Molecular and Cellular Endocrinology 331 (2011) 129–135

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Chemokine (C-C) motif ligand 20 is regulated by $PGF_{2\alpha}$-F-prostanoid receptor signalling in endometrial adenocarcinoma and promotes cell proliferation

Alison E. Wallace[a], Rob D. Catalano[a], Richard A. Anderson[a,b], Henry N. Jabbour[a,*]

[a] MRC Human Reproductive Sciences Unit The Queen's Medical Research Institute, Edinburgh EH16 4TJ, UK
[b] Department of Reproductive and Developmental Sciences, The Queen's Medical Research Institute, Edinburgh EH16 4TJ, UK

ARTICLE INFO

Article history:
Received 15 July 2010
Received in revised form 27 August 2010
Accepted 27 August 2010

Key words:
Endometrial cancer
$PGF_{2\alpha}$
F-prostanoid receptor
Chemokine
CCL20
CCR6

ABSTRACT

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is an inflammatory mediator which signals through a G-protein coupled receptor, the F-prostanoid (FP) receptor. We have previously shown elevated FP receptor expression in endometrial adenocarcinoma, a common gynaecological malignancy in Western countries. In this study, the expression of the chemokine CC motif Ligand 20 (CCL20) was determined to be regulated by $PGF_{2\alpha}$-FP receptor signalling in endometrial adenocarcinoma explants and cell line, and expression of CCL20 and its receptor CCR6 was elevated in endometrial adenocarcinoma compared to non-malignant endometrium. Both CCL20 and CCR6 were localised to neoplastic endometrial epithelial cells. The induction of CCL20 expression by $PGF_{2\alpha}$-FP signalling in an endometrial adenocarcinoma cell line stably expressing the FP receptor (FPS cells) was found to be dependent on the intracellular signalling of Gq, EGFR, ERK, calcineurin and nuclear factor of activated T-cells (NFAT) proteins. The treatment of FPS cells with recombinant CCL20 caused a significant increase in proliferation. Therefore these data demonstrate a role for the FP receptor in regulation of the chemokine CCL20, which can mediate proliferation of endometrial adenocarcinoma epithelial cells.

1. Introduction

Endometrial adenocarcinoma is the most common gynaecological malignancy in Western, developed countries affecting mainly post-menopausal women with a frequency of 15–20 per 100,000 women per year (Doll et al., 2008). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is an inflammatory mediator which signals through a G-protein coupled receptor, the F-prostanoid (FP) receptor. The FP receptor has previously been demonstrated in our laboratory to be elevated in endometrial adenocarcinoma (Sales et al., 2004b), and $PGF_{2\alpha}$ signalling through its receptor has been shown to regulate expression of a number of angiogenic and inflammatory mediators (Sales et al., 2005; Sales et al., 2009; Wallace et al., 2009).

The chemokine (C-C motif) ligand 20 (CCL20; also known as macrophage inflammatory protein-3α) binds to the CC-receptor 6 (CCR6), a GPCR that is expressed on dendritic cells and the epithelial cells of several tissue types including spleen, lymph nodes, appendix and pancreas. Coupling of chemokines to receptors is generally promiscuous between members of the same family, however CCL20 is the only chemokine known to interact with the CCR6 receptor (Schutyser et al., 2003). CCL20 signalling plays an important role in the chemoattraction of immature dendritic cells to sites of inflammation, however novel actions of chemokines on other cell types including epithelial and endothelial cells have recently been discovered, including promotion of angiogenesis, invasion and proliferation (Slettenaar and Wilson, 2006).

Like many other chemokines, elevated expression of CCL20 and CCR6 has been described in several different types of cancer. CCL20 is over-expressed in cancers of the colon (Brand et al., 2006) liver (Rubie et al., 2006), pancreas (Campbell et al., 2005), prostate (Ghadjar et al., 2008) and Hodgkins lymphoma (Baumforth et al., 2008). CCR6 is also upregulated in colon (Brand et al., 2006), liver (Dellacasagrande et al., 2003) and prostate cancer (Ghadjar et al., 2008). Within these cancer types, CCL20-CCR6 signalling has been proposed to influence neoplastic epithelial cell migration or metastasis (Campbell et al., 2005; Dellacasagrande et al., 2003; Rubie et al., 2006) and proliferation (Beider et al., 2009; Brand et al., 2006). Additionally, the influx of immature dendritic cells mediated by CCL20 signalling has been implicated in the promotion of immune tolerance to cancer and therefore tumour growth (Bonnotte et al., 2004; Wang et al., 2008).

The expression of CCL20 has been described in the HHUA endometrial epithelial cell line and more recently primary endometrial epithelial cells (Ghosh et al., 2009), as well as primary endometrial stromal cells (Sun et al., 2002). CCL20 expression in endometrial adenocarcinoma has been identified by a gene array

Abbreviations: FP, F-prostanoid receptor; CCL20, CC motif Ligand 20; CCR6, CC-receptor 6; RCAN1-4, regulator of calcineurin1-4.

* Corresponding author. Tel.: +44 (0) 131 242 6220, fax: +44 (0) 131 242 6197.
E-mail address: H.Jabbour@hrsu.mrc.ac.uk (H.N. Jabbour).

0303-7207/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2010.08.018

comparing normal endometrium with adenocarcinoma samples (Wong et al., 2007); however protein localisation and physiological effects of CCL20 signalling in endometrial cancer have not previously been examined. A link between CCL20 and $PGF_{2\alpha}$ has also not previously been described.

This study describes the expression and localisation of CCL20 and CCR6 in endometrial adenocarcinoma. The roles of $PGF_{2\alpha}$-FP receptor signalling in the regulation of CCL20 and the potential physiological effects of CCL20 expression in endometrial adenocarcinoma were then examined.

2. Materials and methods

2.1. Reagents

Indomethacin, PBS, BSA, AL8810, Tri-reagent and $PGF_{2\alpha}$ were purchased from Sigma Chemical Co. (Dorset, UK). PD98059, AG1478, Cyclosporin A and 4C3MQ were purchased from Calbiochem (Nottingham, UK). CCL20 and CCR6 antibodies were purchased from Santa Cruz Biotechnology (Autogen Bioclear, UK).

2.2. Tissue collection

Endometrial adenocarcinoma tissue and normal endometrial tissue was obtained as detailed in our prior studies (Sales et al., 2004a; Sales et al., 2005). Cancer patients were pre-diagnosed to have adenocarcinoma of the uterus, and diagnosis was confirmed histologically in all cases. Normal endometrial tissue was collected from women undergoing surgery for minor gynaecological procedures with no underlying endometrial pathology. Stage of cycle and grade of cancer was assessed by a pathologist. Ethical approval was obtained from Lothian Research Ethics Committee and written informed consent was obtained from all subjects before tissue collection.

2.3. Cell culture

Wild type (WT) Ishikawa cells and Ishikawa cells engineered to stably express the full length human FP receptor to the levels observed in endometrial adenocarcinomas, referred to as FPS cells, were cultured as described previously (Sales et al., 2005). The optimal concentrations of all chemical inhibitors and antibodies were determined empirically by titration using the manufacturer's guidelines and as described in previous studies (Clipstone et al., 1994; Sales et al., 2008; Takasaki et al., 2004). Cell viability was also determined for inhibitors using the CellTitre 96 AQueous One Solution assay (Promega, Southampton, UK) as described in our previous studies (Sales et al., 2008; Sales et al., 2004b). Cells were treated with vehicle, inhibitor alone, or 100 nM $PGF_{2\alpha}$ alone or in the presence of YM254890 (1 μM), AL8810 (50 μM), PD98059 (50 nM), 43CMQ (1 μM), AG1478 (200 nM) or Cyclosporin A (1 μM) for the time indicated.

2.4. Cell and tissue treatments

To determine the CCL20 expression profile in response to $PGF_{2\alpha}$, WT and FPS cells were serum starved in media containing 3 μg/ml indomethacin for at least 18 h prior to stimulation for 2, 4, 6, 8, 12, 18, 24, 48 and 72 h with fresh serum-free media containing indomethacin and vehicle or 100 nM $PGF_{2\alpha}$ ($n = 5$). Carcinoma tissue was serum starved in media containing 3 μg/ml indomethacin for at least 18 h prior to treatment for 2, 4, 6, 8, and 24 h with fresh serum-free media containing indomethacin and vehicle or 100 nM $PGF_{2\alpha}$ ($n = 8$).

To determine signalling pathways leading to CCL20 expression regulated by $PGF_{2\alpha}$, FPS cells were serum starved in media containing 3 μg/ml indomethacin for at least 18 h and treated for 8 or 24 h respectively with vehicle, inhibitor alone, 100 nM $PGF_{2\alpha}$ alone or 100 nM $PGF_{2\alpha}$ and a panel of chemical inhibitors indicated in Section 2.3 ($n = 7$).

2.5. ELISA

CCL20 protein released into the culture media was assayed by Human CCL20/MIP-3 alpha DuoSet ELISA (R&D Systems, Abingdon, UK). FPS cells were treated as described above, for time indicated in the figure legend. The ELISA was then carried out according to manufacturer's instructions. Optical density of wells was determined by spectrophotometry at 450 nM. Data are presented as mean ± SEM from at least 3 independent experiments.

2.6. Taqman quantitative reverse transcriptase PCR

CCL20 expression in endometrial tissues and FPS cells was measured by quantitative RT-PCR analysis as described previously (Jabbour and Boddy, 2003). RNA samples were then extracted using Tri-reagent following manufacturer's guidelines. RNA samples were reverse transcribed and RT-PCR performed as described previously (Jabbour and Boddy, 2003) using sequence specific primers and probes: CCL20 forward, 5′-TCC TGG CTG CTT TGA TGT CA-3′; reverse, 5′-CCA AGA CAG TCA AAG TTG CT-3′; probe, 5′-TGC TGC TAC TCC ACC TCT GCG GC-3′; CCR6 forward, 5′-TTG GCT ATA CGA AAA CTG TCA CAG A-3′; reverse, 5′-AAG CGT AGA GCA CAG GGT TCA-3′; probe, 5′-TCC TGG CTT TCC TGC ACT GCT GC-3′. Primers and data were analysed and processed using Sequence Detector v1.6.3 (Applied Biosystems). Expression of analysed genes was normalised to RNA loading for each sample using the 18S ribosomal RNA as an internal standard. Results are expressed as fold increase above cells treated with vehicle and inhibitor. Data are presented as mean ± SEM from at least 3 independent experiments.

2.7. Adenoviral and lentiviral constructs

2.7.1. RCAN1-4 adenovirus

An adenoviral construct was used to over-express RCAN-1-4, and was kindly prepared by Dr Pamela Brown (MRC Human Reproductive Sciences Unit, The Queen's Medical Research Institute, Edinburgh EH16 4TJ, UK). cDNA of RCAN-1-4 was prepared as previously described (Maldonado-Perez et al., 2009) and fused to the pDC316 shuttle vector (Microbix Biopharmaceuticals, Toronto, Canada) to create pDC316-RCAN-1-4. The virus was then propagated and purified as previously described (Maldonado-Perez et al., 2009). Efficacy of RCAN1-4 over-expression has been previously described (Maldonado-Perez et al., 2009).

FPS cells were seeded in 6-well plates and incubated for 24 h. Cells were then infected with 5 viruses per cell in complete medium. After 24 h incubation, cells were starved in media containing 3 μg/ml indomethacin for 18 h before treatment with 100 nM $PGF_{2\alpha}$ or vehicle for 8 h ($n = 4$).

2.7.2. RCAN1-4 lentivirus

A short hairpin RNA lentivirus was used to knock down expression of RCAN-1-4 as previously described (Bush et al., 2007), and was the kind gift of Professor Aubrey Thompson (Mayo Clinic, Florida, USA). FPS cells were seeded in 6-well plates and incubated for 24 h. Cells were then infected with 5 viruses per cell in com-

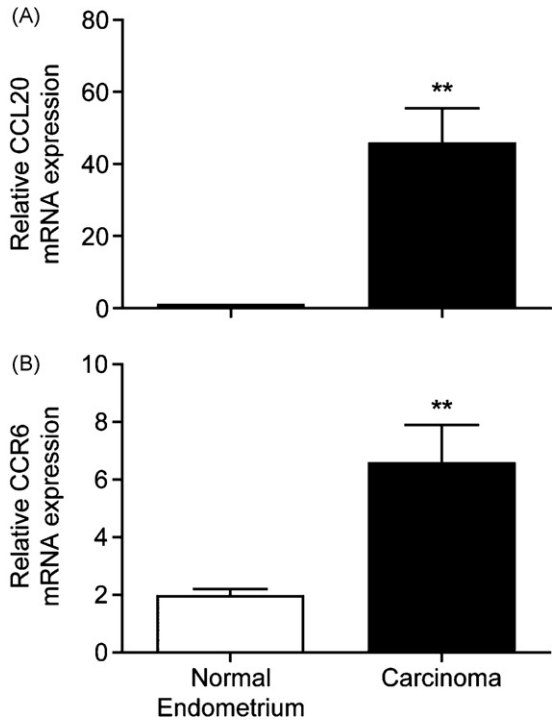

Fig. 1. Expression of CCL20 and CCR6 mRNA in normal endometrium and endometrial adenocarcinoma. (A) CCL20 mRNA was examined by quantitative RT-PCR in pooled normal endometrium tissue (proliferative phase, $n = 9$; early secretory phase, $n = 10$; and mid-late secretory phase, $n = 10$) and pooled endometrial adenocarcinoma tissue (well differentiated, $n = 9$; moderately differentiated, $n = 8$; and poorly differentiated, $n = 10$). CCL20 mRNA was significantly increased in pooled endometrial adenocarcinoma tissue as compared to pooled normal endometrial tissue from all stages of the cycle. (B) CCR6 mRNA was examined by quantitative RT-PCR in normal endometrium (proliferative phase, $n = 10$; early secretory phase, $n = 10$; and mid-late secretory phase, $n = 10$) and endometrial adenocarcinoma (well differentiated $n = 10$; moderately differentiated, $n = 10$; and poorly differentiated, $n = 8$). CCR6 mRNA was significantly increased in pooled endometrial adenocarcinoma tissue as compared to pooled normal endometrial tissue from all stages of the cycle. ** Denotes $p < 0.01$, data are expressed relative to a normal endometrium control and are presented as mean ± SEM.