CCR5 expression is elevated in cervical cancer cells and is up-regulated by seminal plasma

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ABSTRACT: The interplay between inflammation, cervical cancer and HIV acquisition in women is poorly understood. We have previously shown that seminal plasma (SP) can promote cervical tumour cell growth in vitro and in vivo via the activation of potent inflammatory pathways. In this study, we investigated whether SP could regulate expression of chemokine receptors with known roles in HIV infection, in the cervix and in cervical cancer. The expression of CD4 and CCR5 was investigated by RT–PCR analysis and immunohistochemistry. CD4 and CCR5 expression was elevated in cervical cancer tissue compared with normal cervix. Ex vivo studies conducted on cervical tissues and HeLa cells showed that SP significantly increases the expression of CD4 and CCR5 transcripts. Furthermore, it was found that SP also up-regulates CCR5 protein in HeLa cells. The regulation of CCR5 expression was investigated following treatment of HeLa cells with SP in the presence/absence of chemical inhibitors of intracellular signalling, EP2 and EP4 antagonists, prostaglandin (PG) E2 and a cyclooxygenase (COX)-1 doxycycline-inducible expression system. These experiments demonstrated that the regulation of CCR5 expression by SP occurs via the epidermal growth factor receptor (EGFR)-COX-1-PGE2 pathway. This study provides a link between activation of inflammatory pathways and regulation of HIV receptor expression in cervical cancer cells.

Key words: seminal plasma / chemokine receptors / cervical cancer / cyclooxygenase

Introduction

Cervical cancer is the most common gynaecological malignancy in developing countries. The main causal factor for cervical neoplastic transformation and tumorigenesis is infection of the cervical epithelium with high risk variants of the human papillomavirus (HPV) (Arbyn et al., 2011). In 1993, cervical cancer was defined as an AIDS-defining disease in women who are infected with human immunodeficiency virus (HIV) (Levine, 1993), further highlighting HIV as a potent cofactor for developing invasive carcinoma of the cervix (Adefuye and Sales, 2012). The majority of new HIV infections worldwide occur via sexual transmission. In women, this ultimately occurs at the mucosal surface of the genital tract (Prakash et al., 2001).

Little is known of the interplay between HPV-mediated cervical cancer and HIV infection. HIV infects cells via receptors on the host cell surface. Initially, the virus attaches to the surface of the host cells. This can occur via heparin sulphate proteoglycans (Mondor et al., 1998) and can be enhanced by prostatic acid phosphatase-forming amyloid aggregates present in seminal fluid (Kim et al., 2010). The initial step in membrane fusion begins with binding of the viral envelope protein (Env, consisting of a trimer of gp120-gp41 heterodimers) to the CD4 cell surface protein and a chemokine co-receptor present on the host cell (Wilen et al., 2012). Most HIV-1 variants use CCR5 and CXCR4 as the main co-receptor in vivo; however, up to twelve other chemokine co-receptors for HIV infection have been identified in vitro (Doranz et al., 1997; Shimizu et al., 2009).

The epithelial surface of the female reproductive tract expresses all the receptors necessary for HIV infection including CD4, CCR5, CXCR4 (Yeaman et al., 2003, 2004) and various other G protein-coupled co-receptors (GPCRs) known to mediate entry of HIV into cells (including CCR2b, CXCR6 and GPR1). Laboratory studies have shown that cervical epithelial cells can become productively infected and behave as viral reservoirs, to sequester and transfer the virus to activated peripheral blood mononuclear cells in the submucosa (Wu et al., 1997, 2003; Dez-zuti et al., 2001; Maher et al., 2005).

In cervical cancers, expression of chemokine receptors, such as CXCR4, are elevated and are known to play a role in lymph node metastasis during advanced-stage disease (Kodama et al., 2007). These receptors can also be hijacked by HIV for entry. Furthermore, CXCR4 expression can be regulated by HPV oncogenes (Amine et al., 2009).
and prostaglandins (PG) (Sales et al., 2011) in the female genital tract. These observations suggest that HPV infection and inflammation of the cervix can drive expression of HIV co-receptors on cervical epithelial cells.

Cervical inflammation can be mediated by a variety of effectors, including seminal plasma (SP) (Sharkey et al., 2012b) and infection with gram negative sexually transmissible bacteria such as Chlamydia trachomatis and Neisseria gonorrhoea (Anttila et al., 2001; Castle and Giuliano, 2003), which have been shown to increase the risk of HIV transmission (Galvin and Cohen, 2004). These effectors can induce the activation of endogenous PG biosynthesis, by the two cyclooxygenase enzymes, COX-1 and COX-2 (Rizzo, 2011), to mediate inflammatory and tissue remodelling events in the female reproductive tract (Sales et al., 2002b, 2012; Sales and Jabbour, 2003; Jabbour et al., 2009; Sutherland et al., 2012).

In addition to regulation by endogenous PG, the cells lining the vagina and cervix in sexually active women can also be regulated by PG present in seminal fluid (Sales et al., 2002b; Muller et al., 2006; Joseph et al., 2013). It is well documented that PGs are abundant in seminal fluid, and present at concentrations up to 10 000-fold greater than at a site of chronic inflammation; thus PGE2 is the most abundant PG detected in SP (Kelly, 1995; Joseph et al., 2013). In the present study, we investigated the impact of SP on the regulation of receptors involved in HIV infection in the cervix and in HPV 18-positive cervical epithelial cells, as well as the intracellular mechanism underlying its action.

Materials and Methods

Reagents

Phosphate-buffered saline (PBS), bovine serum albumin (BSA), ethylene glycol tetra-acetic acid (EGTA), epidermal growth factor (EGF), prostaglandin (PG) E2, L-161,982 and Tri-reagent were purchased from Sigma Chemical Company (Cape Town, South Africa). AH4809, AG1478, SC560, SN50, NS398 and PD98059 were purchased from Calbiochem (Merck, Darmstadt, Germany). β-Actin (sc-1616), COX-1 (sc-1752), CCR5 (CKR5; sc-6128) and CD4 (sc-19641) antibodies were purchased from Santa Cruz Biotechnology (Whitehead Scientific, Cape Town, South Africa).

Ethics statement

Ethics approval for the study was obtained from the University of Cape Town Research Ethics Committee (REC/REF: 067/2011). Written informed consent was obtained from all subjects before sample collection.

Tissue collection

Cytobrush specimens

Specimens were collected from women (n = 20) attending the Colposcopy Clinic at Groote Schuur Hospital, Cape Town. All women were of reproductive age (median age of 37 years) and reported normal menstrual cycles. Sampling was carried out mid-cycle and any patients who showed signs of bleeding were excluded from the study. Specimens were obtained by cytobrush according to the method described by Musey et al. (1997). Briefly, the cytobrush was inserted within the opening of the cervix inside the uterine cavity (cervical os) and gently rotated one 360 degree turn. The cytobrush was smeared onto a microscope slide for diagnosis and then placed into a 15 ml collection tube containing serum-free DMEM supplemented with 1% penicillin-streptomycin. The tubes were placed on ice and transported to the laboratory. The cells were dispersed from the cytobrushes by gently agitating the tubes to dislodge the cells. Each sample was divided into two aliquots and was treated with vehicle (PBS) or a 1:50 dilution of SP in serum-free DMEM for 24 h. After this, cells were pelleted by centrifugation at 15 000 rpm and RNA was extracted using Tr-riagent (Sigma) following the manufacture’s guidelines and reverse transcription as described previously (Sales et al., 2002a). Data are presented as mean ± SEM.

Cervical cancer and normal biopsies

Cervical cancer tissue specimens were obtained at the time of surgery or biopsy from seven patients (Ca.1 – Ca.7, Table I) who were attending the Gynaecologic Oncology Clinic at Groote Schuur Hospital (Cape Town) and who had previously been diagnosed with pre- or invasive carcinoma of the cervix. The patient age ranged between 29 and 62 years, with a median age of 41 years. Histologically normal cervical tissues were collected from three women (NI – N3) undergoing Wertheim’s hysterectomy for benign gynaecological malignancies at Groote Schuur Hospital (Cape Town). The patient age ranged between 37 and 73 years with a median age of 50.5 years. Sections of tissue were excised from the ectocervix-transformational zone by a specialist pathologist. Tissue sections were placed into a 15 ml collection tube containing serum-free DMEM supplemented with 1% penicillin-streptomycin. The tubes were placed on ice and transported to the laboratory. Each sample was divided equally into aliquots. For explant stimulation experiments, samples were treated with vehicle (PBS) or a 1:50 dilution of SP in serum-free DMEM for 24 h. The site of CD4, CCR5 and leukocyte neutrophil elastase expression was localized in cervical tissues by immunohistochemistry using archival cervical blocks (squamous cell carcinomas n = 5; adenocarcinomas, n = 5 and normal cervix, n = 4) obtained from the Department of Anatomical Pathology, University of Cape Town, South Africa.

Semen donors and preparation

Semen was obtained with informed consent from healthy males attending the Andrology Laboratory of the Reproductive Medicine Unit at Groote Schuur Hospital, Cape Town. Ejaculates were collected in sterile specimen jars following voluntary self-masturbation. Days of sexual abstinence prior to ejaculation were self-reported and sample volume, sperm count, pH and viscosity were noted. All samples were processed within 30 min of collection. The individual ejaculates were transported to the laboratory. For the purpose of our study, seminal plasma (SP; the cell-free supernatant of the ejaculate) was isolated from the ejaculate by centrifugation at 15 000g for 20 min and ejaculates from 10 individual volunteers were pooled. Individual ejaculates were also assayed for their ability to increase CD4 and CCR5 expression (Supplementary data, Fig. S1). The SP was aliquoted and stored at −80°C until required. Results between different pools of ejaculate obtained from different donors were consistent.

Cell culture and treatments

Wild-type HeLa cells, authenticated and verified as cervical adenocarcinoma cells containing HPV 18, were purchased from BioWhittaker (Berkshire, UK). HeLa COX-1 Tet-off cells were constructed as previously described (Sales et al., 2002a). All HeLa Cells were cultured as described previously

<table>
<thead>
<tr>
<th>Sample</th>
<th>Histological type</th>
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<tr>
<td>Ca.2–Ca.6</td>
<td>Squamous carcinoma</td>
<td>0; carcinoma in situ</td>
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<tr>
<td>Ca.7</td>
<td>Squamous carcinoma</td>
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<td>Ca.1</td>
<td>Squamous carcinoma</td>
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RT–PCR and Taqman array analysis

For the Taqman plate array experiments, cells were treated with control (PBS) or 1:50 dilution of SP for 8 h \((n = 5\) independent experiments done in duplicate). RNA was extracted using Trizol reagent (Sigma) following the manufacturer’s guidelines and reverse transcribed as described previously (Sales et al., 2008, 2009, 2010). At the concentrations and time used, the inhibitors showed no adverse effect on cell viability. Fold increase was calculated by dividing the values obtained from the SP/PGE2 only or the SP/PGE2 plus inhibitor treatments by the vehicle only or the vehicle plus inhibitor treatments. For HeLa COX-1 Tet-off experiments, 2 \(\times 10^4\) HeLa COX-1 Tet-off cells were incubated in 3-cm dishes in the absence (to allow for induction of COX-1 transgene expression) or presence (to switch off transgene expression) of 2 \(\mu\)g/ml Doxycycline (DOX; Sigma) in serum-free DMEM for the time indicated before cell lysis and RNA or protein extraction. Cells were supplemented daily with DOX. All in vitro cell culture experiments were carried out in duplicate. For explant stimulation experiments, samples were treated with vehicle (PBS) or 1:50 dilution of SP in serum-free DMEM for 24 h. For low glucose experiments (Supplementary data, Fig. S2), HeLa cells were grown in low glucose DMEM with a final glucose concentration of 5.5 mM, prior to starvation in serum-free low glucose medium and SP stimulation.

| Table II Taqman 96 well plate array. |
|------------------------------|-----------------|-----------------|
| Gene Accession number | Fold change |
| CD4 | NM_000616 | 2.10 |
| CCR5 | NM_000579 | 4.26 |
| CXCR4 | NM_00108540 | – |
| CCR2B | NM_001123041 | 3.10 |
| CCR3 | NM_001164680 | – |
| CCR4 | NM_005508 | – |
| CXCR6 | NM_006564 | 1.23 |
| GPR1 | NM_001098199 | – |
| CCL2 | NM_002982 | 1.63 |
| CXCL2L1 | NM_021006 | 0.43 |
| CCL4 | NM_002984 | – |
| CCL5 | NM_002985 | 1.93 |
| CCL8 | NM_005623 | – |
| CXCL12 | NM_006609 | – |
| TNF | NM_000594 | 9.78 |
| IL-8 | NM_000584 | 14.78 |
| IL-10 | NM_000572 | 0.43 |
| TLR2 | NM_003264 | 32.59 |
| TLR4 | NM_138554 | 0.61 |
| NfκB1 | NM_001165412 | 0.60 |

List of target genes on the Taqman 96 well plate array. The genes include HIV receptors, chemokine co-receptors, inflammatory cytokines, chemokines and cellular co-factors involved in the innate immune response, inflammation and adhesion. Data are presented as fold increase above control. – indicates not detected on the array.

| Table III Primer sequences for real-time PCR. |
|------------------------------|-----------------|
| Gene target | Primer sequence |
| CD4 | CTAAGCTTCAGATGGGCCAAC |
| REV | CACCCACGGTGCTGTCCCTT |
| CCR5 | AGCTATGCAAGTGAAGATC |
| FOR | AGGTCCTCGACTGACGAC |
| CCR5 REV | TCCCCGACCTGACGTC |
| CXCR2b FOR | TGGCTGACTCTAGCTCAAG |
| CXCR2b REV | GGGTCTCAGAGTACGAC |
| CXCR4 FOR | CAGTTGGCCAGCTTCTT |
| CXCR4 REV | CAGTTGGCCAGCTTCTT |
| CXCR6 FOR | GTCTGTCACTGAGTCC |
| CXCR6 REV | CATGAGTGGTGAGGATC |
| GPR1 FOR | TTCTGGATCTATCAGTC |
| GPR1 REV | AGAGCGAAGGGAGAGATG |
| COX-1 FOR | TTGTTGGTGGATACCTC |
| COX-1 REV | ACCTTGAAGGAGTCAGCATG |

Immunohistochemistry

The site of CD4, CCR5 and leukocyte neutrophil elastase expression was localized in cervical tissues by immunohistochemistry using archival cervical blocks (squamous cell carcinomas, \(n = 5\); adenocarcinomas, \(n = 5\) and
normal cervix, n = 4) obtained from the Department of Anatomical Pathology, University of Cape Town. Five-micron paraffin wax-embedded tissue sections were cut and mounted onto coated slides. Sections were dewaxed in xylene, rehydrated in graded ethanol and washed in water followed by TBS (50 mM Tris–HCl, 150 mM NaCl pH 7.4) then blocked for endogenous endoperoxidase (1% H2O2 in methanol). Antigen retrieval was performed by pressure cooking for 2 min in 0.01 M sodium citrate pH 6. Sections were blocked using 5% normal donkey/goat serum diluted in TBS. Subsequently the tissue sections were incubated with polyclonal goat anti-CCR5 (1:200), mouse anti-CD4 (1:200) or rabbit anti-neutrophil elastase (1:400; Abcam 21595) antibodies at 4°C for 18 h. Control tissue was incubated with IgG. After washing with TBS, the tissue sections were incubated with biotinylated donkey anti-goat or goat anti-mouse secondary IgG antibody (Dako) at a dilution of 1:500 at 25°C for 40 min or donkey anti-rabbit Cy3 at a dilution of 1:1000 for neutrophil elastase at 25°C for 60 min. Thereafter the tissue sections incubated with the CCR5 or CD4 antibodies were incubated with streptavidin-biotin peroxidase complex (Dako) at 25°C for 20 min. Colour reaction was developed by incubation with 3,3′-diaminobenzidine (Dako). The tissue sections were counterstained in aqueous haematoxylin, followed by sequential dehydration by graded ethanol and xylene, before mounting and coverslipping. For sections incubated with neutrophil elastase/Cy3, nuclei were counterstained with Dapi (Santa Cruz).

Fluorescent images were visualized and photographed using a Carl Zeiss laser scanning microscope LSM 510 (Jena; Germany). Quantification of CCR5 and CD4 staining was carried out on cervical squamous cell carcinoma (n = 4), adenocarcinoma (n = 4) and normal (n = 4) tissue sections using Image de-convolution (H-DAB) and thresholding analysis on Fiji software (http://fiji.sc/Fiji) as described (Ruifrok and Johnston, 2001; Viel et al., 2013). Briefly, the images were opened in Fiji. The background was subtracted to correct for shadow or colour effects. Images were then de-convolved (H-DAB colour deconvolution). The resulting blue and brown monochromes were converted to grayscale (8-bit thresholded) and % area of staining measured in proportion to the size of the image/tissue. % DAB stained was calculated using the equation below and the data were represented as mean ± SEM.

\[
\% \text{DAB stain} = \frac{\% \text{DAB stain} \times 100}{\% \text{DAB} + \% \text{H stain}}
\]

### Western blot analysis

Cells lysis and immunoblot experiments were conducted as described previously (Sales et al., 2002a). Proteins were revealed and quantified by chemiluminescence and densitometry using a UVP BioSpectrum 500 Imaging System (UVP, Scientific Group, Cape Town, South Africa). For CCR5 quantification, the densitometry of both glycosylated and un-glycosylated forms of the receptor were added together to give a value for total CCR5 expression. Fold induction was determined in SP-treated cells or HeLa COX-1 Tet-off cells incubated without DOX relative to control-treated cells or HeLa COX-1 Tet-off cells incubated with DOX, after normalizing with β-actin, by dividing the expression in the treated group (HeLa COX-1 Tet-off minus DOX) by the expression in the control group (HeLa COX-1 Tet-off plus DOX). Data are presented as mean ± SEM from four independent experiments.

### Statistical analysis

Data were analysed by t-test or one-way ANOVA using Graph Pad Prism 4.0c (Graph Pad, San Diego, CA, USA). Paired T-tests were conducted on the untransformed means of the replicates between treatment (SP/PGE2/EGF) and control groups or inhibitor and inhibitor and treatment group (SP/PGE2) groups. Paired T-tests were conducted on the untransformed means of the replicates between HeLa COX-1 Tet-off cells incubated in the absence versus the presence of DOX for each experiment. Unpaired T-tests were conducted on treatment (SP/PGE2) versus treatment (SP/PGE2) and inhibitor after conversion to fold/percentage increase. One-way ANOVA was used as an additional test to determine significant difference between the various time points for CD4, CCR5, CCR2b, CXCR4, CXCR6 and GPR1 by real-time PCR and/or western blot analysis in response to SP treatment.

### Results

#### CD4 and CCR5 expression is elevated in cervical cancer tissue

Quantitative RT–PCR analysis revealed elevated expression of CD4 (Fig. 1A) and CCR5 (Fig. 1B) in all cervical cancer biopsies (Ca1–Ca7) compared with normal cervical tissues (N1–N3).

Using serial sections, we localized the site of CD4 (Fig. 2A and B) and CCR5 (Fig. 2D and E) expression to the neoplastic epithelial cells in cervical adenocarcinomas (Fig. 2A and D) and squamous cell carcinomas (Fig. 2B and E) with strong immunoreactivity in immune cells adjacent to the epithelium, in the stromal compartment. In contrast to the strong immunoreactive staining for CD4 and CCR5 observed in the cancer tissues, we observed minimal immunoreactivity for CD4 and CCR5 in normal cervix (Fig. 2C and F, respectively) and no immunoreactivity in control sections (Fig. 2I; representative section shown). Quantitative analysis demonstrated a statistically significant increase (P < 0.05) in CD4 and CCR5 protein expression in cervical adenocarcinomas and squamous cell carcinomas compared with normal cervical tissue (Fig. 2, bottom panel), similar to our observations for CD4 and CCR5 mRNA (Fig. 1). Using a specific antibody for leukocyte neutrophil elastase, we confirmed that the immune cells strongly expressing CD4 and CCR5 in the cervical adenocarcinoma (Fig. 2G) and squamous cell carcinomas (Fig. 2H) were infiltrating leukocytes. We did not detect any infiltrating leukocytes in normal cervical tissues by immunohistochemistry and confocal microscopy. The high levels of CD4 and CCR5 expression in the cancer tissues in the same cellular compartment as elevated COX-1 and COX-2 reported in our earlier study (Sales et al., 2002a), suggest that they could be regulated by inflammatory pathways in cervical epithelial cells.

#### Seminal plasma regulates expression of CD4 and CCR5 in the cervix and HPV 18-positive cervical epithelial cells (HeLa)

Since SP has been shown to induce inflammatory and tumourigenic pathways in the vagina (Joseph et al., 2013), cervix and HPV 18-positive cervical cancer cells (HeLa) in vitro and in vivo (Sales et al., 2002b, 2012; Sutherland et al., 2012), we investigated whether SP could regulate expression of CD4 and CCR5 in cervical tissues and HeLa cells. Samples were treated for 24 h with vehicle or a 1:50 dilution of SP and expression of CD4 and CCR5 was determined by real-time PCR. We found that SP significantly elevated expression of CD4 (Fig. 3A, C, E and G; P < 0.05) and CCR5 (Fig. 3B; P < 0.01, 3D; P < 0.01, 3F; P < 0.05 and 3H; P < 0.05) in the cervical specimens obtained from the cervical mucosa by cytobrush sampling (Fig. 3A and B), from cervical biopsy of the ectocervix-transformational zone (Fig. 3C and D), from cervical cancer biopsies (Fig. 3E and F) and in HeLa cells (Fig. 3G and H), compared
with vehicle treated controls. All the experiments described in our study were conducted with pooled SP obtained from 10 healthy individual donors. To assess whether the induction levels of CD4 and CCR5 were donor-dependent, we analysed SP from individual donors. Individual ejaculates were assayed for their ability to increase CD4 and CCR5 expression (Supplementary data, Fig. S1). SP from all ejaculates (without spermatozoa, SP1–SP5; full ejaculate containing spermatozoa, SP6spr and SP7spr; and ejaculates from men post-vasectomy with no detectable sperm present, SP8pv, SP9pv and SP10pv) all increased CD4 and CCR5 expression at 4 and 24 h, albeit with different efficiencies (Supplementary data, Fig. S1). Since the induction levels of CD4 and CCR5 by SP varied between individuals, all subsequent experiments were conducted using pooled SP.

Seminal plasma regulates expression of genes involved in HIV infection and host response in cervical epithelial cells

In addition to CCR5, HIV is known to use alternative chemokine co-receptors to mediate infection of cells (Blaak et al., 2005; Isaacman-Beck et al., 2009; Shimizu et al., 2009). In order to investigate the role of SP in the regulation of HIV receptors and genes with known roles in HIV acquisition and host response in HPV 18-positive cervical epithelial cells, we used a custom Taqman® 96 well gene expression array (Table II). HeLa cells were treated with vehicle or a 1:50 dilution of SP for 8 h and the RNA was subjected to reverse transcription and Taqman array analysis. We considered a fold increase of 2-fold or more as a cut-off point, as determined from our previous microarray experiments to yield a significant difference between treatment and control (Abera et al., 2010). We found that SP induced expression of the HIV receptor CD4, and chemokine co-receptors CCR5 and CCR2b and several genes involved in modulating the inflammatory response and the host adaptive immune response to infection, such as toll-like receptor (TLR)-2, tumour necrosis factor (TNF) and interleukin (IL)-8 (Table II).

In order to validate the results obtained from the Taqman array, HeLa cells were treated with vehicle or a 1:50 dilution of SP for 4, 8, 16 and 24 h and the mRNA expression of CD4 (Fig. 4A), CCR5 (Fig. 4B), CCR2b (Fig. 4C), CXCR4 (Fig. 4D), CXCR6 (Fig. 4E) and GPR1 (Fig. 4F) was determined by quantitative RT–PCR analysis. In keeping with our Taqman array analysis, we found an increase in the expression of CD4, CCR5 and CCR2b mRNA, which was significant at all time points investigated in response to SP treatment compared with control (Fig. 4A, B and C, respectively; P < 0.01). Although we found no increase in CXCR4 mRNA expression at 8 h in our array, SP treatment of HeLa cells significantly elevated CXCR4 expression at 24 h, indicating that a longer exposure of cells to SP is needed to induce CXCR4 (Fig. 4D; P < 0.01). We found CXCR6 expression to be increased significantly in a biphasic manner at 4 h (P < 0.01) and again at 24 h (P < 0.05), with minimal expression observed at 8 and 16 h after SP treatment (Fig. 4E). We found no increase in expression of GPR1 in response to SP treatment, in keeping with our observations for the Taqman array (Fig. 4F). These data indicate that SP could potentially regulate expression of a host of chemokine co-receptors in the cervix, which could mediate infection of cells by HIV strains capable of utilizing CD4-CCR5 or CD4-CXCR4 and alternative CD4-co-receptor combinations, including CD4-CCR2b or CD4-CXCR6.

Seminal plasma regulates expression of CCR5 in HeLa cells via the EGFR and COX-1 pathways

Since CD4 and CCR5 are the main receptors utilized by HIV strains for infection, we focused our analysis on these molecules. Using HeLa cells as a model system to unravel the molecular mechanism mediating the regulation of CCR5 by SP, we further investigated protein expression of CD4 and CCR5 in response to SP treatment by western blot analysis. In contrast to the 4.1 ± 0.5 fold increase in mRNA expression of CD4 in HeLa cells treated with SP (Figs 3G and 4A), we found a marginal 1.4 ± 0.2 fold increase in CD4 protein level in HeLa cells treated with SP (Fig. 5A; P > 0.05). CCR5 protein on the other hand was robustly induced at 8, 16 and 24 h in HeLa cells treated with a 1:50 dilution of SP, compared with control-treated cells (Fig. 5B; P < 0.05), similar to our observations for mRNA.

We next investigated the signal transduction pathways underlying SP-mediated induction of CCR5 expression, using a panel of small
molecule chemical inhibitors of cell signalling. HeLa cells were treated with control, a chemical inhibitor, a 1:50 dilution of SP or SP and an inhibitor for 8 h (Fig. 6A) or 16 h (Fig. 6B). SP treatment of HeLa cells induced a 6.5 ± 0.8 fold increase in CCR5 mRNA (Fig. 6A; P < 0.01) and a 2.8 ± 0.5 fold increase in CCR5 protein (Fig. 6B; P < 0.05) expression respectively compared with control-treated cells. Co-treatment of cells with SP...
Figure 3  Seminal plasma induces expression of CD4 and CCR5 in the cervix. CD4 (A, C, E and G) and CCR5 (B, D, F and H) mRNA expression was determined by real-time RT–PCR analysis of cervical tissues obtained from the cervix with a cervical cytobrush (A and B), from normal cervical biopsy from the ectocervix-transformational zone (C and D), from cervical cancer biopsy (E and F) and the HPV 18-positive cervical epithelial cell line (HeLa; G and H). Cervical cytobrush samples (n = 20), biopsies (n = 5) and HeLa cells (n = 8) were treated with PBS (control) or a 1:50 dilution of seminal plasma for 24 h. mRNA expression, relative to an internal control of HeLa cell cDNA from untreated cells, was determined using the comparative Ct method and all cDNA samples were normalized for RNA loading using GAPDH and β-actin as a loading control for each experiment. Data are presented as mean ± SEM. *, ** indicate significance at P < 0.05 and P < 0.01, respectively.
and inhibitor of epidermal growth factor receptor tyrosine kinase (EGFR; 200 nM AG1478) or COX-1 (10 μM SC560) significantly inhibited SP-mediated increase in CCR5 mRNA expression (Fig. 6A; \( P, 0.001 \)) and protein (Fig. 6B; \( P, 0.001 \)), respectively. However, co-treatment of cells with SP and the NFκB inhibitor (100 μg/ml SN50), extracellular signal-regulated kinase (ERK) inhibitor (50 μM PD98059), COX-2 inhibitor (10 μM NS398) or calcium chelator (1.5 mM EGTA) had no significant effect on SP-induced CCR5 mRNA or protein expression.

**PGE2 regulates expression of CCR5 in HeLa cells**

PGE2 is abundant in SP (Kelly, 1995; Joseph et al., 2013) and has been shown to be the major constituent of SP mediating the activation of inflammatory genes (Joseph et al., 2013). We have previously shown a role for PGE2 and the E-series PG receptors (EP2 and EP4 receptors) in mediating the effects of SP in cervical and endometrial cancer cells via transactivation of the EGFR (Müller et al., 2006; Battersby et al., 2007). Here we sought to determine whether EGF or PGE2 could be the active fraction in SP which is responsible for mediating the elevation of CCR5 in HeLa cells and whether glucose concentrations in the culture medium, which could affect glycosaminoglycan (GAG) synthesis, could impact on CCR5 expression. We treated HeLa cells with 10 ng/ml exogenous EGF (Fig. 7A) or 300 nM PGE2 (Fig. 7B) for 4, 8, 16 and 24 h and subjected the RNA to quantitative real-time PCR analysis. We found that EGF treatment of HeLa cells marginally increased expression of CCR5 only at 16 h of stimulation (Fig. 7A; \( P < 0.01 \)). In contrast, PGE2 treatment rapidly and significantly elevated expression of CCR5 at 4 and...
8 h of stimulation (Fig. 7B; *P* < 0.05), demonstrating that it is a key molecule in SP responsible for the induction of CCR5. We found no significant alteration in CCR5 expression in response to SP in cells grown in low glucose (5.5 mM; Supplementary data, Fig. S2B) medium compared with normal culture medium (20 mM glucose; Fig. 4B). Furthermore, we found that the PGE2-mediated CCR5 increase occurred via the transactivation of the EGFR, since treatment of HeLa cells with the EGFR tyrosine kinase inhibitor AG1478 inhibited the PGE2-induction of CCR5 (*P* < 0.05). PGE2 exerts its effects via E series PG receptors (EP receptors). We have previously highlighted a role for EP2 and EP4 receptors in mediating SP and PGE2 signalling in cervical and endometrial epithelial cells and tissues and confirmed a role for these receptors in transducing SP and PGE2 signalling in HeLa cells (Sales et al., 2002b; Muller et al., 2006; Battersby et al., 2007). Here we show that treatment of HeLa cells with AH6809, a selective EP2 receptor antagonist, or L-161,982, a selective EP4 receptor antagonist (Cherukuri et al., 2007), significantly reduces the SP-mediated CCR5 induction, further confirming a role for the PGE2 component of SP in mediating the CCR5 induction via the EP2 and EP4 receptors (Fig. 7D). These data strongly suggest that the SP-mediated induction of chemokine receptors in the cervix is mediated via the inflammatory PGE2-EP2/EP4 receptor axis.

**Figure 5** Seminal plasma induces expression of CCR5 protein in HeLa cells. (A) CD4 and (B) CCR5 protein expression as determined by western blot analysis. HeLa cells were treated with PBS (control) or a 1:50 dilution of seminal plasma for 8, 16 or 24 h. Immunoblots were revealed and quantified as described in the methods. A representative immunoblot is shown for each with quantification from four individual experiments shown beneath. Data are presented as mean ± SEM. *indicates significance at *P* < 0.05.

**Figure 6** CCR5 mRNA and protein expression is regulated by seminal plasma via the EGFR and COX-1 pathways. (A) CCR5 mRNA and (B) protein expression as determined by real-time RT–PCR and western blot analysis, respectively. HeLa cells were treated for 8 h (A) or 16 h (B) with seminal plasma (1:50) or PBS (control) in the absence/presence of inhibitors of EGFR kinase (AG1478; 200 nM), COX-1 (SC560; 10 μM), NFκB (SN50; 100 μg/ml), calcium (EGTA; 1.5 mM), ERK1/2 kinase (PD98059; 50 μM) or COX-2 (NS398; 10 μM). Data are represented as mean ± SEM from four independent experiments. **,**,**,** indicate significance at *P* < 0.05, *P* < 0.01 and *P* < 0.001, respectively.

8 h of stimulation (Fig. 7B; *P* < 0.05), demonstrating that it is a key molecule in SP responsible for the induction of CCR5. We found no significant alteration in CCR5 expression in response to SP in cells grown in low glucose (5.5 mM; Supplementary data, Fig. S2B) medium compared with normal culture medium (20 mM glucose; Fig. 4B). Furthermore, we found that the PGE2-mediated CCR5 increase occurred via the transactivation of the EGFR, since treatment of HeLa cells with the EGFR tyrosine kinase inhibitor AG1478 inhibited the PGE2-induction of CCR5 (Fig. 7C; *P* < 0.05). PGE2 exerts its effects via E series PG receptors (EP receptors). We have previously highlighted a role for EP2 and EP4 receptors in mediating SP and PGE2 signalling in cervical and endometrial epithelial cells and tissues and confirmed a role for these receptors in transducing SP and PGE2 signalling in HeLa cells (Sales et al., 2002b; Muller et al., 2006; Battersby et al., 2007). Here we show that treatment of HeLa cells with AH6809, a selective EP2 receptor antagonist, or L-161,982, a selective EP4 receptor antagonist (Cherukuri et al., 2007), significantly reduces the SP-mediated CCR5 induction, further confirming a role for the PGE2 component of SP in mediating the CCR5 induction via the EP2 and EP4 receptors (Fig. 7D). These data strongly suggest that the SP-mediated induction of chemokine receptors in the cervix is mediated via the inflammatory PGE2-EP2/EP4 receptor axis.
Expression of CCR5 in HeLa cells can be regulated by an inducible COX-1 Tet-off system

In order to confirm that the COX-1-PGE$_2$ axis can regulate expression of CCR5, we used a doxycycline (DOX)-inducible expression system in HeLa cells (COX-1 Tet-off system) (Sales et al., 2002a). In Hela COX-1 Tet-off cells, the COX-1 transgene is under the control of a DOX-inducible promoter, which contains a tetracycline (DOX) response element (TRE) upstream of the immediate early promoter of cytomegalovirus (as outlined schematically in Fig. 8A). When Hela COX-1 Tet-off cells are maintained in culture medium containing 2 μg/ml DOX, the tetracycline transactivator protein (TetR/VP16) is prevented from binding to the TRE. Under these conditions, induction of the COX-1 transgene is prevented. Removal of DOX from the culture medium results in activation of the COX-1 transgene and biosynthesis of PGE$_2$ (Sales et al., 2002a). We cultured HeLa COX-1 Tet-off cells in serum-free culture medium with or without DOX for 24, 48 or 72 h. Removal of DOX from the culture medium induced a time-dependent increase in COX-1 mRNA and protein levels (Fig. 8B) reaching a maximum after 72 h (3.8 ± 0.7 and 4.5 ± 0.8 fold increase in COX-1 mRNA and protein expression, respectively; Fig. 8B; P < 0.001), consistent with our previous study (Sales et al., 2002a). Coincident with the induction of COX-1 expression in HeLa cells, we observed a 3.8 ± 0.9 and 2.9 ± 0.8 fold increase in expression of CCR5 mRNA and protein, respectively (Fig. 8B; P < 0.001), consistent with our previous study (Sales et al., 2002a). Coincident with the induction of COX-1 expression in HeLa cells, we observed a 3.8 ± 0.9 and 2.9 ± 0.8 fold increase in expression of CCR5 mRNA and protein, respectively (Fig. 8B; P < 0.001), consistent with our previous study (Sales et al., 2002a).

Discussion

Cervical cancer displays all the hallmarks of a chronic inflammatory disease and over the past two decades, the COX-PG pathway has
emerged as a central regulator of inflammatory and tumourigenic events in the female reproductive tract (Jabbour et al., 2009; Rizzo, 2011). In cervical cancers, COX-1 and COX-2 expression is elevated (Ryu et al., 2000; Sales and Katz, 2012), where they have been shown to mediate the induction of inflammatory chemokines, cytokines and angiogenic genes via the autocrine/paracrine regulation of prostaglandin.
E2 and the E-series prostaglandin receptors (EP2 and EP4) (Sales et al., 2002a). Interestingly, in the present study we found high levels of CD4 and CCR5 in cervical cancer tissues localized in the same cellular compartment in which we had previously detected high levels of COX-1 (Sales et al., 2002a) and PGE2 (Sales et al., 2001), raising the possibility that CD4 and CCR5 could be regulated by inflammatory pathways.

Recent studies have shown that in addition to endogenous regulation of inflammatory pathways by the COX-PG axis, SP and SP prostaglandins can directly regulate inflammatory and tumorigenic pathways in cervical (Sales et al., 2002b, 2012; Sutherland et al., 2012) and vaginal cells (Joseph et al., 2013). Inflammation and lesions in the mucosal barrier are considered a significant risk factor for HIV infection (Castle and Giuliano, 2003; Galvin and Cohen, 2004). More recently, SP has been shown to enhance the susceptibility of cells to HIV-1 infection (Kim et al., 2010), however, the role of inflammatory mediators and SP on pathways involved in HIV infection in the cervix have yet to be fully elucidated. We hypothesized that SP and inflammatory pathways could regulate chemokine receptors and pathways with known roles in HIV infection, in cervical epithelial cells and cervical cancer.

We found that in normal cervical tissues obtained by cytobrush sampling of the cervical os or from the ectocervix-transformation zone at biopsy as well as in cervical cancer tissue and with HPV 18-positive cervical cancer cell line (HeLa), treatment with SP significantly elevated the expression of the HIV receptor CD4 and chemokine co-receptor CCR5. We used pools of SP from 10 individuals for the current study. In addition, we showed that although there are individual variations in the potency of CCR5 induction between SP samples, the samples irrespective of whether they were whole ejaculate, centrifuged to remove sperm cells or ejaculate from post-vasectomy patients, with no detectable sperm, all had the ability to induce CCR5 expression (Supplementary data, Fig. S1). This variability in SP effect is consistent with the reported donor specific effects of SP on HIV infection (Kim et al., 2010) and suggests that in addition to viral load and other factors, individual variation in SP potency in inducing CD4 and CCR5 may also affect HIV susceptibility and transmission. Moreover we found by Taqman PCR profiling that, in addition to CCR5, SP could also potentially regulate expression of other chemokine receptors and pathways that have been shown in vitro to mediate HIV infection, such as CCR2b, CXCR4 and CXCR6 (Blak et al., 2005; Isaacman-Beck et al., 2009; Shimizu et al., 2009).

The epithelial compartment of several tissues in the human body, including the gastrointestinal tract, prostate and cervix have all been implicated in the uptake and transport of HIV to submucosal leukocytes (Meng et al., 2002; Wu et al., 2003). Cervical epithelial cells can behave as viral reservoirs, to sequester and transfer virus to activated peripheral blood mononuclear cells in the submucosa (Dezzutti et al., 2001; Gupta et al., 2002; Wu et al., 2003). Furthermore, SP can enhance HIV infection (Kim et al., 2010) and several studies have shown that levels of CCR5 in cells positively correlate with HIV infectivity and levels of cellular activation in vivo (Wu et al., 1997; Ostrowski et al., 1998; Stoddart et al., 2010). It is thus plausible that any mechanism that enhances CCR5 expression, or indeed expression of other HIV co-receptors, could enhance HIV susceptibility. In sexually active women, this could be enhanced by exposure of the cervix to SP.

We explored the SP-mediated regulation of CCR5 in more detail. In accordance with our observation for SP regulation of CCR5 mRNA expression, we found a similar robust increase in CCR5 protein in HeLa cells in response to SP, indicating a good correlation between SP-mediated CCR5 mRNA and protein expression. To determine whether the high (20 mM) glucose concentrations in culture media, which may have profound effects on glycansynexo synthesis and mediated downstream signalling, could affect CCR5 expression, we cultured HeLa cells in low glucose conditions (5.5 mM) prior to stimulation with 1:50 dilution of SP. We found that SP induced CCR5 expression to a similar extent in low glucose compared with the high glucose present in our normal culture conditions (Supplementary data, Fig. S2).

We explored the molecular mechanism whereby SP regulates CCR5 expression using a panel of small molecule chemical inhibitors. We found that SP regulated CCR5 expression via the EGFR and COX-1 pathways. These pathways have been shown to regulate inflammatory and angiogenic genes in cervical and endometrial cancers and are regulated by SP and SP prostaglandins (Muller et al., 2006; Battersby et al., 2007). SP is rich in a variety of cytokines and inflammatory mediators, including epidermal growth factor (EGF), transforming growth factor beta (TGFβ) and PGE2 (Hirata et al., 1987; Kelly, 1995; Robertson, 2005; Politch et al., 2007; Sharkey et al., 2012a). Recently the PGE2 fraction of SP has been shown to be the main fraction responsible for inducing inflammatory gene expression in vaginal cells (Joseph et al., 2013). In our study, we found that although EGF could modestly induce CCR5 expression, PGE2, which is present at very high concentrations in SP (Kelly, 1995; Joseph et al., 2013), had a greater effect on CCR5 expression. We previously showed that PGE2 concentrations in pooled ejaculates were in the order of 43.5 ± 8.7 μg/ml (Muller et al., 2006). In our present study, consistent with previous studies from our laboratory, we have used 300 nM PGE2 for our experiments, which is equivalent to the PGE2 concentration in a 1:50 dilution of SP as used in our previous study (Muller et al., 2006). We thus believe that the difference in induction of CCR5 by SP, compared with PGE2, is due to the 10-fold higher concentration of PGE2 in the SP in our present study. Nonetheless, we have confirmed that the SP-mediated induction of CCR5 occurs by PGE2 via the E-series PG receptor (EP2 and EP4)-mediated induction of the EGFR, since induction of CCR5 could be abolished with the EP2 receptor antagonist, AH6809, or the EP4 receptor antagonist, L-161,982, or the EGFR tyrosine kinase inhibitor, AG1478. It is thus likely that SP and PGE2 induce intracellular second messenger scaffolds to transactivate the EGFR in HeLa cells via ligand-receptor activation of the E-series PG receptors (EP2 and EP4), as reported previously (Muller et al., 2006; Battersby et al., 2007). These data highlight the EP2 and EP4 receptors as potential targets to prevent SP- or PGE2-mediated signalling in cervical adenocarcinoma cells.

We previously showed that SP can induce COX-1 expression (Sutherland et al., 2012). Induction of COX-1 can in turn induce EP2 and EP4 receptor expression and mediate PGE2 autocrine/paracrine signalling (Sales et al., 2002a). To confirm the role of COX-1 in regulating CCR5 expression, we used an inducible COX-1 model system in HeLa COX-1 Tet off cells (Sales et al., 2002a). We show here that induction of COX-1 induces expression of CCR5 in HeLa cells to a similar level observed for SP, indicating that activation of this inflammatory pathway can regulate CCR5 expression directly, and further confirms that COX-1 induction is an intermediate step in the SP-mediated induction of CCR5 in HeLa cells. Furthermore, inhibition of COX-1 with the COX-1 inhibitor, SC560, or maintaining HeLa COX-1 Tet-off cells in DOX, to maintain the transgene in its inactive state, abolished induction of CCR5 expression in HeLa cells.
Inhibition of the inflammatory COX enzyme pathway over the past two decades has elicited significant attention in terms of therapeutic intervention strategy for a host of inflammatory diseases. Our observations for the role of COX-1 in regulating HIV receptor expression suggest that administration of non-steroidal anti-inflammatory drugs (NSAIDs) to suppress COX-enzyme expression, or selective prostaglandin receptor antagonists to inhibit receptor-mediated signal transduction, in sexually active women might also suppress inflammatory pathways that regulate HIV receptor expression and susceptibility to HIV infection. Taken together, our data show for the first time a potential role for SP in regulating HIV chemokine co-receptors in cervical epithelial cells and cervical cancer, via the inflammatory cyclooxygenase—prostaglandin receptor pathway. These findings implicate SP in the regulation of the mucosal epithelium in sexually active women and suggest that it may enhance susceptibility to HIV infection.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

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**Authors’ roles**

K.J.S. conceived and designed the study, played a role in the acquisition, analysis and interpretation of the data and drafted and revised the article. A.A.K. contributed to the study design, interpretation of results and drafting and revising of the article. A.A. and L.N. played a role in the acquisition and analysis and interpretation of data and drafting of the article. All authors approved the final version of the manuscript to be published.

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**Conflict of interest**

None declared.

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