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 also be 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/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 trichomonas 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 isoforms of the COX enzyme, COX-1 and COX-2, have been characterized (Hla and Neilson, 1992; Herschman, 1994; Vane et al., 1998). Both COX isoforms 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 APC∆¹⁶ 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 a paracrine manner. This in turn may elevate expression of 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 (Berkeley, 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 Kedzie, 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 HeLa cells by the calcium phosphate transfection method. Cell transfections were performed on the first day after seeding. The culture medium was replaced with serum-free medium or additional proteins.

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 cAMP accumulation was measured by enzyme-linked immunosorbent assay (ELISA) using a cAMP kit.

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 quantitation. SDS–PAGE. The protein content in the supernatant fraction was determined by standard methods.

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pH 6.8, 4% SDS, 5% 2-mercaptoethanol, 20% glycerol and 0.05% bromophenol blue), boiled for 5 min at 95°C and run on a 4–20% SDS–polyacrylamide gel. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Watford, UK) and subjected to immunoblot analysis. Membranes were blocked for 1 h at 25°C in blocking solution [5% skimmed milk powder diluted in washing buffer (50 mmol/l Tris–HCl, 150 mmol/l NaCl and 0.05% (v/v) Tween-20)]. Thereafter, membranes were incubated overnight with either goat anti- COX-1 (1:500; sc-1752; Autogenbioclear, Wilshire, UK), COX-2 (1:500; sc-1745; Autogenbioclear) or β-actin (1:500; sc-1616; Autogenbioclear) primary IgG antibodies diluted in blocking solution. The following day, membranes were washed and incubated for 1 h with rabbit anti-goat secondary antibody coupled to horseradish peroxidase at a dilution of 1:30 000 (Sigma) in washing buffer. Proteins were revealed by chemiluminescence (ECLplus kit; Amersham, Aylesbury, UK) following the manufacturer’s instructions. Fold induction of protein expression in HeLa cells grown in the presence of PGE2 or seminal plasma was determined relative to cells maintained in culture medium alone by scanning densiometric analysis. After normalizing to β-actin, the expression in cells grown in the presence of PGE2 or seminal plasma was divided by the expression in cells maintained in culture medium alone for the same period of time. The molecular weights of the COX and actin protein were determined as being ~72 and ~46 kDa respectively, from the relative mobility on SDS–PAGE compared with the molecular weight standard. Data are presented as mean ± SEM from three independent experiments.

**PGE2 stimulation and cAMP measurement**

EP receptor signalling was assessed by measuring cAMP accumulation following stimulation of cells with either seminal plasma or PGE2. Cells (2x10⁶) were plated in 6-well dishes containing 4 ml/well of complete medium. Cells were allowed to attach overnight. Thereafter, the culture medium was removed and replaced with serum-free medium containing 3-isobutyl-1-methylxanthine (IBMX; Sigma) to a final concentration of 1 mmol/l. cAMP concentrations were determined using protein assay kits (Bio-Rad). The data in this study were analysed by ANOVA using StatView 5.0 (Abacus Concepts, Berkeley, CA, USA).

**Statistical analysis**

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

**Results**

**Seminal plasma and PGE2 induces COX-2 expression in HeLa cells**

The effects of seminal plasma and PGE2 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 PGE2 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 PGE2 (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 PGE2 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.

**Up-regulation of EP receptor mRNA expression by seminal plasma and PGE2**

The effects of seminal plasma (Figure 3A) and PGE2 (Figure 3B) on mRNA expression on the four subtypes of PGE2 receptors, namely EP1–EP4, were investigated by real-time quantitative RT–PCR analysis. Treatment of HeLa cells with seminal plasma for 24 h significantly induced the mRNA expression of EP1, EP2 and EP4 receptors (5 ± 1.2, 12.5 ± 3.6- and 7.8 ± 2.26-fold induction respectively; Figure 3A, solid bars; P < 0.05), but had no significant effect on the expression of the EP3 receptor mRNA. Co-treatment of HeLa cells with indomethacin abolished the up-regulated mRNA expression of EP1, EP2 and EP4 receptors (Figure 3A, open bars; P < 0.01). There was no significant alteration in expression of EP1, EP2 or EP3 receptor mRNAs in response to treatment with PGE2 for 24 h (Figure 3B, solid bars; P > 0.05). However, PGE2 significantly induced the expression of the EP4 receptor mRNA (4.1 ± 2.26-fold induction, solid bars; P < 0.05). Co-treatment of cells with the dual COX enzyme inhibitor, indomethacin, abolished the induced expression of EP4 receptor mRNA (P < 0.01), but failed to elicit any significant effect on the mRNA expression of EP1, EP2 or EP3 receptors (Figure 3B, open bars; P > 0.05).

**cAMP production in HeLa cells in response to seminal plasma and PGE2**

cAMP signalling in HeLa cells was determined following stimulation of HeLa cells with seminal plasma or exogenous PGE2 (Figure 4). A rapid accumulation of cAMP was observed after 5 min of stimulation with seminal plasma or PGE2; the accumulation was
greater in seminal plasma stimulated cells compared with PGE2 stimulated cells (31 ± 10.2 versus 11.33 ± 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 PGE2

HeLa cells were transiently transfected with either the EP2 or EP4 receptor cDNA or control cDNA. Real-time quantitative RT-PCR analysis revealed a 5.31 ± 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 PGE2 (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 PGE2 (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 PGE2 in neoplastic epithelial and endothelial cells (Jabbour et al., 2001; Sales et al., 2001), suggesting that COX enzyme products such as PGE2 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 PGE2 as determined by real-time quantitative RT-PCR and Western blot analysis. The positive feedback effect of PGE2 in inducing expression of COX-2 has been demonstrated in several
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manner to enhance COX-2 expression. Moreover, the mechanism of action of seminal plasma and PGE₂ in inducing expression of COX-2 may involve differential regulation of COX-2 transcription and mRNA stability.

PGE₂ exerts its autocrine/paracrine effect by binding to prostanoid G-protein-coupled transmembrane receptors of which four pharmacologically classified subtypes have been described (namely EP1–EP4). We investigated the effect of seminal plasma and PGE₂ on the expression of the subtypes of EP receptors in HeLa cells. Treatment of HeLa cells with seminal plasma or PGE₂ significantly up-regulated the mRNA expression of the EP1, EP2 and EP4 receptors or EP4 receptor respectively. Co-treatment of HeLa cells with the dual COX enzyme inhibitor, indomethacin, significantly inhibited the up-regulation of receptor mRNA expression, suggesting that the observed up-regulation was a result of enhanced COX-2 expression in HeLa cells, mediated by seminal plasma or exogenous PGE₂. Previous studies have demonstrated up-regulated EP receptor expression in cervical and endometrial carcinomas coincident with elevated cAMP signalling (Jabbour et al., 2001; Sales et al., 2001). In addition, a direct role for EP receptors in tumorigenesis has been reported recently in mediating proliferation and tumorigenesis in colon carcinoma cells (Sheng et al., 2001), as well as accelerating intestinal polyp formation in APC∆716 knock-out mouse models (Sonoshiba et al., 2001). It is likely that in sexually active women, exposure to seminal plasma prostanoids may act in a similar manner in cervical carcinomas to enhance or sustain tumorigenesis following ligand–receptor binding and activation of intracellular signal transduction pathways.

One of the signal transduction pathways associated with EP receptor function is the cAMP pathway. Treatment of HeLa cells with seminal plasma or PGE₂ resulted in a rapid cAMP accumulation, which was augmented in the cells stimulated with seminal plasma compared with cells stimulated with PGE₂. In order to determine whether seminal plasma prostanoids were partially responsible for the observed cAMP signalling in HeLa cells, we transiently transfected HeLa cells with the EP2 or EP4 receptor cDNA [which activate adenylyl cyclase and cAMP signalling (Ashby, 1998)] and subsequently treated the cells with seminal plasma or PGE₂. A rapid accumulation of cAMP was observed in EP2/EP4 transfected cells, and was greater than that detected in control transfected cells. Taken together, these data confirm that seminal plasma prostanoids, including PGE₂, may modulate cervical tumorigenesis in an autocrine/paracrine manner via the COX-2/PGE₂ biosynthetic pathway. It is noteworthy that seminal plasma is a heterogeneous fluid and the difference in kinetics observed between PGE₂ and seminal plasma in regulating COX-2 and EP receptor expression and signalling could suggest that other factors and effectors of signal transduction pathways may be functioning in synergy with PGE₂ to modulate neoplastic cervical epithelial cell function. These factors may include other prostanoid derivatives of the E series (PGE and 19-hydroxy-PGE), which may be acting in combination to shift the kinetics of signal transduction in cervical carcinoma 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/PGE 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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