Seminal plasma activates cyclooxygenase-2 and prostaglandin E₂ receptor expression and signalling in cervical adenocarcinoma cells

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Enhanced cyclooxygenase (COX) expression and prostaglandin E₂ (PGE₂) synthesis are regarded as promoters of neoplastic cell proliferation and angiogenesis. Expression of COX-2 and synthesis of PGE₂ are up-regulated in cervical carcinomas. In sexually active women, growth and invasiveness of neoplastic cervical epithelial cells may be also under the direct influence of PGE₂ present in seminal plasma. The aims of this study were to investigate the effect of seminal plasma and PGE₂ on the expression of COX-2 and expression and signalling of the PGE₂ receptor subtypes (EP1–EP4) in HeLa (cervical adenocarcinoma) cells. Treatment of HeLa cells with seminal plasma or PGE₂ resulted in up-regulation of COX-2 expression (P < 0.05). In addition, seminal plasma induced the mRNA expression of EP1, EP2 and EP4 receptors, whilst PGE₂ treatment of HeLa cells induced the expression of the EP4 receptor (P < 0.05). This was coincident with a rapid accumulation of adenosine 3',5'-cyclic monophosphate (cAMP) in HeLa cells stimulated with seminal plasma or PGE₂, which was greater in seminal plasma stimulated cells compared with PGE₂ stimulated cells (P < 0.05). Subsequently, we investigated whether the effect of seminal plasma on cAMP signalling in HeLa cells was mediated via the cAMP-linked EP2/EP4 receptors. Stimulation of HeLa cells with seminal plasma or PGE₂ resulted in an augmented cAMP accumulation in cells transfected with the EP2 or EP4 receptor cDNA compared with control transfected cells (P < 0.05). These data suggest that, in sexually active women, seminal plasma may play a role in modulating neoplastic cell function and cervical tumorigenesis.

Key words: cervical adenocarcinomas/cyclooxygenase/EP receptors/prostaglandin/semen/ seminal plasma

Introduction

Uterine cervical cancer is a disease of multifactorial aetiology, generally found amongst sexually active women. Current evidence suggests that sexually transmitted infection of the benign cervical epithelium with human papilloma virus (HPV) is the main cause of the disease, however, several other factors including venereally transmitted disease, genital warts, genital herpes and trichomonal infection have also been recognized to play a role in the development and progression of cervical tumorigenesis (Brinton and Fraumeni, 1986; Brinton et al., 1987; McInerney, 1987; zur Hausen, 1991; Brinton, 1992; Munoz et al., 1992).

Recent studies have demonstrated that reproductive tract carcinomas, including carcinomas of the ovary, uterus and cervix, may be regulated by cyclooxygenase (COX) enzyme products (Dore et al., 1998; Ryu et al., 2000; Tong et al., 2000; Jabbour et al., 2001; Klimp et al., 2001; Kulkarni et al., 2001; Sales et al., 2001, 2002). Two distinct isozymes of the COX enzyme, COX-1 and COX-2, have been characterized (Hla and Neilson, 1992; Herschman, 1994; Vane et al., 1998). Both COX isozymes catalyse the rate-limiting step in the conversion of arachidonic acid to eicosanoids, a class of compounds which includes prostaglandins, thromboxanes and leukotrienes (DeWitt, 1991). COX-1 and COX-2 expression and prostaglandin E₂ (PGE₂) synthesis are elevated in numerous solid epithelial tumours, including carcinomas of the cervix (Sales et al., 2001, 2002), suggesting that both COX enzymes and their synthesized products, such as PGE₂, may be contributory towards the neoplastic process.

The biological actions of PGE₂ have been attributed to its interaction with G-protein-coupled seven-transmembrane-domain receptors (GPCRs) which belong to the rhodopsin superfamily of serpentine receptors (Coleman et al., 1994). Four main sub-types of PGE₂ receptors have been identified (EP₁, EP₂, EP₃, EP₄) and are known to utilize alternate, and in some cases opposing, intracellular pathways (Ashby, 1998). Recently, a direct role for PGE₂ and EP receptors has been ascertained in colorectal carcinomas. In this model, enhanced proliferative and tumorigenic effects are mediated by PGE₂ following interaction with the EP4 receptor (Sheng et al., 2001). Similarly, another study has demonstrated a role for PGE₂ and EP2 receptor in accelerating intestinal polyp formation in APC₇¹⁶ knock-out mouse models (Sonoshita et al., 2001). In cervical and endometrial carcinomas, elevated expression of the EP2 and EP4 receptors and enhanced adenosine 3',5'-cyclic monophosphate (cAMP) signalling has also been observed (Jabbour et al., 2001; Sales et al., 2001), further supporting the idea that prostanooids such as PGE₂, may regulate neoplastic cell function in reproductive tract carcinomas in an autocrine/paracrine manner.

In addition to endogenously synthesized PGE₂, sexually active women also come into contact with PGE₂ present in seminal plasma.
Prostaglandins are present in seminal plasma at 10,000-fold greater concentrations than that detected at the site of inflammation, and PGE2 is one of the predominant types detected (Templeton et al., 1978). Little is known of the effect of seminal plasma and seminal plasma prostaglandins, including PGE2, on the neoplastic cervical epithelium of sexually active women. Cervical tumorigenesis may thus be regulated in an autocrine/paracrine manner by PGE2 present in seminal plasma via PGE2–EP receptor interactions, initiation of intracellular signalling pathways and transcription of target genes involved in enhancing or sustaining cervical tumorigenesis.

The aims of this study were to determine the effect of seminal plasma and PGE2 on the expression of COX-2 and on the expression and signalling of the PGE2 receptors in HeLa cervical adenocarcinoma cells. In cervical carcinomas elevated PGE2 may act in an autocrine/paracrine manner via cAMP-linked PGE2 receptors to mediate an effect on target genes, such as COX-2. Thus, it would use the cAMP-dependent protein kinase pathway to activate adenylate cyclase and increase intracellular cAMP. This in turn may elevate expression of COX-2 via the cAMP regulatory element (CRE) on the COX-2 promoter. This positive feedback loop between COX-2 and PGE2 may potentiate the progression of the disease, which may be further enhanced in sexually active women.

Materials and methods

Semen donors and preparation

Semen was obtained from healthy male volunteers by masturbation. The collected ejaculates were pooled and incubated at room temperature for 30 min prior to overlaying on a 100–50% percoll gradient. Seminal plasma was isolated from the pooled ejaculate by percoll density gradient centrifugation at 500 g for 20 min. The seminal plasma was added immediately to HeLa cell cultures at a final concentration of 1:50. At this dilution, seminal fluid has been reported to exert no effect on HeLa cell viability (Jeremias et al., 1997). Approval for the study was obtained from the University of Cape Town Research Ethics Committee, and informed consent was obtained from all patients prior to sample collection.

Cell culture

HeLa-S3 cells were purchased from BioWhittaker (Berkshire, UK) and were routinely maintained in Dulbecco’s modified Eagle’s medium nutrient mixture F-12 with Glutamax-1 and pyridoxine, supplemented with 10% fetal bovine serum and 1% antibiotics (stock 500 IU/ml penicillin and 500 µg/ml streptomycin) at 37°C and 5% CO2 (v/v). All experiments and cell treatments were performed on HeLa cells using culture medium containing no fetal bovine serum (serum-free conditions) or additional proteins.

Cell transfections

Cells were plated in 12-well dishes in complete medium and were allowed to attach and grow overnight. The EP2 receptor cDNA (a kind gift from Dr Karen Gedzie, Allergan, CA, USA) or EP4 receptor cDNA (a kind gift from Dr Mark Abramovitz, Merck Frosst Centre for Therapeutic Research, Canada) or empty vector (pcDNA3.1; Invitrogen, De Schelp, Netherlands) were transfected into the HeLa cell line at ~50% confluency using pfx-5 (Invitrogen) diluted in Optitrem (Gibco, Life Technologies, Paisley, UK). Cells were incubated for 4 h at 37°C in 5% humidified CO2. Thereafter, the medium was replaced with fresh complete medium. Cells were allowed to grow for 48 h. Transfected cells were then stimulated either with a 1:50 dilution of seminal plasma or 300 nmol/l PGE2 and cAMP accumulation was measured by enzyme-linked immunosorbent assay (ELISA) using a cAMP kit.

Real-time quantitative RT–PCR

Real-time quantitative RT–PCR was performed to determine COX-2 and EP receptor expression in HeLa cells. HeLa cells were grown in the presence or absence of a 1:50 dilution of seminal plasma or 300 nmol/l PGE2 and in the presence or absence of the dual COX enzyme inhibitor, indomethacin (used at a final concentration of 3 µg/ml to exclude the effects of any endogenously synthesized PGE2). Cells were harvested with Tri-Reagent (Sigma; Sigma Chemical Company, Dorset, UK) as per the manufacturer’s protocol after 24, 48 and 72 h, for determination of COX-2 expression, and after 24 h for determination of EP receptor expression. For validation studies of EP2 and EP4 receptor expression in transfected cells, HeLa cells were grown for 48 h following transfection and harvested with Tri-Reagent (Sigma). RNA samples were reverse transcribed using M-MLV (5.5 mmol/l), dNTPs (0.5 mmol/l each), random hexamers (1.25 µmol/l), oligo-dT (1.25 µmol/l), RNase inhibitor (0.4 IU/µl) and multiscribe reverse transcriptase (1.25 IU/µl; all from PE Biosystems, Warrington, UK). The mix was aliquoted into individual tubes (16 µl/tube) and template RNA was added (4 µl/tube of 250 ng/µl RNA). Samples were incubated for 60 min at 25°C, 45 min at 48°C and then at 95°C for 5 min. A reaction mix was made containing Taqman buffer (5.5 mmol/l MgCl2, 200 µmol/l dATP, 200 µmol/l dCTP, 200 µmol/l dGTP, 400 µmol/l dUTP), ribosomal 18S forward and reverse primers and probe (all at 50 nmol/l), forward and reverse primers for COX-2 or EP1, EP2, EP3 or EP4 receptor (300 nmol/l), COX-2 or EP1, EP2, EP3 or EP4 receptor probe (200 nmol/l), AmpliErase UNG (0.01 IU/µl) and AmpliTaq Gold DNA Polymerase (0.025 IU/µl; all from PE Biosystems). A volume of 48 µl of reaction mix was aliquoted into separate tubes for each cDNA sample and 2 µl/replicate of cDNA was added. After mixing, 23 µl of sample was added to the wells on a PCR plate. Each sample was added in duplicate. A no template control (containing water) was included in triplicate. Wells were sealed with optical caps and the PCR run on an ABI Prism 7700 using standard conditions (initial holding steps of 50 and 95°C for 2 and 10 min respectively, followed by 40 cycles of 95 and 60°C for 15 s and 1 min respectively). COX-2 and EP receptor primers and probe for quantitative PCR were designed using the PRIMER express program (PE Biosystems). The sequence of the COX-2 primers and probe were: forward, 5′-CCCTTCCTCTGTGCTTG TG-3′; reverse, 5′-ACAACTCATTTGAATCCAGGAGC-3′; probe (FAM labelled), 5′-TGGCCGCATCTCCTTTGGGTTCA-3′; reverse, 5′-GCAACCAACACACGATTG-3′; probe (FAM labelled), 5′-GAGATGGTGCGCCAGCTGTT-3′; reverse, 5′-GCCACCACACACGATTG-3′; probe (FAM labelled), 5′-CAGCAGATGGCCAGCACACGATTG-3′. The sequence of the EP2 receptor primers and probe were: forward, 5′-GAGGATGATGGCCAGCTGTT-3′; reverse, 5′-GACCGCTACTCCAGCCTGATC-3′; probe (FAM labelled), 5′-CCACCTGGCTGCTGCTTACTGTTGTC-3′. The sequence of the EP3 receptor primers and probe were: forward, 5′-GACGGGATTCTCAGTGATG-3′; reverse, 5′-TCTGTCGCTCGTGTGCTTCCGCTC-3′; probe (FAM labelled), 5′-CTGGTCGCTCGTGTGCTTCCGCTC-3′. The sequence of the EP4 receptor primers and probe were: forward, 5′-AGCGGCCGTCATCTACTCAGT-3′; reverse, 5′-AGAGGACCCGGCGAGAAT-3′; probe (FAM labelled), 5′-AGCGGGCTCTCAGCTTCCCTCTC-3′. The sequence of the EP5 receptor primers and probe were: forward, 5′-AGCGCCCATTGACTCATCAGT-3′; reverse, 5′-TGCGTACTCATGACTCATCAGT-3′; probe (VIC labelled), 5′-TGCGTACCATTCCAGACTCATCAGT-3′. Expression of COX-2 and EP receptors was normalized to RNA loading for each sample using the 18S ribosomal RNA as an internal standard. Fold induction of COX-2 and EP receptor expression was calculated by dividing the relative expression of COX-2 or EP receptors in cells incubated with seminal plasma or PGE2 by the relative expression of COX-2 and EP receptors in cells maintained in culture medium alone. Fold induction of EP2 and EP4 receptor expression in transfected cells was calculated by dividing the relative expression of EP2 or EP4 in cells transfected with EP2 or EP4 receptor cDNA by the relative expression of EP2 or EP4 in cells transfected with control cDNA. The data are presented as mean ± SEM from three independent experiments.

Western blotting

Cells were seeded in 5 cm dishes and allowed to attach overnight. The following day, the culture medium was replaced with serum-free medium or serum-free medium containing either a 1:50 dilution of seminal plasma or 300 nmol/l PGE2. Cells were grown for 24, 48 and 72 h and harvested by lysis in protein lysis buffer (150 mmol/l NaCl, 10 mmol/l Tris–HCl pH 7.4, 1 mmol/l EDTA, 1% Triton X-100, 0.1% SDS) and centrifugation. The clarified lysate was removed to a new tube for protein quantification and SDS–PAGE. The protein content in the supernatant fraction was determined using protein assay kits (Bio-Rad, Hemel Hempstead, UK). A total of 20 µg of protein was resuspended in 20 µl of sample buffer (125 mmol/l Tris–HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol). Protein samples were run on a 10% gel at 150 V for 1 h and were transferred to a PVDF membrane at 100 V for 1 h. Membranes were blocked in 5% non-fat milk in TBST (Tris-buffered saline containing 0.05% Tween-20) for 1 h and incubated overnight with rabbit polyclonal antibodies to COX-2 or EP receptors diluted 1:1000 in TBST. Membranes were incubated with donkey anti-rabbit conjugated with horseradish peroxidase for 1 h and were visualized with an enhanced chemiluminescence reagent (Amersham).
COX-2 protein expression in HeLa cells following treatment with seminal plasma (Figure 1A) for 24 h or PGE 2 (Figure 1B) for 72 h: COX-2 mRNA expression was determined by real-time quantitative RT–PCR analysis. HeLa cells were co-treated with the dual COX enzyme inhibitor indomethacin (open bars). Fold induction was determined by dividing the relative COX-2 expression in cells grown in the presence of seminal plasma or PGE 2 by the relative COX-2 expression in cells grown in culture medium alone.

Statistical analysis
The data in this study were analysed by ANOVA using StatView 5.0 (Abacus Concepts, Berkeley, CA, USA).

Results
Seminal plasma and PGE 2 induces COX-2 expression in HeLa cells
The effects of seminal plasma and PGE 2 on COX-2 expression in HeLa cells were investigated by real-time quantitative RT–PCR (Figure 1A, B) and Western blot analysis (Figure 2A, B). HeLa cells were treated with seminal plasma or PGE 2 or maintained untreated in culture medium (control cells) for 24, 48 and 72 h respectively. Real-time quantitative RT–PCR analysis revealed a 20.25 ± 5.38- and 4.3 ± 0.75-fold induction of COX-2 mRNA in HeLa cells treated with seminal plasma (Figure 1A) for 24 h or PGE 2 (Figure 1B) for 72 h respectively (P < 0.05). Co-treatment of HeLa cells with 3 μg/ml indomethacin (a dual COX enzyme inhibitor) showed no significant reduction in induced COX-2 mRNA expression. Western blot analysis revealed 8 ± 3.78- and 3.7 ± 0.78-fold induction of COX-2 protein expression in HeLa cells following treatment with seminal plasma for 24 h (Figure 2A) or PGE 2 for 72 h (Figure 2B) respectively (P < 0.05). No significant elevation in COX-1 protein was observed as determined by Western blot analysis. Cells were normalized for protein loading against β-actin on the same blot.
Figure 2. Western blot analysis of 20 µg of total protein isolated from HeLa cells treated with either a 1:50 dilution of seminal plasma (A) or 300 nmol/l prostaglandin E\(_2\) (PGE\(_2\)) (B) for 24, 48 or 72 h. The proteins were loaded onto a 10% SDS-gel, electrophoresed and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The immunoblot was probed with antibody raised against human COX-1, COX-2 or β-actin.

greater in seminal plasma stimulated cells compared with PGE\(_2\) stimulated cells (31 ± 10.2 versus 11.3 ± 1.92 pmol cAMP/mg protein respectively; \(P < 0.05\)).

cAMP production in EP2 or EP4 receptor transfected HeLa cells in response to seminal plasma or PGE\(_2\)

HeLa cells were transiently transfected with either the EP2 or EP4 receptor cDNA or control cDNA. Real-time quantitative RT–PCR analysis revealed a 5.3 ± 0.58- and 6.7 ± 0.91-fold overexpression of EP2 and EP4 receptor mRNA expression in HeLa cells transfected with EP2 or EP4 receptor cDNA respectively, after 48 h (Figure 5A; \(P < 0.05\)). cAMP signalling via the EP2/EP4 receptors was ascertained following transient transfection of HeLa cells with either the EP2 or EP4 receptor cDNA or control cDNA, and stimulation with seminal plasma (Figure 5B) or PGE\(_2\) (Figure 5C). Treatment of HeLa cells with seminal plasma (Figure 5B) resulted in a rapid accumulation of cAMP after 5 min in cells transfected with the EP2 or EP4 receptor cDNAs (33.3 ± 1.25 and 26 ± 2.92 pmol cAMP/mg protein respectively; \(P < 0.05\)) compared with cells transfected with control cDNA (20 ± 2.3 pmol cAMP/mg protein). Similarly, stimulation of transfected HeLa cells with 300 nmol/l PGE\(_2\) (Figure 5C) revealed a rapid augmented accumulation of cAMP in cells transfected with the EP2 or EP4 receptor cDNAs (25.69 ± 1 and 34.63 ± 6.5 pmol cAMP/mg protein respectively; \(P < 0.05\)) compared with cells transfected with control cDNA (13.3 ± 0.64 pmol cAMP/mg protein).

Discussion

COX enzyme expression is elevated in numerous reproductive tract carcinomas, including cervical carcinoma, endometrial adenocarcinoma and ovarian adenocarcinoma (Dore et al., 1998; Ryu et al., 2000; Tong et al., 2000; Jabbour et al., 2001; Klimp et al., 2001; Kulkarni et al., 2001; Sales et al., 2001, 2002). In cervical and endometrial carcinomas, enhanced expression of COX enzymes is associated with an elevated synthesis of PGE\(_2\) in neoplastic epithelial and endothelial cells (Jabbour et al., 2001; Sales et al., 2001), suggesting that COX enzyme products such as PGE\(_2\) may be involved in neoplastic cell transformation and reproductive tract tumorigenesis.

This study reports up-regulated expression of COX-2, but not COX-1, in HeLa (cervical adenocarcinoma) cells by seminal plasma and PGE\(_2\) as determined by real-time quantitative RT–PCR and Western blot analysis. The positive feedback effect of PGE\(_2\) in inducing expression of COX-2 has been demonstrated in several

Figure 3. Fold induction of prostaglandin E\(_2\) (PGE\(_2\)) receptor mRNA expression in HeLa cells grown for 24 h in the presence of a 1:50 dilution of seminal plasma (A, solid bars) or 300 nmol/l PGE\(_2\) (B, solid bars). PGE\(_2\) receptor mRNA expression was determined by real-time quantitative RT–PCR. In parallel, HeLa cells were co-treated with indomethacin (open bars). Fold induction was determined by dividing the relative receptor expression in cells grown in the presence of seminal plasma or PGE\(_2\) (in the presence or absence of indomethacin) by the relative receptor expression in cells grown in culture medium alone.

Figure 4. Adenosine 3′,5′-cyclic monophosphate (cAMP) accumulation in HeLa cells following treatment with a 1:50 dilution of seminal plasma (closed diamonds) or 300 nmol/l prostaglandin E\(_2\) (PGE\(_2\)) (closed squares).
Seminal plasma regulates COX-2 and PGE2 receptor expression

Figure 5. Fold induction of EP2 and EP4 receptor expression in HeLa cells following transfection (A). Adenosine 3',5'-cyclic monophosphate (cAMP) accumulation in HeLa cells transiently transfected with either EP2 receptor (closed squares), EP4 receptor (closed triangles) or control cDNA (closed diamonds) and treated with a 1:50 dilution of seminal plasma (B) or 300 nmol/l prostaglandin E2 (PGE2) (C).

model systems (Maldve et al., 2000; Munir et al., 2000). PGE2 may mediate its potential role in enhancing tumorigenesis through immunosuppression (DeWitt, 1991), inhibiting apoptosis (Tsuji and DuBois, 1995), increasing metastatic potential of epithelial cells (Tsuji et al., 1997) and promoting angiogenesis (Tsuji et al., 1998; Jones et al., 1999). To date, most studies have focussed on a role for endogenously produced PGE2 in modulating tumorigenesis. However, PGE2 is one of the predominant prostanoids detected in seminal plasma (Templeton et al., 1978), with concentrations of up to 53 μmol/l described in the seminal plasma of fertile men (Huleihel et al., 1999). This suggests that in sexually active women, neoplastic cervical epithelial cells may also be modulated in a paracrine manner by PGE2 present in seminal plasma. This was demonstrated by our laboratory to elucidate the differential effects of the various components of seminal plasma, on normal and HPV-transformed cervical epithelial cells. Further studies into the elucidation of the effect of the various components of seminal plasma, on normal and HPV-transformed cervical epithelial cells, may augment our understanding of the role of seminal plasma in progression of cervical pathologies in sexually active women. These studies are currently underway in our laboratory to elucidate the differential effects of the various components of seminal plasma on modulation of the COX/PGE2 biosynthetic pathway.

Thus, in conclusion, enhanced ligand–receptor binding of seminal plasma prostanoids, specifically prostanoids of the E series, brought about by prolonged exposure of neoplastic cervical epithelial cells to seminal plasma, may modulate cervical tumorigenesis by enhancing transcription of target genes such as COX-2 and prostanoid receptors.
This in turn initiates signal transduction pathways, such as the cAMP pathway, and transcription of target genes that may modulate cervical tumorigenesis.

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References


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