

ANALYSIS

Inflammatory pathways in cervical cancer – the University of Cape Town's contribution

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Cervical cancer is the leading gynaecological malignancy in southern Africa. The main causal factor for development of the disease is infection of the cervix with human papillomavirus. It is a multi-step disease with several contributing co-factors including multiple sexual partners, a compromised immune system and cervical inflammation caused by infections with *Chlamydia trachomatis* or *Neisseria gonorrhoeae*. Inflammation involves extensive tissue remodelling events which are orchestrated by complex networks of cytokines, chemokines and bio-active lipids working across multiple cellular compartments to maintain tissue

homeostasis. Many pathological disorders or diseases, including cervical cancer, are characterised by the exacerbated activation and maintenance of inflammatory pathways. In this review we highlight our findings pertaining to activation of inflammatory pathways in cervical cancers, addressing their potential role in pathological changes of the cervix and the significance of these findings for intervention strategies.

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Cervical cancer is the second most common cancer in women worldwide and the leading gynaecological malignancy in women in Africa.¹ In 2008 the International Agency for Research on Cancer estimated that 493 243 women are newly diagnosed with cervical cancer annually. Of these, more than 273 000 die each year.^{1,2} It is estimated that around 80 000 women, of whom 60 000 die each year, live in Africa. Because there are inadequate cancer registries in many African countries, it is likely that these figures are a gross under-representation.

Infection of the cervix with high-risk human papillomavirus (HPV) is regarded as the main causal factor in cervical cancer.³ There are more than 150 genotypes of HPV with around 40 known to infect the anogenital tract, giving rise to genital warts or neoplastic lesions.³ Recently, 2 prophylactic vaccines against the main high-risk HPV variants, 16 and 18, have been introduced by Merck & Company (Gardasil) and GlaxoSmithKline (Cervarix). These induce an immune response that blocks initial HPV infection and confers protection against cancer associated with HPV 16 and 18 and some closely related variants. These have limited benefit for women already infected with high-risk HPV and in addition are out of reach of the majority of women in Africa due to the high costs involved. Although HPV infection initiates disease, cervical cancer is a multi-step process, with other contributing factors, including multiple sexual partners, tobacco carcinogens, a weakened immune system and sexually transmitted infection by human immunodeficiency virus (HIV), *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, thought to contribute to the aetiology.

HPV initially infects basal keratinocytes and epithelial cells and uses the host's cellular machinery for replication and persistence.⁴

The HPV genome consists of 3 domains, a non-coding upstream region, an early region containing open reading frames E1, E2, E4, E5, E6 and E7, and a late region encoding the major and minor capsid proteins.⁴ In the vast majority of women, infections and HPV-induced lesions are transient and are naturally resolved. However, approximately 10 - 20% of women fail to eliminate the virus.⁴ In these cases, persistence of infection, viral integration and activation of inflammatory pathways have been linked to neoplastic transformation and malignant progression.^{4,5} In this review, we highlight our findings relating to the activation of inflammatory pathways in cervical cancers and address their role in disease progression.

Persistent HPV infection and inflammation

By broad definition, inflammation involves tissue remodelling events brought about by alterations to epithelial, vascular and immune cell function. These are orchestrated by specific molecular pathways involving a host of cytokines, chemokines, growth factors and lipid mediators.⁶ Compelling evidence has shown that the majority of cancers arise from sites of chronic irritation, infection and inflammation,⁷ solidifying the concept that chronic unabated inflammation is critical for tumour progression.

Persistent HPV infection and integration of E6 and E7 oncogenes into the host genome is considered key to development of cervical cancer.⁴ The HPV E6 and E7 early genes encode oncoproteins responsible for cervical neoplastic transformation³ by inactivating tumour suppressors as well as promoting the accumulation of genetic mutations.^{3,4} Although E6 and E7 oncogenes appear to be the main HPV genes involved in transformation, recent studies have highlighted an important role for E5 oncogene in tumorigenesis and immune cell modulation⁸ and regulation of late viral functions together with the E4 oncogene. In addition, E1 and E2 oncogenes encode replication factors and are thought to play a role in HPV persistence by allowing episomal copies of the virus to be maintained in the nucleus and partitioned into daughter cells during mitosis.⁹

Immune evasion is an essential aspect of HPV persistence and development of cervical cancer. Since there is no viraemia or cytolysis associated with initial viral infection of the cervix, there is no activation of the innate immune system and no inflammation.

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Despite this, the virus actively induces mechanisms to evade immune detection and ensure its success by deregulating the interferon pathway⁴ and via the down-regulation of pattern recognition receptors such as Toll-like receptor 9, thereby allowing infection to proceed undetected.⁴ The virus requires actively dividing cells and active host cellular machinery for replication and persistence.⁴ Once established, persistent infections promote alterations in the release of inflammatory cytokines which in turn can alter immune cell infiltration and inflammation. Alterations in immune responsiveness and elevated systemic levels of inflammatory cytokines have been observed in older women (about 50 years of age) with persistent HPV infection.⁵ Since this is the age group most likely to present with cervical cancer in the clinic, it is likely that sustained elevation in systemic cytokine release contributes to HPV-mediated tumorigenesis.

Although the direct association between HPV infection and inflammation is controversial, transgenic mouse models, expressing the early genes from HPV 16 under the control of the human keratin 14 promoter, have shown that HPV-induced lesions release the chemokine CCL2 which enhances macrophage recruitment into tumours via CCR2.¹⁰ In human neoplastic cervical epithelial cells, HPV 16 E5, E6 and E7 oncogenes have been shown to induce the inflammatory cyclo-oxygenase (COX)-prostaglandin axis, by elevating expression of the immediate early oncogene COX-2.¹¹ These findings provide a direct link between HPV oncogenes and activation of potent inflammatory cascades, with known roles in promoting cancer. Thus, although HPV is not associated with inflammation at the initial point of infection, it is likely that following integration and transformation, persistent HPV infection drives inflammatory pathways, such as the COX-prostaglandin pathway in neoplastic epithelial cells, to promote immune cell infiltration, inflammation and tumour progression.

The inflammatory cyclo-oxygenase-prostaglandin pathway

COX enzymes, of which there are 2 isoforms in humans (COX-1 and COX-2), catalyse the rate-limiting conversion of arachidonic acid to the unstable intermediate prostaglandin H_2 , which in turn is converted by terminal prostaglandin synthase enzymes to specific classes of prostaglandins.¹² For many years COX-1 was considered to be constitutively expressed in tissues at low levels, generating prostaglandins for normal physiological functions, whereas COX-2 was considered to be an immediate early gene involved in pathology.¹² Studying tissue biopsies, we showed that COX-1 and COX-2 expression were both significantly elevated in the neoplastic epithelial and vascular endothelial cells of cervical cancers of all grades and stages.^{13,14} These findings highlighted a role for both COX isoforms in pathology of the cervix. In order to elucidate the role of COX-1 in cervical cancers, we used an *in vitro* model system where we stably expressed the COX-1 gene in cervical adenocarcinoma (HeLa) cells under the control of a tetracycline-inducible promoter (HeLa COX-1 TET-OFF system).¹⁴ Induction of COX-1 expression in HeLa cells caused a rapid and sustained elevation in the expression of COX-2 and terminal PGE synthase (PTGES), resulting in the biosynthesis of PGE₂.¹⁴ Furthermore, the PGE₂ was produced by both COX-1 and COX-2, indicating that they can contribute equally, or work synergistically, to promote cervical cancer.

The selectivity for prostaglandin production is determined by the terminal prostaglandin synthase enzyme present in cells expressing COX-1 and COX-2.¹² Santin and colleagues¹⁵ showed that the terminal PTGES enzyme, which converts PGH₂ to PGE₂, is significantly over-represented in invasive cervical cancers. This is consistent with our observations of elevated biosynthesis of PGE₂ in cervical cancers,¹³

suggesting a dominance of this prostaglandin in cervical cancer. PGE₂ exerts its biological role via 4 subtypes of E-series prostanoid G protein-coupled receptors (PTGER1-4).¹⁴ These receptors are often co-expressed on the same cell. We found that cervical cancers expressed elevated PTGER2 and PTGER4 in addition to elevated expression of COX enzymes and biosynthesis of PGE₂. Until recently, the molecular mechanisms regulating prostaglandin receptor expression in cervical cancer cells were unknown. However, *in vitro* studies have shown that HPV oncogenes and PGE₂ can regulate the expression of prostaglandin receptors. For example, the HPV 16 E5 oncogene has been shown to regulate expression of PTGER4 in a cervical cancer cell line in a PGE₂-cAMP-dependent manner.⁸ We have shown that PGE₂, either directly¹⁶ or following induction of COX-1 and COX-2 in HeLa cells, using the HeLa COX-1 TET-OFF system, can regulate prostaglandin receptor (PTGER2/PTGER4) expression.¹⁴ These findings suggest that in cervical cancers the elevated PGE₂ could regulate neoplastic cervical cell function in an autocrine/paracrine manner via the elevated PTGER2 and PTGER4 receptors. Indeed, our studies using tissue biopsies showed that cAMP levels were augmented in cervical cancer biopsies, relative to normal cervix, treated *ex vivo* with PGE₂.¹³

Taken together, our findings demonstrate that following HPV infection and viral integration in cervical epithelial cells, activation of viral oncogenes induces COX enzyme expression, PGE₂ biosynthesis and PTGER expression. In turn, PGE₂ via PTGER can regulate tumour cell function via cAMP signalling.

Regulation of vascular function and immune cell recruitment by the COX-prostaglandin pathway

In several *in vitro* and *in vivo* model systems employing cell lines and rodents, overexpression of PGE₂ as a consequence of elevated COX enzyme expression has been shown to promote tumorigenesis. This occurs by inducing tissue remodelling within the tumour by inhibiting apoptosis, enhancing cellular proliferation, facilitating tumour metastases and elevating angiogenesis.¹² We have shown that PGE₂, either directly, or biosynthesised following induction of COX-1 and COX-2 in HeLa cells, elevates the expression of potent pro-angiogenic factors such as basic fibroblast growth factor 2, vascular endothelial growth factor (VEGF) and angiopoietins.^{14,17} 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 to facilitate tumour growth, as well as alter vascular permeability to allow extravasation of leucocytes and macrophages into the surrounding tissues.⁶

Macrophage infiltration into cervical tumours has been positively correlated with tumour vascularity¹⁸ and women with advanced-stage invasive cancer have higher blood neutrophil counts than those with early-stage disease.¹⁹ Although the precise mechanism for immune cell recruitment into the cervix in humans has not been elucidated, prostaglandins biosynthesised by COX enzymes in epithelial, stromal and vascular cells have been shown to induce the expression of a host of cytokines and chemokines. These can in turn act in an autocrine or paracrine manner in the cervix to enhance inflammation by promoting tissue remodelling and recruitment of immune cells via chemotaxis and extravasation, which in turn can promote disease progression.⁶

In order to allow for leucocyte extravasation, changes in the vasculature and angiogenesis are required. This involves tissue remodelling of the extracellular matrix, a process facilitated by matrix metalloproteinases (MMPs).⁶ Several studies have correlated transcription of HPV E6 and E7 with transcription of MMPs,²⁰ suggesting that HPV oncogenes can drive tissue and vascular

remodelling. Indeed, micro-array analysis has shown that HPV 16 E6 oncoprotein regulates several genes involved in tissue differentiation and remodelling, which are important for inflammation and tumour progression.²¹ Whether HPV oncogenes directly regulate these genes involved in tissue remodelling events, or drive their transcription via intermediary pathways such as the COX-prostaglandin pathway, remains to be determined.

Nonetheless, our studies, and others, highlight a mechanism whereby activation of a chronic inflammatory pathway following HPV infection and cellular transformation can induce tissue remodelling events in cervical epithelial cells. Disease progression is promoted by altered vascular function and angiogenesis via the increased biosynthesis and signalling of PGE₂.

Seminal fluid as a regulator of cervical inflammation and cancer

The main route of HPV transmission is via exposure of the cervix to virus present in seminal fluid and in the infected partner's skin during coitus. In addition to being a vehicle for the dissemination

of HPV, seminal fluid contains a diversity of molecules that include cytokines, angiogenic factors, proteases, protein kinases, transporter proteins, structural molecules and immune response proteins.²² Based on our research, we have proposed that the inflammatory environment of cervical cancers can be further modulated by these mediators present in seminal fluid.^{16,17} Deposition of seminal fluid into the female reproductive tract elicits a wave of cytokine release and recruitment and activation of leucocytes.²³ Little is known about the effect of seminal fluid on the neoplastic cervical epithelium. However, it can promote the release of MMPs which can alter the integrity of the epithelial barrier at the endocervical canal and can enhance metastases²⁴ and promote the release of local pro-inflammatory mediators to regulate immune cell recruitment.²³

We have shown that seminal plasma can induce expression of COX-1 and COX-2 and the E-series prostaglandin receptors (PTGER1, PTGER3 and PTGER4) in normal cervical tissue explants (Fig. 1A) and neoplastic cervical epithelial cells.¹⁶ Furthermore, in addition to the inflammatory COX-prostaglandin receptor axis, seminal plasma induces the expression of inflammatory cytokine interleukin (IL)-6, chemokines (IL-8 and growth-regulated oncogene (GRO) alpha) and VEGF in cervical tissue explants (Fig. 1B). These observations have been confirmed by Sharkey *et al.*,²³ who have shown that seminal fluid induces an inflammatory response in the cervix in humans after coitus, characterised by the influx of leucocytes and dendritic cells into the epithelium and stromal compartments and an accompanying increase in inflammatory cytokines such as IL-6 and IL-8. These data provide robust evidence for a regulatory role of seminal fluid on the cervical micro-environment in favour of inflammation which might facilitate disease progression.

We earlier discussed the role of PGE₂, produced by elevated COX enzyme expression in cervical cancers. PGE₂ is abundant in seminal fluid, present at concentrations of up to 10 000-fold greater than at the site of chronic inflammation. We have shown that the PGE₂ in seminal fluid can enhance the biosynthesis and release of VEGF from cervical cancer cells via the PTGER4-mediated transactivation of the epidermal growth factor receptor and extracellular signal-regulated kinase signalling pathways.¹⁷ The elevated synthesis and release of VEGF in turn can regulate vascular permeability to facilitate extravasation of immune cells from the vasculature into the tumour, as well as promote angiogenesis in cervical cancers.¹⁷ Taken together, our observations, as outlined in Fig. 2, suggest that repeated exposure of neoplastic cervical epithelial cells to seminal fluid can promote tissue remodelling events associated with inflammation. These exogenous inflammatory stimuli can act together with inflammatory stimuli, regulated endogenously by HPV oncogenes and COX enzymes, to augment cervical cancer progression.

Therapeutic management strategies

In Africa a large proportion of women have HPV infections; the majority of women with cervical cancer present with advanced-stage disease and poor prognosis. Treatment of early-stage cervical cancer is generally surgical, often combined with radiation and/or chemotherapy. However, radiation and chemotherapy are not available in all African countries and it is evident that adequate national screening programmes to detect HPV and early cervical cancer precursors are needed.

In a number of tumour model systems, including colon cancer cells implanted into nude mice and carcinogen-induced tumours in rats, the application of non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX enzyme inhibitors exhibit dramatic anti-cancer activity.¹² This is mediated partially by reducing PGE synthesis in the COX-2-overexpressing cells, which in turn down-regulates the

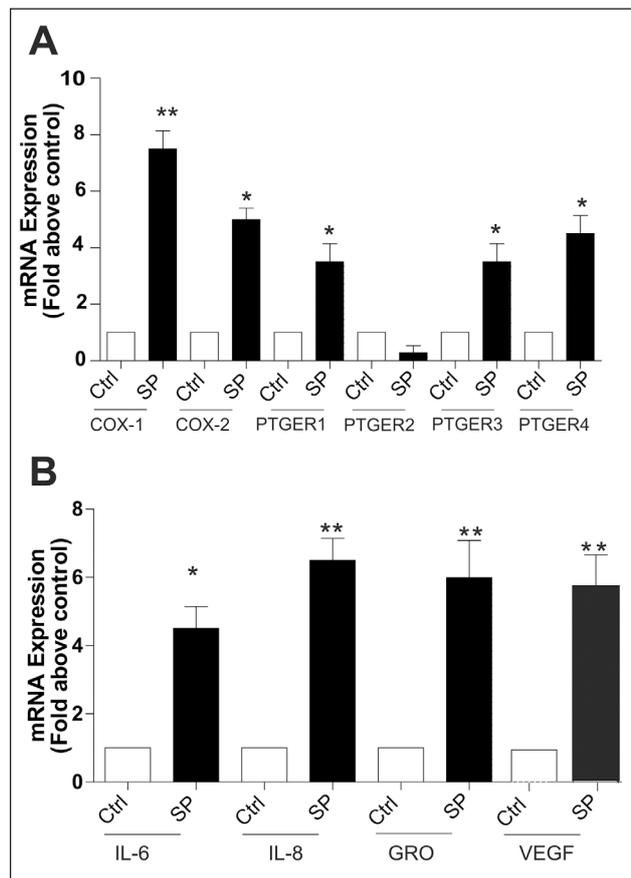


Fig. 1. Regulation of inflammatory pathways in cervical tissue explants by seminal plasma. Human cervical explants were obtained with informed written patient consent as described in our study.¹⁴ Tissue sections were finely chopped and incubated with a 1:100 dilution of seminal plasma or control for 24 hours.¹⁶ (A) The expression of COX-1, COX-2 and the E-series prostaglandin receptors (PTGER1-4), and (B) inflammatory cytokine interleukin (IL)-6 and chemokines IL-8 and growth-regulated oncogene (GRO) alpha and vascular endothelial growth factor (VEGF) were determined by Taqman quantitative RT-PCR analysis. Data shown is from 4 individual experiments using tissue taken from 4 different patients and are expressed as mean \pm SEM (* and ** denote statistical significance $p < 0.05$ and $p < 0.01$, respectively).

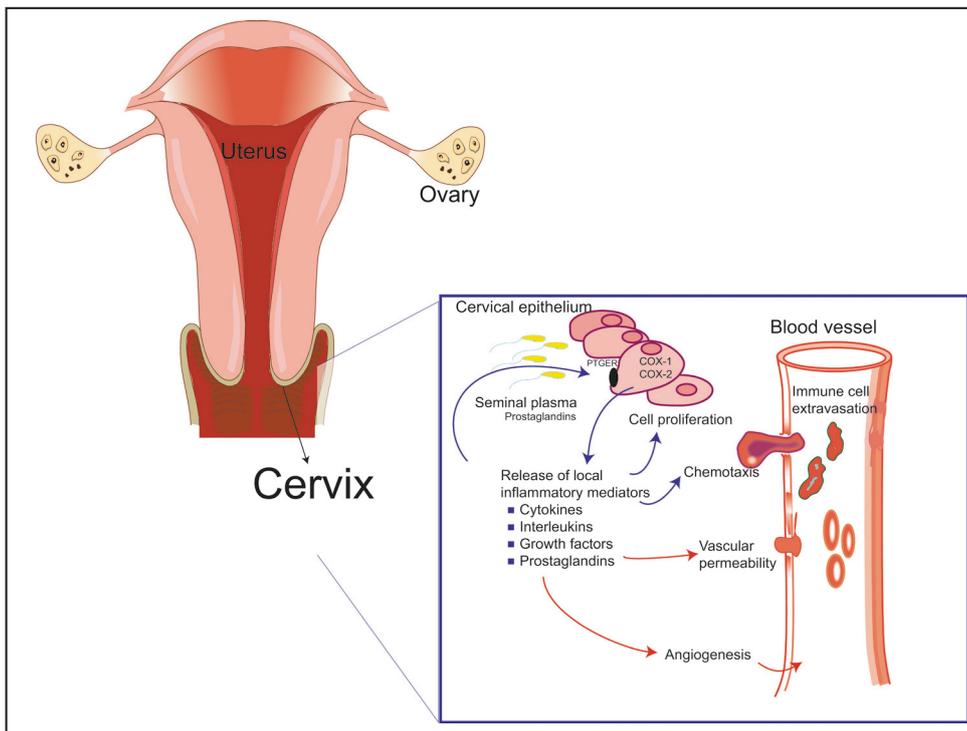


Fig. 2. A schematic diagram highlighting pathways involved in inflammation and cervical cancer progression. Seminal plasma and prostaglandins elevate expression of COX-1 and COX-2 in cervical epithelial cells. This can occur via prostaglandin G protein-coupled receptors (PTGER), causing the cells to release a host of local inflammatory mediators including cytokines, interleukins, growth factors and prostaglandins. These mediators activate numerous pathways which act synergistically to control tissue remodelling and tumour progression. For example, inflammatory mediators are released to facilitate cellular proliferation and extravasation of immune cells into the tumour tissue by chemotaxis in response to stimuli from local inflammatory mediators. In parallel, angiogenic factors and vascular permeability factors promote the remodelling of the vasculature thereby facilitating angiogenesis.

survival, metastatic, and angiogenic potentials of the cancerous tissue.¹² Our observations of elevated biosynthesis and signalling of PGE₂ in cervical cancers prompt us to suggest that inhibition of PGE₂ secretion by the application of COX enzyme inhibitors may suppress growth and invasiveness of cervical carcinomas. One of the most widely available and cheapest NSAIDs is aspirin. Recent clinical trials have shown that long-term aspirin treatment can be beneficial in colorectal cancer.²⁵ It is tempting to speculate that such anti-inflammatory agents may similarly prove beneficial and cost-effective for preventing progression of cervical cancer.

Our observations of the role of seminal plasma in regulating potent inflammatory and angiogenic pathways in neoplastic cervical epithelial cells suggest use of barrier contraceptives as a method of preventing disease, not only as a barrier against HPV transmission, but as a method of preventing the inflammatory actions of seminal fluid on the neoplastic cervical micro-environment. In the absence of barrier contraceptives, our research has highlighted the potential advantages of using prostaglandin receptor antagonists to prevent the activation and signalling of prostaglandin receptors by PGE₂ present in seminal fluid. Potentially, these antagonists could be utilised in combination therapy with COX enzyme inhibitors such as aspirin to prevent endogenous production of PGE₂ in the cervical tumour as well as the exogenous actions of prostaglandin present in the seminal fluid.

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