Seminal plasma induces angiogenic chemokine expression in cervical cancer cells and regulates vascular function

Kurt J. Sales a,⁎, 1, Jason R. Sutherland a, 1, Henry N. Jabbour b, Arieh A. Katz a,⁎

a MRC/UCT Research Group for Receptor Biology, Institute of Infectious Disease and Molecular Medicine and Division of Medical Biochemistry, Faculty of Health Sciences, University of Cape Town, 7925, South Africa
b MRC Human Reproductive Sciences Unit, The Queen’s Medical Research Institute, The University of Edinburgh, Edinburgh, EH16 4TJ, UK

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ABSTRACT

Cervical cancer is one of the leading gynecological malignancies in women. We have recently shown that seminal plasma (SP) can regulate the inflammatory cyclooxygenase-prostaglandin pathway and enhance the growth of cervical epithelial tumours in vivo by promoting cellular proliferation and alteration of vascular function. This study investigated the molecular mechanism whereby SP regulates vascular function using an in vitro model system of HeLa cervical adenocarcinoma cells and human umbilical vein endothelial cells (HUVECs). We found that SP rapidly enhanced the expression of the angiogenic chemokines, interleukin (IL)-8 and growth regulated oncogene alpha (GRO) in HeLa cells in a time-dependent manner. We investigated the molecular mechanism of SP-mediated regulation of IL-8 and GRO using a panel of chemical inhibitors of cell signalling. We found that treatment of HeLa cells with SP elevated expression of IL-8 and GRO by transduction of cyclooxygenase enzymes and nuclear factor kappa B. We investigated the impact of IL-8 and GRO, released from HeLa cells after treatment with SP, on vascular function using a co-culture model system of conditioned medium (CM) from HeLa cells, treated with or without SP, and HUVECs. We found that CM from HeLa cells induced the arrangement of endothelial cells into a network of tube-like structures via the CXC2 receptor on HUVECs. Taken together our data outline a molecular mechanism whereby SP can alter vascular function in cervical cancers via the pro-angiogenic chemokines, IL-8 and GRO.

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1. Introduction

Chronic inflammation has been linked to increased cancer risk in numerous organs including the liver and colon [1,2]. Non-steroidal anti-inflammatory drug use and suppression of the inflammatory cyclooxygenase (COX)-prostaglandin (PG) axis have been shown over the last two decades to have beneficial effects in reducing inflammation and have been associated with an inverse risk of developing cancer. These observations have been confirmed by recent clinical trials that have shown that long term aspirin treatment can be beneficial for reducing the burden of colorectal cancer [3].

In sub-Saharan African countries, cancer of the uterine–cervix is the leading gynecological malignancy [4,5]. The main etiological factor associated with cervical cancer is infection of the cervix by human papillomavirus (HPV) [6]. Following infection, HPV oncogenes have been shown to regulate the COX-PG axis in cervical cancer cells to promote inflammation, persistent infection and tumorigenesis [7,8]. We and others have shown that the inflammatory COX-PG axis is elevated in cervical cancers [9]. COX enzymes, of which there are two isoforms in humans, namely COX-1 and COX-2, catalyses the rate limiting conversion of arachidonic acid to PG [9]. Following their biosynthesis, PG are actively transported from the cell, where they act locally in an autocrine/paracrine manner via PG receptors [9]. We have shown that PG, biosynthesised following induction of COX-1 and COX-2 in cervical adenocarcinoma cells, elevates the expression of potent pro-angiogenic factors such as basic fibroblast growth factor 2 (FGF2), vascular endothelial growth factor (VEGF) and angiopoietins [9,10]. Following their biosynthesis and release from neoplastic cervical epithelial cells, angiogenic factors can then exert a paracrine activity on endothelial cells to enhance blood supply via angiogenesis to facilitate tumour growth [9]. These observations have led us to propose that suppression of the inflammatory COX-PG axis with affordable non-steroidal anti-inflammatory drugs like aspirin could be beneficial for preventing cervical cancer progression in resource-poor countries in Africa by suppressing potent inflammatory and angiogenic pathways that can promote disease progression.

⁎ Corresponding authors at: MRC/UCT Research Group for Receptor Biology, Room N2.02 Wernher and Beit North, Institute of Infectious Disease and Molecular Medicine and Division of Medical Biochemistry, UCT Faculty of Health Sciences, Observatory 7925, South Africa.
E-mail addresses: kurt.sales@uct.ac.za (K.J. Sales), arieh.katz@uct.ac.za (A.A. Katz).
1 These authors contributed equally to this work.

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In addition to HPV regulation of the COX-PG axis, we have recently shown that seminal plasma (SP) can elevate the COX-PG axis and enhance the growth of cervical cancer cells in vitro and in nude mouse xenografts in vivo [11]. In our recent study, we found that the SP-induced tumour growth in nude mice was associated with an alteration in the vasculature, with xenograft tumours arising from animals injected with SP yielding significantly larger blood vessels compared with controls [11]. These observations led us to propose that the enhanced tumour growth we observed in xenografts arising from mice injected with SP was due to an increase in the capacity of blood vessels to supply the tumour with nutrients and oxygen [11]. Our observations imply that in sexually active women, repeated exposure of neoplastic epithelial cells to seminal fluid could enhance tumorigenesis by inducing inflammatory and angiogenic pathways in the cervical epithelium.

Several angiogenic factors have been shown to regulate blood vessel branching, sprouting and maturation to facilitate tumour angiogenesis, including VEGF and FGF2 [12,13]. In the present study, we investigated the regulation of two potent pro-angiogenic chemokines, interleukin (IL)-8 (also called CXCL8) and growth regulated oncogene alpha (GRO; also called CXCL1) by SP and their role in regulating endothelial cell function in vitro. Both IL-8 and GRO are related proteins, which belong to the C-X-C motif superfamily of chemokines and function via the same G protein-coupled receptor, CXCR2, present on endothelial cells [14–16]. Here we show that SP induces the expression of IL-8 and GRO in a time-dependent manner via the epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK)–mediated induction of COX enzymes in cervical cancer (HeLa) cells. Using a co-culture model system of conditioned medium from HeLa cells, treated with or without SP and endothelial cells, we found that the IL-8 and GRO can regulate vascular function in vitro via the CXCR2 receptor on endothelial cells.

2. Materials and methods

2.1. Human ethics

Ethics approval for the study was obtained from the University of Cape Town Research Ethics Committee (REC/REF: 152/99). Written informed consent was obtained from all subjects before sample collection.

2.2. Reagents

Culture medium, penicillin–streptomycin and fetal-calf serum (FCS) were purchased from Highveld Biological (PTY) Limited (Cape Town, South Africa). PBS, BSA, Percoll and Tri-reagent® were purchased from Sigma Chemical Company (Cape Town, South Africa). Anti-phospho-p42/44 ERK (9101) and anti-p42/p44 ERK were purchased from Cell Signalling Technologies/New England Biolabs (Hertfordshire, UK). SB225002 was purchased from Calbiochem (Nottingham, UK). PBS, BSA, Percoll and Tri-reagent® were purchased from Sigma Chemical Company (Cape Town, South Africa). PBS, BSA, Percoll and Tri-reagent® were purchased from Sigma Chemical Company (Cape Town, South Africa). Anti-phospho-p42/44 ERK (9101) and anti-p42/p44 ERK were purchased from Cell Signalling Technologies/New England Biolabs (Hertfordshire, UK). SB225002 was purchased from Calbiochem (Nottingham, UK).

2.3. Cell culture

Wild type HeLa-S3 cells, authenticated and verified as cervical adenocarcinoma cells, were purchased from BioWhittaker (Berkshire, UK) and were maintained as described [11]. Conditioned medium (CM) was prepared by seeding HeLa cells at a density of 2 × 10^6 cells in a 75 cm flask. Cells were treated with 20 μl of serum free DMEM in the presence of 1:100 dilution of SP or vehicle (PBS) for 24 h to create HeLa SP conditioned medium (SP CM) or vehicle conditioned medium (V CM). Conditioned medium from four independent experiments was pooled, aliquoted and stored at −20°C until required for network assays. For HeLa cell experiments, cells were incubated in the presence of vehicle (PBS) or 1:100 dilution of SP or SP and inhibitors of EGFR kinase (AG1478; 200 μM), ERK1/2 kinase (PD98059; 50 μM), COX-1 (SC560; 10 μM), COX-2 (NS398; 10 μM) or nuclear factor kappa B (NF-κB, SN50; 100 μg/ml) for the time indicated. Human umbilical vein endothelial cells (HUVECs) (Lonza, Walkersville, USA) were cultured in Endothelial Basal Medium (EBM-2) with 2% FCS and growth supplements (VEGF, FGF, PGDF, IGF, EGF, ascorbic acid, heparin and gentamicin) subsequently referred to as Endothelial Growth Medium (EGM-2) (Lonza, Walkersville, USA). Under experimental conditions HUVECs were incubated with EBM-2 plus 1% FCS with the addition of ascorbic acid and gentamicin (EBM1%) (Lonza, USA).

2.4. Semen donors and preparation

Semen was obtained from 10 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. Seminal plasma (SP) was isolated from the pooled ejaculate by density gradient centrifugation as described previously [11]. The seminal plasma was aliquoted and stored at −80°C until required and used at a concentration of 1:100 for in vitro studies [11].

2.5. Taqman quantitative RT-PCR

HeLa cell RNA was extracted and reverse transcribed as described previously [11]. Quantitative RT-PCR was performed under standard operating conditions using an ABI Prism 7500 Real-time PCR machine (Applied Biosystems, Warrington, UK), using sequence-specific primers and 6-carboxyfluoresceine-labelled probes [11,15,16]. RNA loading of all target genes was normalised using the 18S ribosomal RNA as an internal standard. Data were analysed using the comparative Ct method and processed using Sequence Detector v1.6.3 (Applied Biosystems). All results were calculated relative to an endogenous control of HeLa cell cDNA included in each experiment. Fold increase was determined by dividing the relative expression of the treatment group by the relative expression of the control group. Data are presented as mean ± SEM.

2.6. Western blot analysis

Immunoblot analysis was carried out using a LI-COR Odyssey™ infrared imager (LI-COR Biosciences, Cambridge, UK) [15]. Briefly 20 μg of total protein was isolated from HeLa cells and resolved on a sodium dodecyl sulphate polyacrylamide gel and transferred to PVDF membrane. Immunoblots were blocked in Odyssey Blocking buffer™ (LI-COR Biosciences) before overnight incubation with primary rabbit–phospho-p42/44 and mouse p42/44 antibodies (diluted 1:1000 in Odyssey blocking buffer) at 4°C. The following day, blots were washed and incubated with the goat anti-mouse IRDye™ 800 (1:10,000) (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) and goat anti-rabbit Alexafluor 680 (1:5000) (Invitrogen, Paisley, UK) for 60 min at room temperature. Immunoreactive proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences). ERK1/2 phosphorylation was calculated by dividing the value obtained from the phosphorylated ERK1/2 channel (700 nm) by the value obtained from total ERK1/2 channel (800 nm) and expressed as fold above vehicle controls. Results are expressed as mean ± SEM.

2.7. ELISA

Secreted IL-8 and GRO were measured in the HeLa cell CM using a commercially available enzyme linked immunosorbent assay (R&D Systems, Oxford, UK). CM was prepared as described in Section 2.3. A control flask containing 1:100 dilution of SP in serum free medium alone (without HeLa cells) was prepared in parallel to the HeLa cell CM described in Section 2.3 to investigate the levels of IL-8 and GRO.
in the SP. The ELISA was carried out as per the manufacturer’s protocol on individual batches of CM prior to pooling for network assays. Data are presented as mean±SEM from four independent experiments.

2.8. Tube-like structure (endothelial network) assays

Endothelial network (tube-like structure; TLS) formation was measured using 12-well Transwell plates (Corning Costar, Cambridge, UK). The upper chambers were coated with 80μl of growth factor (GF)-reduced Matrigel (BD Biosciences, MA, USA) in the absence/presence of the CXCR2 antagonist SB225002 (100nM) and incubated at 37°C for 30 min to allow thin gel formation. HUVECs were plated in the lower chamber at 37°C for 30 min to allow thin gel formation. HUVECs were plated in the presence of the CXCR2 antagonist SB225002 (100nM) and incubated for 24h in EBM 1%. Subsequently, wells were fixed with 100% ice cold methanol and stained with haematoxylin. To assess the ability of HUVECs to form networks of TLS, Subsequently, wells were fixed with 100% ice cold methanol and stained with haematoxylin. To assess the ability of HUVECs to form networks of TLS, five photomicrographs (×10 magnification) of each well were captured using an inverted microscope and camera (Axiovert 200, Carl Zeiss, Germany). TLS formation was quantified by counting the number of network branch points (as indicated by the arrow in Fig. 5) per photomicrograph by an investigator blinded to treatment. Experiments were repeated four times in duplicate. Fold difference was determined by dividing the value obtained from SP or SP CM treated cells by the value obtained from V CM treated cells. Data are represented as percentage increase in network formation with V CM = 100% and are presented as mean±SEM.

2.9. Statistical analysis

The data in this study was analysed by t-test or one-way ANOVA using Graph Pad Prism 4.0c (Graph Pad, San Diego, CA). Paired T-tests were conducted on untransformed means of the replicates between SP and control or inhibitor and SP and each experiment. Unpaired T-tests were conducted on SP versus SP and inhibitor after conversion to fold/percentage increase. ANOVA was used to determine significant difference between the various time points for IL-8 and GRO by real-time PCR and phosphorylation of ERK1/2 in response to SP treatment.

3. Results

3.1. Seminal plasma induces expression of IL-8 and GRO in HeLa cells

We investigated whether exposure of neoplastic cervical epithelial cells to SP would induce expression of the angiogenic chemokines IL-8 and GRO. HeLa cervical adenocarcinoma cells were used as a model system and were treated with 1:100 dilution of SP or vehicle (PBS) for 4, 8, 12 and 24h and the mRNA expression of IL-8 and GRO was determined by quantitative RT-PCR analysis. We found that SP significantly elevated expression of IL-8 and GRO at all time points investigated with maximal expression observed for IL-8 after 12h (Fig. 1A; 250.3±20.7 fold increase compared to vehicle treatment; P<0.01) and for GRO after 8h of SP treatment (Fig. 1B; 18.4±2.5 fold increase compared to vehicle treatment; P=0.01).

3.2. IL-8 and GRO expression in HeLa cells is regulated by SP via the EGFR-ERK-COX-NFκB pathway

We investigated the pathway whereby SP mediates the induction of IL-8 and GRO in HeLa cells using a panel of small molecule chemical inhibitors of cell signalling. HeLa cells were treated for 8h with vehicle (Fig. 2A and B; white bar) or 1:100 dilution of SP (Fig. 2A and B; black bar) in the presence of chemical inhibitors (Fig. 2A and B; dark grey bar) of EGFR kinase (AG1478; 200nM), ERK1/2 kinase (PD98059; 50μM), COX-1 (SC560; 10μM), COX-2 (NS398; 10μM) or nuclear factor kappa B (NFκB; SN50; 100μg/ml). We found that all chemical inhibitors significantly reduced the SP-mediated induction of IL-8 in HeLa cells with an average percentage inhibition of 65%, 70%, 55%, 98% and 70% for AG1478, PD98059, SC560, NS398 and SN50 respectively (Fig. 2A; dark grey bar; P<0.01). Similarly we found that AG1478, PD98059, SC560, NS398 and SN50 inhibited SP-mediated induction of GRO in HeLa cells by 65%, 70%, 50%, 98% and 60% respectively (Fig. 2B; dark grey bar; P<0.01). Treatment of cells with inhibitor alone (Fig. 2A and B; light grey bar) had no significant effect on expression of IL-8 and GRO in HeLa cells compared with vehicle treated cells (Fig. 2A and B; white bar).

We have previously shown that SP can mediate signalling to ERK1/2 via transactivation of the EGFR [17]. We used immunoblot analysis to investigate whether the EGFR, COX and NFκB pathways lay upstream or downstream of ERK1/2 in the present study. HeLa cells were treated with vehicle or 1:100 dilution of SP for 0, 5, 10, 20, 30 and 60 min and ERK1/2 phosphorylation was measured by immunoblot analysis. We found a rapid time-dependent increase in ERK1/2 phosphorylation, reaching a maximum after 10 min of stimulation (Fig. 3A; 7.5±1.2 fold increase above control; P<0.05). We next treated HeLa cells with vehicle or 1:100 dilution of SP alone or in the presence of chemical inhibitors of EGFR kinase (AG1478), ERK1/2 kinase (PD98059), COX-1 (SC560), COX-2 (NS398) or NFκB (SN50) for 10 min and measured ERK1/2 phosphorylation. We found that SP-mediated ERK1/2 phosphorylation was significantly reduced by an average of 80% and 98% respectively in cells co-treated with SP and inhibitors of EGFR kinase (Fig. 3B; P<0.05) and ERK1/2 kinase (Fig. 3B; P<0.01). We found that co-treatment of HeLa cells with SP and inhibitors of EGFR kinase (Fig. 3B; P<0.05) and ERK1/2 kinase (Fig. 3B; P<0.01). We found that co-treatment of HeLa cells with SP and inhibitors of EGFR or COX-2 or NFκB had no inhibitory effect on ERK1/2 phosphorylation in the presence of SP (Fig. 3B). These findings suggested that the EGFR pathway lay upstream of ERK1/2, whereas the COX-NFκB pathways lay downstream of ERK1/2.

Since our data suggest that SP sequentially transactivates the EGFR and then activates ERK1/2 to up-regulate IL-8 and GRO via the COX-NFκB pathway, we investigated the regulation of COX-1 and
COX-2 mRNA expression by SP. HeLa cells were treated for 8h with vehicle (Fig. 4A and B; white bar) or 1:100 dilution of SP (Fig. 4A and B; black bar) in the presence of our panel of chemical inhibitors (Fig. 4A and B; dark grey bar). We found that SP significantly induced COX-1 (Fig. 4A; black bar; P<0.05) and COX-2 (Fig. 4B; black bar; P<0.05) mRNA expression. Moreover we found that COX-1 mRNA expression was inhibited by an average of 65% and 70% respectively by co-treatment of HeLa cells with EGFR kinase (AG1478; 200nM), ERK1/2 kinase (PD98059; 50μM), COX-1 (SC560; 10μM), COX-2 (NS398; 10μM) or NFκB (SN50; 100μg/ml). COX-2 mRNA expression was inhibited by an average of 65% and 70% respectively by co-treatment of cells with inhibitors of COX-1 (SC560), COX-2 (NS398) or NFκB (SN50; Fig. 4A) had no significant effect on reducing the SP-mediated induction of COX-1. In contrast, SP-mediated induction of COX-2 mRNA expression was significantly reduced by an average of 60%, 98% and 97% respectively by co-treatment of HeLa cells with SP and the EGFR kinase (AG1478), ERK1/2 kinase (PD98059), and COX-2 (NS398; Fig. 4B; P<0.01) inhibitors. Co-treatment of cells with inhibitors of COX-1 (SC560) or NFκB (SN50; Fig. 4B) had no effect on reducing the SP-mediated induction of COX-2. These data suggest that SP sequentially activates EGFR and ERK1/2 signalling to induce inflammatory COX enzyme expression and regulate IL-8 and GRO expression via the NFκB pathway.

3.3. IL-8 and GRO, released from HeLa cells in response to SP treatment, regulates vascular function

We measured IL-8 and GRO in the CM by ELISA. We found IL-8 to be present at 8.5±2.3pg/ml in the diluted SP (1:100), consistent with other studies [18]. In contrast, we did not detect GRO, at the dilution of SP used in this study, by ELISA. Relative to diluted SP in culture medium alone, we found a 3.1±0.5 fold increase in IL-8 levels in the SP CM. In addition, we found that SP treatment of HeLa cells for 24h increased the secretion of IL-8 and GRO proteins by 6.0±4.5 and 9.8±2.3 fold, respectively in HeLa cell CM compared to vehicle treatment (P<0.05). To assess the effects of the CM on vascular function, we used an in vitro endothelial cell tube-formation (network formation) assay [12]. For this we used a co-culture system of conditioned medium (CM) from HeLa cells treated with vehicle (V CM) or 1:100 dilution of SP for 24h (SP CM) or serum-free culture medium containing 1:100 dilution of SP alone (SP only) and HUVECs. Treatment of HUVECs with SP CM significantly increased endothelial cell network formation, as indicated by the ability of HUVECs to form stabilised polygonal structures comprised of three dimensional capillary-like tubes (as indicated by the arrow in Fig. 5), compared to V CM-treated cells (Fig. 5; P<0.001). Treatment of HUVECs with SP CM in the presence of the CXCR2 antagonist SB225002 inhibited endothelial network formation to the levels observed for V CM treated HUVECs, confirming that these alterations in endothelial cell function were mediated by IL-8 and GRO via their common receptor CXCR2 (Fig. 5). Interestingly, co-culture of HUVECs with medium containing 1:100 dilution of SP alone also significantly increased network formation, but to a lesser extent compared with CM from HeLa cells treated with SP (Fig. 5; P<0.05).

4. Discussion

Inflammation involves tissue remodelling events which act across multiple cellular compartments in the face of infection or injury in order to regulate homeostasis. If left unchecked, unabated inflammation results in pathology. Indeed, chronic inflammation is estimated to contribute to more than 25% of all new cancer cases globally...
Our data over the past 10 years have shown that the COX-PG pathway, a critical pathway regulating inflammation and cancer, is significantly up-regulated in cervical carcinomas [9]. Moreover, we have shown that SP can regulate this pathway in vitro and can enhance the growth of neoplastic cervical cells xenografted in nude mice in vivo [11,17]. Here we demonstrate that SP can induce the expression of the potent chemokines, IL-8 and GRO in cervical cancer (HeLa) cells. Chemokines have emerged as important regulators of cell proliferation, angiogenesis and inflammation [20–24]. Seminal plasma has been shown to regulate the release of inflammatory cytokines, including IL-8 and GRO from normal cervical and vaginal cells [25]. These modulate inflammation within the cervical compartment after coitus by regulating the influx of leukocytes into the epithelial and stromal compartments [25,26]. Although the precise role of the post-coital inflammatory response is unclear, it has been proposed to facilitate immune tolerance to male transplantation antigens contained within the ejaculate and prime the uterus for conception [26]. However, in sexually active women with neoplastic cervical lesions, activation of these inflammatory and pro-tumorigenic pathways by SP could put women at risk of disease progression by exacerbating the release of inflammatory mediators and inducing the polarization and recruitment of tumour associated macrophages and neutrophils into the cervix. These have been shown to promote tissue remodeling, angiogenesis and tumorigenesis [27,28].

We investigated the pathways whereby SP regulates IL-8 and GRO expression in HeLa cells, using a panel of small molecule chemical inhibitors targeted to specific signal transduction pathways with known roles in regulating cell growth, differentiation and angiogenesis. We found that SP induced IL-8 and GRO mRNA expression via the EGFR-mediated activation of ERK1/2 signalling since inhibitors of EGFR kinase and ERK1/2 kinase inhibited SP-mediated induction of IL-8 and GRO. Although we have not isolated the precise ligand in the SP responsible for activation/transactivation of the EGFR in our study, SP is rich in epidermal growth factor (EGF), transforming growth factor beta (TGFβ), inflammatory cytokines and prostaglandins [18,29–31]. It is feasible that the EGFR could be phosphorylated and activated directly by the EGF in the SP to induce IL-8 and GRO expression. Alternatively, PGE2, which is present at high concentrations in SP can transactivate the EGFR via the E-series PG receptors (EP2 and EP4 receptors) as we have previously shown [17,32]. In our present study, the net effect of SP signalling to IL-8 and GRO in cervical cancer cells is most likely mediated by a combination of ligands, such as EGF activating its receptor directly and PGE2, transactivating the EGFR via the phosphorylation of intracellular tyrosine kinases [17,32].

Fig. 4. Regulation of COX-1 and COX-2 expression in HeLa cells by seminal plasma. (A) COX-1 and (B) COX-2 mRNA expression as measured by quantitative RT-PCR analysis. HeLa cells were treated for 8h with SP (1:100) or control in the presence/absence of chemical inhibitors of EGFR kinase (AG1478), ERK1/2 kinase (PD98059), COX-1 (SC560), COX-2 (NS398) or NFκB (S N 5 0 ) . b significantly different from a, c is significantly different from a and b; P<0.05; ns: not significantly different for SP versus SP and inhibitor. Data are represented as mean±SEM from 4 independent experiments.

Fig. 5. The effect of SP conditioned medium on endothelial network (TLS) formation. The ability of endothelial cells to differentiate into polygonal networks of three-dimensional tube-like structures (endothelial network formation) was investigated using HUVECs stimulated with vehicle conditioned medium (V CM), SP conditioned medium (SP CM), 1:100 dilution of SP in culture medium alone or SP CM in the presence of CXCR2 antagonist SB225002. (*P<0.05, **P<0.01, ***P<0.001). The arrowhead indicates a network branch point within the polygonal capillary network. Data are represented as percentage increase compared to V CM and presented as mean±SEM from 4 independent experiments.
Furthermore, in our present study we show that activation of EGFR and ERK1/2 signalling leads to the elevation of COX-1 and COX-2 expression, as an intermediate step in the SP-mediated induction of IL-8 and GRO. We have recently shown that SP treatment of HeLa cells elevates COX-1 and COX-2 expression in vitro and in HeLa cells xenografted in nude mice in vivo [11]. Moreover, we have shown that the PG released as a consequence of elevated COX enzyme expression in HeLa cells can regulate tumorigenic and angiogenic gene expression in vitro [10,33]. However, the present study is the first to demonstrate that SP regulates chemokine expression in cervical cancer cells by first inducing the inflammatory COX-PG pathway. Furthermore our study also shows that COX-2 expression, but not COX-1, in response to SP treatment is autoregulated, since co-treatment of HeLa cells with SP and the selective COX-2 inhibitor NS398 abolished the SP-mediated induction of COX-2. The auto-regulation of COX-2 expression via a positive feedback loop has been demonstrated in several model systems and is proposed to be the mechanism behind the role of COX-2 in regulating chronic inflammatory events and tumourgenesis [33,34]. Interestingly we found that the NFκB inhibitor SN50 abolished IL-8 and GRO expression, but not COX-1 or COX-2, in response to SP. These data suggest that IL-8 and GRO are regulated by NFκB via the COX-PG pathway. Indeed PGE2 has recently been shown to up-regulate IL-8 expression in the endometrium via NFκB [13]. Furthermore, constitutive activation of NFκB leading to activation of chemokines has been associated with cellular transformation, tumourgenesis and angiogenesis in several solid tumours in humans [24]. It is therefore plausible that repeated exposure of neoplastic cervical epithelial cells to SP could promote tumourgenesis by elevating NFκB signalling and inducing chemokine expression.

In our recent study, we found that tumours arising from HeLa cell xenografts in nude mice grew larger and had larger blood vessels in animals injected with seminal plasma, compared with control animals [11]. In order to investigate a potential mechanism behind the regulation of vasculature in HeLa cell xenografts by SP, we investigated whether the IL-8 and GRO, released from HeLa cells in response to SP, could modulate vascular function using an in vitro co-culture system. A critical process required for angiogenesis is endothelial cell stabilisation and differentiation into three dimensional capillary networks [35–37]. Recently, Maybin and colleagues showed that IL-8 can increase angiogenesis in menstrual endometrial explants ex vivo by modulating endothelial cell outgrowths [13]. Here we used an endothelial cell tube-formation (network) assay to determine the role of IL-8 and GRO on vascular function in vitro. This assay measures the ability of endothelial cells to form polygonal networks comprising three dimensional capillary-like tubular structures (TLS) when cultured on a thin gel of basement membrane extract. We show that conditioned medium from HeLa cells treated with SP contains elevated IL-8 and GRO and promotes the formation of HUVECs into polygonal endothelial networks, which were more stable and dense compared with HUVECs incubated with VCM. Interestingly, incubation of HUVECs with SP alone could also increase TLS formation but to a lesser extent than the SP conditioned medium. This is possibly due to the lower levels of IL-8 found in SP compared with SP conditioned medium. IL-8 and GRO act via a common protein-coupled receptor CXCR2, present on endothelial cells [24]. Using a specific non-peptide antagonist of CXCR2, which prevents the binding of IL-8 and GRO to their receptor, we show that conditioned medium from SP treated HeLa cells, which contains both IL-8 present in SP as well as IL-8 and GRO biosynthesised in HeLa cells in response to SP signalling, promotes endothelial cell network formation via CXCR2 on endothelial cells, since incubation of HUVECs with SP CM and the CXCR2 antagonist disrupted endothelial TLS formation. Stabilised networks of endothelial tube-like structures would then allow other pro-angiogenic factors such as VEGF and FGF2 to interact with endothelial cells to promote proliferation and angiogenesis. These findings suggest a potential mechanism for the alteration of the vasculature we observed in HeLa cell xenografts in nude mice injected with SP, in our previous study [11].

5. Conclusions

In conclusion, our study as summarised schematically in Fig. 6, outlines the regulation of pro-angiogenic chemokines IL-8 and GRO by SP in neoplastic cervical epithelial cells and their role in vascular function. SP induces ERK1/2 via transactivation of the EGFR. This in turn induces biosynthesis and signalling of PG to NFκB via upregulation of COX enzyme expression. IL-8 and GRO, once released from neoplastic epithelial cells can induce the arrangement of endothelial cells into networks of tube-like structures to alter vascular function. This alteration of vascular function can be further enhanced by the endogenous IL-8 present in the SP. These findings highlight that suppression of the inflammatory COX-PG axis can attenuate the release of pro-inflammatory cytokines from cervical epithelial cells exposed to SP. Our findings further suggest that investigation of non-steroidal anti-inflammatory drugs as interventional strategies for preventing cervical inflammation and tumourigenesis is warranted.

Conflict of interest

There are no conflicts of interest to declare.

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![Fig. 6. Schematic summary highlighting the role of SP in regulating vascular function.](image)

Our findings demonstrate that SP activates ERK1/2 signalling via the transactivation of the EGFR. This induces expression of inflammatory COX-1 and COX-2 genes resulting in production of PG and activation of PG receptors [10,33]. This pathway then regulates COX-2 expression in an autoregulatory manner and regulates IL-8 and GRO via NFκB [13]. In turn, IL-8 and GRO act via a common receptor, CXCR2 on endothelial cells to alter vascular function by promoting the formation of endothelial cell tube-like structures and network formation. In addition endogenous IL-8 present in SP can enhance the effect of the IL-8 and GRO, released from HeLa cells in response to SP signalling, on endothelial network formation.
the study design; nor in the collection, analysis and interpretation of data; or in the writing of the report; or the decision to submit the article for publication.

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