyethylenimine (PEI) in serum-free Opti-modified Eagle's medium (Opti-MEM) (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After 24 h, the medium containing DNA plasmids or siRNA were replaced with fresh RPMI1640 with 5% FBS, and the cells were treated with iloprost.

2.4. Cell growth assay

The cell viability and proliferation was assessed using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. In brief, the cells (8×10^4) were seeded in each well containing 50 μL of RPMI medium in a 96-well plate. After 24 h, various concentrations (0.1–100 nM) of iloprost were treated. After 48 h, 25 μL of MTT (5 mg/mL stock solution) was added, and the plates were incubated for an additional 4 h. The medium was discarded, and the formazan blue, which was formed in the cells, was dissolved by 50 μL of DMSO. The optical density was measured at 540 nm using a microplate spectrophotometer (SpectraMax; Molecular Devices, Sunnyvale, CA, USA).

2.5. Transwell-migration assay

Cell migration assay was carried out by a Boyden chamber with a 8-μm pore size polyvinylpyrrolidone-free polycarbonate (PVPF) filters. The filters were washed thoroughly in phosphate buffer saline (PBS) and dried immediately before use. After transfection with DNA plasmid or siRNA or treatment with antagonist (CAY10449), cells were re-suspened in medium RPMI 1640 containing 1% FBS with or without various concentrations of iloprost (1 and 10 nM) and added to the top chamber for 12 or 24 h. Culture medium containing 5% FBS was then added to the bottom chamber. The cells that migrated to the lower surface of the membrane were fixed with methanol for 10 min and stained with 0.5% crystal violet for 30 min. Non-migratory cells were removed using a cotton swab, whereas migratory cells on the underside of the filter were counted using an inverted microscope. All experiments were done in triplicate, and a minimum of 5 fields per filter was counted.

2.6. Invasion assay

Invasion assay was performed by modified Matrigel Boyden chamber with a 8-μm pore size PVPF filters. Briefly, polycarbonate filters were coated with Matrigel at a concentration of 1 μg/mL and placed in a Boyden chamber. After transfection with DNA plasmid or siRNA or treatment with antagonist (CAY10449), cells were re-suspended in medium RPMI 1640 containing 1% FBS with or without various concentrations of iloprost (1 and 10 nM) and added to the top chamber. Culture medium containing 5% FBS was then added to the bottom chamber. The cells were incubated at 37 °C and allowed to invade through the Matrigel barrier for 18 or 24 h. Following incubation, filters were fixed with methanol for 10 min and stained with 0.5% crystal violet for 30 min. Non-invading cells were removed using a cotton swab, whereas invading cells on the underside of the filter were counted using an inverted microscope. All experiments were done in triplicate, and a minimum of 5 fields per filter was counted.

2.7. Western blot analysis

Cells were washed with ice-cold PBS and extracted in protein lysis buffer (Intron Biotechnology, Seoul, Korea). Protein concentrations were determined by the Bradford assay. The cell lysates were mixed with 5× SDS sample buffer, boiled for 4 min, and then separated on 10–12% SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% non-fat dry milk for 30 min, washed, and incubated overnight at 4 °C with specific primary antibodies in Tris-buffered saline containing 5% non-fat dry milk and 0.1% Tween-20 (TBS-T). The membranes were washed three times to remove the primary antibodies and incubated for 2 h with a horseradish peroxidase-conjugated secondary antibody (1:1000–2500). After washing three times with TBS-T, immunopositive bands were visualized by enhanced chemiluminescence (ECL; Abclon, Seoul, Korea) and analyzed using ImageQuant Las-4000 (GE Healthcare Life Science, Milwaukee, WI, USA).