EXPRESSION AND FUNCTIONAL ROLE OF CYCLOOXYGENASE ENZYMES IN CERVICAL CARCINOMA

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DECLARATION

Except where due acknowledgement is made by reference, the studies undertaken herein are the unaided work of the author. No portion of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Kurt J. Sales
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ABBREVIATIONS

AA	Arachidonic acid
ANOVA	Analysis of variance
AMPS	Ammonium persulphate
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
APC	Adenomatous polyposis coli
Asn	Asparagine
ATP	Adenosine triphosphate
bp	Base pairs
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
COX	Cyclooxygenase
cAMP	Adenosine 3', 5'-cyclic monophosphate
cDNA	Complementary deoxyribonucleic acid
CIN	Cervical intraepithelial neoplasia
cpm	Counts per minute
CRE	cAMP response element
ddH_2O	Double distilled water
DAB	3,3'-diaminobenzidine
DNA	Deoxyribonucleic acid
DOX	doxycycline
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
FIGO	International federation of obstetricians and gynaecologists
g	gravity
H_2O	Water
HPV	Human papillomavirus
Hyg	Hygromycin
IBMX	3-isobutyl-1-methylxanthine
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
JNK	c-Jun amino-terminal kinase
kb	Kilobases
kDa	Kilodaltons
LPS	Lipopolysaccharide
MAPK	Mitogen-associated protein kinase
MEK	Mitogen-associated protein kinase/Extracellular signal-regulated kinasekinase
NFκB	nuclear factor-kappaB
nm	nonometer

NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PG	Prostaglandin
PGES	Prostaglandin E synthase
PMSF	phenylmethylsulphonylfluoride
PPAR	Peroxisome proliferator-activated receptors
PVDF	Polyvinylidene difluoride
Rb	Retinoblastoma
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
SAPK	stress-activated protein kinase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SIL	Squamous intraepithelial lesion
Taq	Thermus aquaticus
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylenediamine
Tet	Tetracycline
Tris	Trizma base
VEGF	Vascular endothelial growth factor
X-Gal	5-bromo-4-chloro-indoyl β-D-galacto-pyranoside

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ABSTRACT

Cervical cancer is considered an important clinical problem in sub-Saharan Africa. Recent studies have suggested that epithelial tumors may be regulated by cyclooxygenase enzyme products. The purpose of this thesis was to determine the expression, localisation and possible functional role of cyclooxygenase enzymes in cervical carcinomas. The initial aim of the study was to determine whether cyclooxygenase-1 and cyclooxygenase-2 expression and prostaglandin E_2 synthesis are up-regulated in cervical cancers. Real-time quantitative reverse-transcriptase polymerase chain reaction and Western blot analysis confirmed cyclooxygenase-1 and cyclooxygenase-2 ribonucleic acid and protein expression in all cases of squamous cell carcinoma and adenocarcinoma investigated. In contrast, minimal expression of cyclooxygenase-1 or cyclooxygenase-2 was detected in histologically normal cervix. Immunohistochemical analyses localised the site of cyclooxygenase-1 and cyclooxygenase-2 expression and prostaglandin E_2 synthesis to neoplastic epithelial cells of all squamous cell carcinomas and adenocarcinomas studied. Immunoreactive cyclooxygenase-2 and prostaglandin E_2 were also co-localised to endothelial cells lining the microvasculature. Minimal cyclooxygenase-1, cyclooxygenase-2 and prostaglandin E_2 immunoreactivity were detected in normal cervix. In order to establish whether prostaglandin E_2 has an autocrine/paracrine effect in cervical carcinomas, we investigated the expression of two subtypes of prostaglandin E_2 receptors, namely EP2 and EP4, by real-time quantitative reverse-transcriptase polymerase chain reaction. Expression of EP2 and EP4 receptors was detected in cervical squamous cell carcinoma and adenocarcinoma and was significantly higher than that detected in histologically normal cervix. The functionality and signalling of the EP2/EP4 receptors was assessed by investigating adenosine 3', 5'-cyclic monophosphate generation following *in vitro* culture of cervical cancer biopsies and normal cervix in the presence or absence of 300nM prostaglandin E_2. Adenosine 3', 5'-cyclic monophosphate production was detected in all carcinoma tissue following treatment with exogenous prostaglandin E_2 and was significantly higher in carcinoma tissue than that detected in normal cervix. To explore events associated with cyclooxygenase-1 up-regulation, we developed a doxycycline-regulated expression

system (Tet-Off system) in HeLa (cervical carcinoma) cells. Overexpression of cyclooxygenase-1 in HeLa cells resulted in induced expression of cyclooxygenase-2 and prostaglandin E synthase concomitant with increased prostaglandin E_2 synthesis. Treatment of HeLa cells overexpressing cyclooxygenase-1 with the dual cyclooxygenase inhibitor indomethacin or selective cyclooxygenase-2 inhibitor NS-398 significantly reduced prostaglandin E_2 synthesis. Indomethacin treatment abolished the up-regulation of expression of cyclooxygenase-2 and prostaglandin E synthase in HeLa cells, but NS-398 treatment only partially abolished the up-regulated expression of cyclooxygenase-2 and prostaglandin E synthase suggesting that the observed up-regulation was mediated by enzyme products from both COX enzymes. In order to assess whether enhanced prostaglandin E_2 synthesis, following cyclooxygenase-1 induction, would act in an autocrine/paracrine manner, we investigated the effect of cyclooxygenase-1 on the expression of the different isoforms of prostaglandin E receptors (EP1-4). We found that the adenosine 3', 5'-cyclic monophosphate-linked prostaglandin E_2 receptors (EP2/3/4) were significantly up-regulated by cyclooxygenase-1 overexpression. This was coincident with enhanced adenosine 3', 5'-cyclic monophosphate-responsiveness of cyclooxygenase-1 overexpressing cells to administration of exogenous prostaglandin E_2 ligand. Finally, overexpression of cyclooxygenase-1 was associated with enhanced expression of the angiogenic factors; basic fibroblast growth factor, vascular endothelial growth factor, angiopoietin-1 and angiopoietin-2. This up-regulation of angiogenic factor expression was abolished by indomethacin and partially reduced by NS-398. These results confirm that cyclooxygenase-1, cyclooxygenase-2, EP2, EP4 expression and prostaglandin E_2 synthesis are up-regulated in cervical carcinomas and suggest that prostaglandin E_2 may regulate neoplastic cell function in cervical carcinoma tissue in an autocrine/paracrine manner via the EP2/EP4 receptors. Furthermore, these data indicate that cyclooxygenase-1 up-regulation modulates the expression of pro-angiogenic factors that may act in an autocrine/paracrine manner to enhance and sustain tumorigenesis in neoplastic cervical epithelial cells. It is likely that similar mechanisms may act *in vivo* to modulate tumorigenesis of cervical carcinomas.

CHAPTER 1

GENERAL INTRODUCTION

1. INTRODUCTION

Malignant neoplastic transformation is a multifactorial process resulting from aberration in cell regulatory circuits governing cell proliferation and homeostasis. More than 100 distinct cancers have been reported to occur in various cells and organs of the human body. Although the initiating response may be different for each type of cancer, all tumors share certain common characteristics, namely: self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis and apoptosis evasion (1). Numerous studies have suggested that epithelial tumors may be regulated in an autocrine/paracrine manner by cyclooxygenase (COX) enzyme products (2-10). This thesis focuses on a possible regulation of neoplastic cell function in cervical carcinomas by COX enzymes. The aim of this introduction is to provide a brief outline of the significance, possible causes, prevention, treatment and histopathological classification of uterine cervical carcinomas, and the roles of cyclooxygenase enzymes and prostaglandins in mediating events associated with physiology and pathophysiology.

1.1. Epidemiology Of Cervical Cancer in South Africa

Cancer of the uterine cervix is one of the leading causes of cancer-related death in women world-wide (11-16). Cervical cancer is particularly common in less developed countries, including South and Central America, Southeast Asia and Sub-Saharan Africa, where 80 % of the world's cervical cancers occur (13). The prevalence of cervical cancer in South African women is reported as being the highest in the world, occurring on average in 60 out of every 100 000 women (12, 15, 17). In certain groups of South African women screened at the primary health care clinic in Soweto, Johannesburg, the detection rate of invasive cervical cancers is 1.8 per 1000 women (18). This is very high compared with countries such as Finland, which are regarded as having one of the lowest incidence of cervical cancer, occurring only in 3.8 per 100 000 women (19), and Columbia (which is regarded as having a high incidence of cervical cancer) where the incidence is 48.2 per 100 000 women (20). Currently, the National Cancer Registry

estimates that 1 in 4 South Africans will develop cancer in their lifetime (16). Cancer of the cervix is the most common cancer in Black (31.2 %) and Coloured (22.9 %) South African women, second most common cancer in Asian (8.9 %) and fourth most common cancer in White South African women (2.7 %) (15, 16). The lifetime risk of developing cervical cancer is 1:34 for black women and 1:93 for white women (16). The peak incidence of cervical cancer in women is between 50 and 54 years of age, with more than 80 % presenting with cervical carcinoma at 40 years of age or older (21). Although cervical dysplasia and cervical carcinoma are diseases generally associated with middle-aged women, statistics from the annual reports of the cytopathology laboratory at Groote Schuur Hospital, Cape Town show increased incidence of cervical neoplasia in the 29 to 39 year age group (12). Cancer of the cervix is thus regarded as an important clinical problem in South Africa.

1.2. Histology Of The Uterine Cervix

The function of the cervix is to admit spermatozoa to the genital tract to allow for pregnancy during ovulation as well as to protect the uterus against bacterial infection. The cervix forms part of the lower uterus. It is fibromuscular in origin and is continuous with the vagina below and uterus above, connecting the vagina and uterus via the endocervical canal. A representative uterine cervical section is illustrated in Figure 1.1. The endocervical canal is lined by columnar epithelium, whilst the exocervix is covered by stratified squamous epithelium. The junction between the squamous epithelium of the cervix and the columnar epithelium originating from the endocervical glands is referred to as the squamocolumnar junction. During adolescence and pregnancy, the squamous epithelium (metaplastic squamous epithelium) replaces the columnar epithelium by metaplasia to form a new squamocolumnar junction. The area between the new and old squamocolumnar junction is referred to as the transformation zone. It is proposed that carcinogens act at the transformation zone to cause cervical neoplasia (22, 23).

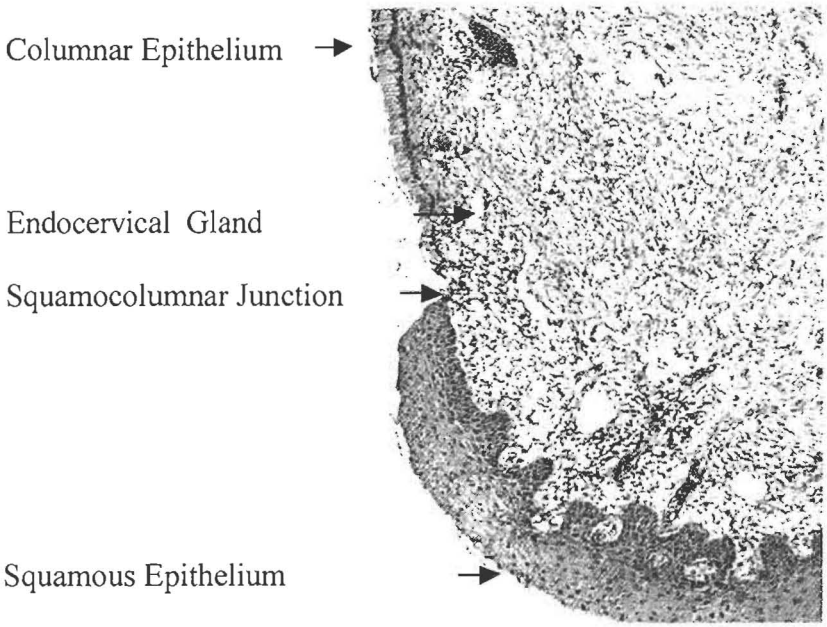


Figure 1.1. Histology of the Uterine Cervix. Haemotoxylin- and eosin-stained section (Magnification X 200) (24).

1.3. Pathology Of Pre-invasive lesions (Cervical Intraepithelial Neoplasia)

Cervical cytology and histology are the tools used for clinical diagnosis of cervical cancer in most countries (13, 22, 23). Histopathological confirmation after colposcopy with biopsy is used to determine the extent of pre-malignant disease. The colposcope, a stereoscopic binocular low power microscope, is used to visualise epithelium of the cervix. Colposcopy is used to evaluate the cervix to determine the size of the lesion, the area of cervix involved, and to evaluate the transformational zone, as the entire squamocolumnar junction is at risk for developing neoplastic lesions. Directed biopsy is used to sample tissues directly to gain a magnified examination of abnormality of the cervix (22). Depth of invasion, measured in millimetres, is one of the criteria used by histopathologists to distinguish between *in situ* and invasive cancer (13, 22). Changes in the metaplastic processes at the transformation zone leading to precursor lesions in the cervical epithelium may lead to dysplasia and give rise to invasive cervical cancer *in situ* (13, 22, 23). Although regression can occur, the greater the degree of dysplasia, the greater the chance of progression to invasive cancer.

Much controversy still surrounds the histopathologic classification of cervical cancer precursors. The World Health Organisation (WHO) classification system uses the terminology 'dysplasia' and 'carcinoma *in situ*' to refer to precursor lesions (25). In the 1960's Richart introduced the terms cervical intraepithelial neoplasia (CIN), which was later modified (26). The nomenclature CIN grades I to III are used to describe pre-invasive epithelial lesions or various categories of dysplasia and carcinoma *in situ*. CIN grade I is equivalent to mild dysplasia in which undifferentiated cells (cells which are atypical with hyperchromatic nuclei and increased nuclear-cytoplasmic ratio demonstrating increased mitotic index) occupy approximately the lower one third of the epithelium. CIN grade II is equivalent to moderate dysplasia where undifferentiated cells replace two thirds of the thickness of normal epithelium. CIN grade III denotes severe dysplasia and carcinoma *in situ*. Severe dysplasia describes a condition in which undifferentiated cells replace all but one or two of the most superficial cell layers of the cervical epithelium. When undifferentiated cells replace the entire surface of the

epithelium, the diagnosis of carcinoma *in situ* is made. All degrees of dysplasia are pre-invasive - meaning that the basement membrane (stromal epithelial junction) remains intact. The Bethesda System (27) of cytologic diagnosis uses low-grade squamous intraepithelial lesions (LoSIL) to describe lesions previously referred to as dysplasia or CIN1 and high-grade squamous intraepithelial lesions (HiSIL) to denote severe dysplasia and carcinoma *in situ*. There is currently no consensus as to which terminology should be used (22, 23, 28).

1.3.1. Low-Grade Squamous Intraepithelial Lesions

Low-grade lesions are characterised by disorganisation of the basal cell and parabasal layers of the epithelium, crowding and overlapping of cells and loss of polarity and koilocytosis (atypical nuclei and cytoplasmic cavitation) - indicative of human papillomavirus (HPV) infection. Low-grade squamous intraepithelial lesions are associated with infection by most HPV types (27, 29).

1.3.2. High-Grade Squamous Intraepithelial Lesions

High-grade squamous intraepithelial lesions are characterised by coarse granular chromatin, mitotic bodies, and loss of normal cell polarity and high nuclear: cytoplasm ratio. The effects of HPV are less prominent in high-grade squamous intraepithelial lesions than the low-grade and the HPV types associated with it are classified as being of intermediate- or high-oncogenic risk (27, 29).

1.4. Symptoms And Classification Of Cervical Cancer

Symptoms of cervical cancer include abnormal vaginal bleeding, persistent sero-sanguinous foul-smelling discharge, disruption of normal bladder or bowl function and leg oedema. Once the basement membrane is invaded, the neoplastic process is termed invasive. Carcinoma is defined according to the international federation of obstetricians and gynaecologists (FIGO; 30) staging upon physical examination (22, 23). The World Health Organisation (WHO) recognises three general categories of cervical cancer. These are squamous cell carcinoma, adenocarcinoma and other epithelial tumors including less common types such as adenosquamous carcinoma, glassy cell carcinoma, adenoid basal cell carcinoma as well as carcinoid-like and small cell carcinoma (25). Approximately 60 % to 80 % of cervical carcinomas are squamous cell carcinoma. Adenocarcinomas and to lesser extent adenosquamous carcinomas and rarely sarcomas, lymphoma and melanoma account for approximately 20 % of the invasive cervical carcinoma and display a variety of types and sub-types (23, 25).

1.4.1. International Federation Of Obstetricians And Gynaecologists (FIGO) Staging of Cervical Carcinomas.

I-Carcinoma confined to cervix

IA-Pre-clinical carcinomas of the cervix (diagnosed by microscopy only)

IAi-Minimal microscopically evident stromal invasion

IAii-Lesions detected microscopically that can be measured to a depth of not more than 5mm of invasion from the base of epithelium. The horizontal spread should not be more than 7mm.

IB-Lesions of greater dimension than stage IAii

IIA-Extends to upper 2/3 of the vagina

IIB-Extends to paracervical tissue

IIIA-Extends to lower 1/3 of the vagina

IIIB-Pelvic sidewall extension or uretal obstruction on intravenous pyelogram

IVA-Bladder or rectal mucosal involvement

IVB-Distant metastasis.

1.4.2. Adenocarcinoma

Adenocarcinoma of the cervix arises from glandular epithelium lining the endocervical canal and the endocervical glands, and is considered to be multifocal in origin. The WHO has classified adenocarcinoma into 5 subtypes: 1) endocervical adenocarcinoma, 2) endometrial adenocarcinoma, 3) clear cell carcinoma, 4) adenoid cystic carcinoma and 5) adenosquamous carcinoma. Patients with adenocarcinoma of the cervix have risk factors that are similar to those associated with endometrial adenocarcinoma including obesity, diabetes, hypertension and nulliparity. HPV 16 and 18 are the most frequently detected types of HPV in adenocarcinoma and squamous cell carcinoma, with HPV 18 more frequently detected in adenocarcinoma than squamous carcinoma (31).

1.4.3. Squamous Carcinoma

Squamous cell carcinomas arise in the metaplastic squamous cells lining the exocervix. As with adenocarcinoma, squamous cell carcinoma is associated with HPV infection, early and frequent child bearing, multiple sexual partners, multiple pregnancies, cigarette smoking and human immunodeficiency virus (HIV) infection (22, 23). Small stage I lesions are generally visible on speculum examination. Frequently, these give rise to barrel carcinoma, especially when from endocervical canal origin. Tumors are classified according to FIGO staging.

1.5. Causes, Prevention and Treatment of Cervical Cancer.

Current evidence indicates that the main cause of cervical cancer is by infection of the uterine cervix with HPV, particularly type 16, 18, 31, 33, 35 and 45 which are transmitted sexually (20, 32). In addition to HPV, women presenting with cervical carcinoma show a high incidence of venereally transmitted disease, genital warts, genital herpes and trichomonas infection (33-36). The incidence of cervical cancer is approximately 10 times greater in developing countries, whilst the occurrence of HPV is only 2 to 3 times greater in developing countries compared with developed countries (20). This suggests that factors other than HPV may be associated with cervical neoplasia and cancer progression.

1.5.1. Human Papillomavirus (HPV) As A Causative Agent For The Development Of Cervical Cancer

Extensive laboratory and epidemiological evidence have established that HPV is a causative agent for the development of invasive cervical cancer and its precursor lesions. Papillomaviruses together with simian virus (SV-40) and polyoma virus are classified as members of the Papovaviridae family. All members of the family are classified as DNA tumor viruses (37). Papillomaviruses are double-stranded DNA viruses of approximately 8000 base pairs in length. The viral genome is divided into three sections:

A non-coding upstream regulatory region (URR) of about 400 base pairs in length for regulation of transcription of viral proteins and virions (38); an early region downstream of the URR containing six open reading frames important for viral replication; and a late region of two open reading frames encoding for the major and minor capsid proteins.

Human papilloma viruses preferentially infect the epithelia of the mucosa and skin forming benign epithelial proliferations at the site of infection. To date, 70 different human papilloma viruses have been identified. HPV types 16, 18, 45 and 56 are viruses classified as being of high oncogenic risk, as these are viruses found to be most frequently associated with genital tract carcinoma. Other groups of HPV including types 5, 8, 9, 15, 17 are genital tract viruses found to cause squamous intraepithelial lesions (SIL) of the cervix (39). Various other types of HPV are associated with SIL and invasive carcinomas of the cervix, however these occur infrequently or are classified into low oncogenic risk groups.

Cervical carcinomas infected with the high-risk HPV types 16 and 18, express the E6 and E7 HPV oncogenes, which when transcribed give rise to the E6 and E7 HPV oncoproteins, which neutralize cellular tumor suppressor function (40). The HPV-18 URR controls cell type-specific expression of the E6 and E7 viral oncoproteins in cervical carcinoma (HeLa) cells (41). E6 and E7 oncoproteins interact with and inactivate the intracellular tumor suppressor proteins p53 and retinoblastoma (Rb) and a correlation is known to exist between the expression of the viral oncogenes and development of cervical carcinoma (42), since dormant tumor-suppressor pathways can be reactivated by inactivation of the viral oncoproteins (40). The E6 oncoprotein binds to the cellular tumor-suppressor protein p53 forming a complex, and directs its degradation through the ubiquitin pathway (43, 44). Thus E6 expression results in the loss of p53 function in cells, including stimulation of apoptosis and inhibition of the antiapoptotic protein bcl-2 (45). The p53 protein is mutated in many human tumors but is usually wild-type in early cervical tumors suggesting the inactivation of p53 by the E6 oncoprotein is analogous to an inactivating mutation. Two forms of p53 are expressed in patients presenting with cervical carcinoma, namely: the arginine form and the proline

form. The arginine form of p53 is more susceptible to degradation by the HPV E6 oncoprotein than the proline form. Patients with this polymorphism are more likely to be susceptible to cervical neoplastic transformation by HPV than the remainder of the population, and patients with two copies of the arginine form have a seven fold higher risk of developing cervical cancer than those carrying the proline form (44). The E7 oncoprotein binds to and inactivates the cellular tumor-suppressor protein retinoblastoma as well as stimulates intracellular cytokines such as interleukin-1α (IL-1α). IL-1 modulates several inflammatory signalling pathways by activating downstream effectors including the mitogen-associated protein kinase (MAPK) and COX pathway (46-49).

1.5.2. Other Risk Factors Implicated As Causative Agents For Developing Cervical Cancer

In addition to HPV infection, various other factors have been identified as causative agents for the development or progression of cervical carcinomas. These factors, significant in the aetiology of cervical intra-epithelial neoplasia, includes long term cigarette smoking, oral contraceptives, multiple sexual partners and sexually transmitted infection by herpes simplex virus type-2 and chlamydial infection (11, 14, 23, 35). In sexually active women, the neoplastic cervical epithelium may be regulated by seminal plasma. Repeated exposure of neoplastic cervical epithelial cells to seminal plasma has been shown to promote the release of matrix metalloproteinases (MMPs), which would in turn cause degradation of the extracellular matrix and enhance metastasis and cervical tumorigenesis (50). In addition, repeated exposure to prostaglandins present in human seminal fluid may accelerate the progression of disease by reducing the body's natural auto-immune response, as prostaglandins mediate their role in tumor development partly through their immunosuppressive ability (51, 52). This may be augmented by factors such as the age at first intercourse as the immature cervix is covered by an immature metaplastic squamous epithelium, which is thought to be highly susceptible to neoplastic transformation (11, 23, 35). Taken together, this suggests that

although infection of the cervical epithelium with HPV may be the initiator of neoplastic cell transformation, the progression to cancer is multifactorial.

1.5.3. Cytology Screening As A Modality For Preventing Cervical Cancer.

Cervical cytological screening can detect precursor intraepithelial lesions of the cervix thereby allowing appropriate medical treatment and enhanced prognosis. Certainly in more developed countries, cytological screening has been efficacious in the management of cervical cancer as it increases the diagnosis of pre-invasive disease, thereby lowering the incidence of invasive disease. However in developing countries, there are no well-organised screening programs resulting in detection of most cervical cancers at advanced stages (13). Although many women are screened in developing countries, these women tend to be those at relatively low risk (21, 53). The development of an organised screening programme could help control cervical cancer (21, 54) and the maintenance of such programmes coupled to regular screenings and better socio-economic status could reduce the prevalence of cervical cancer in developing countries (15, 54-56). Large scale cytological testing is however very costly. Recently, the possibility of a 2-stage cervical cancer-screening programme in which 2 screening tests were performed sequentially (the second test performed only if the first result was positive - followed by treatment if both test results are abnormal) was investigated in South Africa (57). More simply, the two-stage screening involved direct visual inspection first, followed by cytologic testing, human papillomavirus DNA testing, or cervicography thereafter. Data arising from this study showed a reduction in false diagnosis and demonstrated that the implementation of such a screening programme would be efficacious in diagnosing and controlling disease in countries operating with limited resources.

1.5.4. Treatment Modalities.

The main therapeutic modalities currently used for the treatment of invasive cancer of the cervix are radical hysterectomy and radiation therapy, either alone or in combination. Chemotherapy is used to a lesser extent and is generally reserved for cases where the invasive cancer is too far advanced for treatment by radical surgery or radiation therapy. Radical hysterectomy is most successful in patients with FIGO stage 1a(ii), Ib and IIa tumors – where the tumor has not spread beyond the cervix to the parametria or lymph nodes. The larger the tumor, the greater the risk of metastases to the lymph nodes. Such cases are preferentially treated with radiation therapy rather than radical hysterectomy. Radiation therapy results in a good prognosis, however acute side effects such as perforation of the uterus, acute haemorrhagic cystitis, vaginal stenosis and obstruction of the small bowl are known to occur. A significant proportion of advanced stage disease is not cured by radiation therapy and increased interest is being shown towards the use of chemotherapy in such cases (58).

1.6. Cyclooxygenase Enzymes And Prostaglandins

Over the past 10 years, a role for cyclooxygenase (COX) and prostaglandins (PG) in cell neoplasias has been established. Although the existence of prostaglandins had been known for many years, the mechanisms involving prostaglandin biosynthesis were initially outlined in 1967 by Hamberg and Samuelsson. The term prostaglandin endoperoxide (PGH) synthase or COX was coined to describe the enzyme responsible for catalysing the conversion of fatty acids to prostaglandins (59). Several prostaglandins were subsequently isolated (60, 61). It was known that non-steroidal anti-inflammatory drugs (NSAIDs) inhibited COX activity (255) and this lead to the purification of COX-1 in 1976 as the key enzyme in the prostaglandin biosynthetic pathway (62). Subsequently the mode of action of NSAIDs was attributed to inhibition of a single cyclooxygenase. The role of NSAIDs in health and disease was altered by the discovery of a second inducible form of cyclooxygenase, called COX-2 (63, 64, 256, 257).

Initial observations and epidemiological studies showed that administration of NSAIDs on a regular basis reduced the mortality rate of patients suffering from colorectal cancer. The administration of NSAID aspirin on a continuous basis was noted to reduce the risk of colorectal disease by 40 % to 50 % demonstrating a negative correlation between NSAID use and development of colorectal cancer (65). Treatment of patients suffering from Familial Adenomatous Polyposis (FAP), an autosomal dominant disorder characterized by the formation of hundreds of colorectal adenomas and eventual colorectal cancer, with NSAIDs showed a marked reduction in the amount of colorectal polyps developed (66). The mechansim by which NSAIDs reduced the formation of polyps was unknown at the time, however it was postulated that NSAIDs affected eicosanoid biosynthesis by inhibiting COX. It was discovered that COX-2 was up-regulated from 2 to 50 fold in 85 % to 90 % of colorectal adenomas and adenocarcinomas and an association between COX-2 and colon cancer was established (4, 10, 65, 67). Studies in animal models of colorectal carcinogenesis showed a decrease in the size and number of colorectal polyps upon administration of selective COX-2 inhibitors (68). These data suggested a role for COX-2 in polyp formation and colorectal disease, making the COX-2 enzyme a likely target in treatment of patients with colon cancer. In cultures of rat intestinal epithelial cells, transfection with COX-2 cDNA and consequently increased synthesis of PGs decreased the apoptotic rate. The apoptotic rate was restored to normal by administration of the NSAID sulindac (69). Similar results were observed *in vivo* with chemically induced colon cancers in rats (70). *In vitro* studies further demonstrated that COX-2 overexpression in colon cells mediate tumor progression by enhancing cell proliferation (71) and the transcription of angiogenic factors which act on endothelial cells to enhance tubular formation and angiogenesis (8). Subsequently, several investigators discovered up-regulated COX-2 expression in epithelial carcinomas of the head and neck, oesophagus, pancreas, lung, prostate and bladder (3, 72-79). The up-regulation of COX-2 in epithelial tumors suggests a common theme might exist for the role of COX-2 in epithelial cell neoplasia. More recently a potential role for COX-1 in tumorigenesis has been established by the observation that endothelial cells transfected with COX-1 become tumorigenic (80). Up-regulated expression of COX-1 has been reported in human ovarian adenocarcinoma (81), murine

models of lung tumorigenesis (82), human prostate cancer (78), human breast cancer (83) and human gastric ulcers (84). As both COX isoforms are up-regulated in selected epithelial tumors, it is feasible that under certain circumstances both COX enzymes may function in enhancing or maintaining the neoplastic state.

1.6.1. Arachidonic Acid Metabolism

COX-enzymes catalyse the conversion of arachidonate in the cell to eicosanoids. Arachidonic acid is an essential fatty acid either obtained from the diet or by desaturation and elongation of dietary linoleic acid. Arachidonic acid metabolites, are collectively referred to as eicosanoids, and includes prostaglandins, thromboxanes and leukotrienes (85).

Eicosanoid biosynthesis, outlined in Figure 1.1, is controlled by the rate-limited release of arachidonic acid from plasma membrane phospholipids by phospholipase A2 (PLA2), as most polyunsaturated fatty acids in cells are esterified to phospholipids (85, 86). Following its release from intracellular stores, arachidonic acid is oxidised by either cytochromes P450 to epoxyarachidonic acids, or COX to prostaglandin H_2 (PGH_2) or by lipoxygenases to leukotrienes and lipoxins (85). As depicted in Figure 1.1, the NSAIDs such as aspirin exert their mode of action on eicosanoid biosynthesis by inhibiting COX and subsequent production of PG.

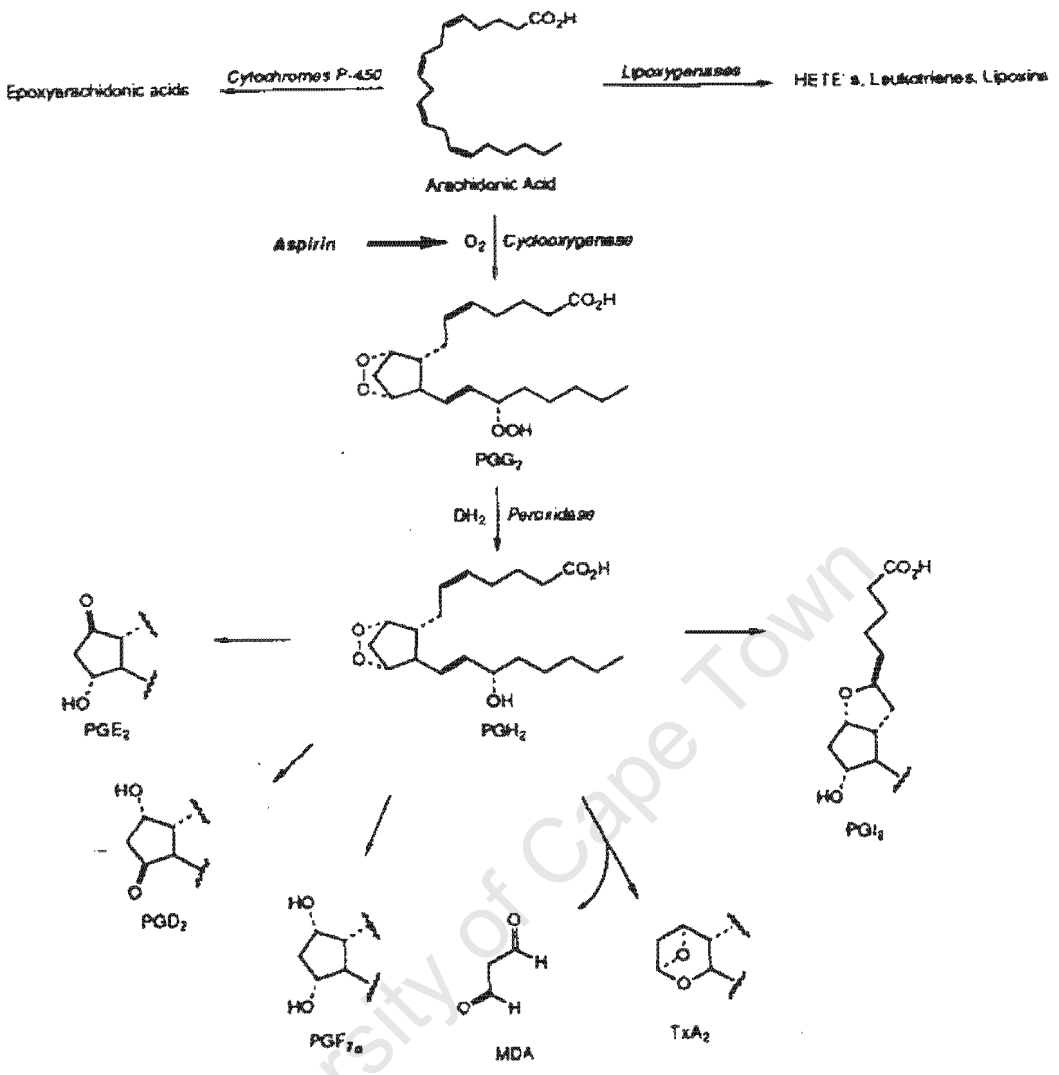


Figure 1.1. Schematic representation of the arachidonic acid cascade (85).

1.6.2. COX-1 and COX-2

COX catalyses the rate limiting step in the conversion of arachidonic acid to PGH_2 (86). The cyclooxygenase activity both cyclizes arachidonic acid and adds the 15-hydroperoxy group to form prostaglandin G_2 (PGG_2). The hydroperoxy group of PGG_2 is reduced to the hydroxy group of PGH_2 (62, 85). This intermediate serves as the substrate for terminal prostanoid synthases. These are named according to the prostaglandin they produce such that prostaglandin D_2 is synthesized by prostaglandin-D-synthase, prostaglandin E_2 (PGE_2) by prostaglandin-E-synthase and prostaglandin $F_{2\alpha}$ by prostaglandin-F-synthase (85-88). The physiological response to arachidonic acid oxygenation is determined by the levels of terminal prostanoid synthase enzymes in the cells and tissues as each prostaglandin has its own range of biological activities and may be cell-type specific (89).

Two cyclooxygenase isoenzymes, COX-1 and COX-2 have been identified (62, 85, 90, 91). COX-1 expression is considered to be constitutive, as basal levels of COX-1 mRNA and protein are observed to be present and generate prostaglandins for normal physiological functions (10, 67, 92). However, in some cell types including pulmonary artery endothelial cells COX-1 levels are increased during differentiation (93, 94). COX-2 expression is up-regulated in human gastric ulcers (84) and numerous epithelial cancers as mentioned in section 1.6. This suggests that the increased level of prostaglandins and other eicosanoids present in cancer tissue is a consequence of up-regulated COX-2. More recently, it has been suggested that both COX isoforms can be up-regulated (95). *In vitro* models have demonstrated that the transcription of both COX isoenzymes can be induced by arachidonic acid, prostaglandins, vascular endothelial growth factor (VEGF), forskolin (an activator of adenylate cyclase) and dibutryl-cAMP (a cAMP analogue) (95, 96). In addition COX-1 expression is up-regulated in certain epithelial tumours (section 1.6) suggesting a role for both COX enzymes in tumorigenesis.

The primary structures of COX-1 and COX-2 from various species are known (97). The COX-2 gene, located on chromosome 1, is an immediate early-response gene

that is rapidly induced following stimulation of quiescent cells by growth factors, oncogenes, carcinogens and tumor-promoting phorbal esters (4, 5, 90, 92, 98). The human COX-2 gene is approximately 8.3 kb in length, whereas human COX-1 originates from a larger 22 kb gene located on chromosome 9. There is an 80 % to 90 % sequence similarity between COX-1 and COX-2 from different species (99). Murine and human COX genes are virtually superimposable, exhibiting 90 % sequence homology (85). COX-1 and COX-2, within the same species, are 60 % to 65 % homologous based on the amino acid composition (90, 99) and differ mainly in the regions corresponding to the N and C termini. One of the most unique differences between the two COX isoforms is the extended C terminus tail of COX-2 brought about by the insertion of 18 amino acids, inserted 6 residues from the C-terminus (99). Post translational processing of the COX-2 transcript results in the expression of protein homodimers of 72 kDa or heterodimers of 72 kDa and 74 kDa compared with translation of the COX-1 transcript which results in protein homodimers of 72 kDa subunits (90, 100). Both COX isoforms exist as dimers both structurally and functionally. Dimerisation is necessary for the catalytic processes (99). Both isoforms of COX are N-glycosylated at Asn^{68}, Asn^{144} and Asn^{410}. Glycosylation is necessary at Asn^{410} and either Asn^{68} or Asn^{144} for expression of the cyclooxygenase and peroxidase activities. Moreover COX-2 is N-glycosylated at Asn^{580} 50 % of the time explaining the presence of the 72 kDa and 74 kDa COX-2 subunits (69, 101, 102). Glycosylation of COX-2 at Asn^{580} does not alter activity (100). Both COX isoforms are integral membrane heme-glycoproteins that use arachidonic acid as substrates. The COX-2 active site is approximately 20 % larger than that of COX-1 and of slightly different shape (99). COX-2 will thus accept a wider range of fatty acids as substrates than COX-1 (91). COX-1 and COX-2 also differ in their ultrastructural locations. Both enzymes are present on the lumenal surfaces of the endoplasmic reticulum and inner and outer membranes of the nuclear envelope, however COX-2 appears to be more associated with the nuclear envelope than COX-1 (99, 103). Thus, although the two COX enzymes catalyse the same reaction, their respective patterns of distribution throughout the cell suggest that distinct biosynthetic pathways for the respective products may exist (104). The predominantly nuclear localisation of

COX-2 raises the possibility that it may be involved in gene transcription or regulation at the nuclear level. This modulation of cellular function may occur via nuclear peroxisome proliferator-activated receptors (PPARs) (105).

1.6.3. Inhibition of COX

COX enzymes are the pharmacological targets of the nonsteroidal anti-inflammatory drugs (NSAIDs). The interactions of the NSAIDs with the COX active sites have been extensively reviewed (106). All classical NSAIDs, such as aspirin, ibuprofen, sulindac, indomethacin and peroxicam inhibit both COX isoforms, but may bind more tightly to COX-1 than COX-2 due to differences in size and shape of the active site. Selective COX-2 inhibitors exhibit specificity towards COX-2 (85, 106). Generally, inhibition of COX enzymes by NSAIDs conforms to three inhibitory mechanisms, such as simple reversible inhibition as demonstrated by ibuprofen; time-dependent reversible inhibition as demonstrated by naproxen, indomethacin and meclofenamic acid and irreversible covalent inhibition as demonstrated by aspirin (107). Glucocorticoids such as dexamethasone (DEX) also inhibit prostaglandin production by reducing the amount of substrate available (108, 109).

Both COX isoenzymes catalyse the two-step conversion of arachidonate to PGH_2. All NSAIDs bind only in the active site and prevent the biosynthesis of PGH_2 without affecting the peroxidase activity of the enzyme (106). Aspirin (acetyl salicylate) competitively inhibits COX by covalently modifying it. Aspirin transfers its acetyl moiety to the single serine hydroxyl group (Ser^{530}) of COX. This occludes the substrate-binding site from arachidonic acid, thus inhibiting COX activity (85, 110). The affect of aspirin results in irreversible inhibition, requiring *de Novo* protein synthesis (85, 86, 110). By contrast other NSAIDs are competitive inhibitors, which form tight complexes with COX. These complexes eventually dissociate, and COX levels return to basal.

As mentioned in section 1.6, NSAID therapy reduces the risk of colorectal disease amoungst users. The NSAID sulindac reduces the size and number of colorectal

polyps in individuals with FAP who have undergone colectomy and ileoproctrostomy, and also reduce tumors in affected individuals who have not had surgical treatment (4, 66) and is thus more efficacious in preventing colon carcinomas than the NSAIDs indomethacin, ketoprofen, ibuprofen and aspirin in suppressing both invasive and non-invasive adenocarcinoma of the colon (111). Sulindac does not induce polyp regression in the stomach, small intestine or ileum (85). Discontinuation of sulindac treatment in individuals resulted in recurrence of tumors. NSAIDs also boost mechanisms involved in tumor immune surveillance and induce interferon, altered leukocyte migration and T-lymphocyte functions as well as inhibition of angiogenesis (112).

Non-selective NSAID therapy has been shown to cause gastrointestinal ulceration, platelet dysfunction and kidney damage in at least 1 % of users (99, 113). The pathology of NSAID therapy is attributed to the inhibition of COX-1, which is predominantly expressed in the stomach lining (99). COX-2 selective NSAIDs have little effect on COX-1 but dramatically inhibits the production of inflammatory prostaglandins brought about by COX-2 (99). In addition, several epithelial tumors overexpress COX-2. COX-2 produces prostaglandins, which inhibit apoptosis and stimulate angiogenesis and invasiveness (8, 69). The development of COX-2 inhibitors including celecoxib, rofecoxib and meloxicam appear to be safer than NSAIDs and may prove to be useful anti-cancer therapies. These inhibitors specifically target COX-2 and have exhibited dramatic antineoplastic activity in a number of tumor model systems investigated thus far, including: colon cancer cells implanted into nude mice, tumor production in APC mutant mice and carcinogen-induced tumors in rats (2, 68, 114-116).

1.6.4. COX Biosynthetic Signalling Pathways

Studies using COX knock-out mice (117) and selective COX inhibitors (106) have established that distinct biosynthetic pathways and physiological functions may be performed by the respective COX enzymes. Selective synthesis of prostanoids between COX-1 and COX-2 has been observed (118). The differences in intracellular distributions of the two COX enzymes suggests that COX-1 prostaglandins function via

the traditional cell-membrane bound GPCRs whereas COX-2 can function via cell-membrane GPCRs as well as nuclear receptors (99). Differences in cell signalling may be brought about by the different functions performed by the COX isoenzymes. COX-1 generally performs a homeostatic function maintaining cell physiology, whereas COX-2 is inducible and mediates responses towards inflammation, stress, hypotonicity and infection (99).

Both COX-1 and COX-2 display similar reaction kinetics towards arachidonic acid, although a greater concentration of arachidonic acid is generally required for activation of COX-1 (99). Arachidonic acid is released from membrane glycerophospholipids by three phospholipase A2 (PLA2) forms (87, 119). The activation of PLA2 can occur by any signalling pathway that results in the activation of intracellular calcium or MAP kinases (99). No specificity has been found for interaction with each specific PLA2 and COX isoenzyme (99). Coupling of PLA2 to COX is dependent on amplitude of stimuli. In HEK293 cells, bradykinin activation of PLA2 leads to the production of PGE_2 via both COX-1 and COX-2, however stimulation of the same cells with IL-1β results in products formed only via COX-2 (119). This indicates that the prostaglandin produced and consequent response depends on the effector stimulus. In ionophore-stimulated rat fibroblasts, calcium-dependent phospholipases are stimulated, resulting in activation of high levels of free arachidonic acid resulting in the production of prostaglandins by COX-1 (120). Preferential biosynthesis of prostaglandins by COX-1 may be reliant on the relative expression of COX in the cell. In many cells, COX-2 levels are typically only 20 % to 30 % of COX-1 (99). Thus altered patterns of expression of COX in the cell could lead to patho-physiology by causing an elevated biosynthesis and release of prostanoids.

1.6.5. Regulation Of COX

COX-1 is generally constitutively expressed in cells and tissues. In selected cells and tissues including endothelium, monocytes, platelets, renal collecting tubes and seminal vesicles COX-1 is reported to be constitutively expressed at high levels (99). Expression is known to increase in differentiating cells, suggesting that COX-1 is developmentally regulated (97). The COX-1 gene has a TATA-less promotor region containing multiple start sights for transcription. COX-1 expression is controlled by the Sp1 transcription factor. The Sp1 *cis*-regulatory element on the COX-1 promotor binds the *trans*-activating Sp1 protein. Deletion of the SP1 site is associated with significant reduction in basal levels of COX-1 (99).

COX-2 expression is rapidly induced following stimulation of cells with growth factors and mediators of inflammation such as IL-1 (47, 121). The contribution of COX-2 towards the development of cancers, especially cancers of the colon suggests that it is regulated differently from COX-1 (99). The stimulus necessary for inducing COX-2 expression differs depending on cell type and physiological activity. In the kidney COX-2 is up-regulated in response to increases in salt concentration, whereas in granulosa cells COX-2 is induced in response to follicle-stimulating hormone and luteinizing hormone (99). Signal transduction pathways linked to the transcriptional activation of COX-2 may differ depending on the stimulus. These pathways may be shared or convergent and include the NFκB and MAPK pathways (122, 123).

The MAPK signalling pathways are the more major signalling pathways employed by growth factors and oncogenes (99). The MAPK signalling cascade is divided into three pathways: ERK1/2, JNK/SAPK, and p38 MAPK (123). The MAPK pathways can be activated by various effectors such as IL-1, tumor necrosis factor alpha (TNFα), lipopolysaccharide (LPS) and phorbal esters. Inhibitors of MAPK pathways or dominant negative mutant kinases cause a reduction in COX-2 expression (99). In LPS-stimulated monocytes, COX-2 expression is inhibited following treatment of cells with inhibitors to ERK1/2 MAPK. In addition, administration of the p38 MAPK

inhibitor rapidly destabilises COX-2 mRNA. This suggests that COX-2 expression and posttranscriptional regulation is dependent on kinase signalling via the p38 MAPK and/or ERK1/2 MAPK in LPS-stimulated monocytes (48, 99, 124). Stimulation of cells with IL-1 induces COX-2 expression via the JNK/SAPK and p38 MAPK pathways (48, 99).

Five major regulatory elements flanking the 5'-region of the COX-2 gene have been identified as rigorous regulators of COX-2 transcription. These regulatory elements are: overlapping E-box and ATF/CRE sequences, NF/IL-6 CAAT enhancer binding sites and two NFκB binding sites (99). The NFκB signalling pathway can be activated by various effectors including hypoxia, endothelin, IL-1β and tumor necrosis factor alpha (TNFα) in various cell types (99). Mutations in the NFκB *cis*-regulatory region causes attenuated activation of COX-2 in response to TNFα stimulation as well as reduced binding of NFκB-like proteins, suggesting that NFκB regulates COX-2 expression in response to certain activators (99). The ATF/CRE site is activated by hetero-and homodimers of the c-fos, c-jun and ATF families (125) and the cAMP regulatory binding protein (CREB). Mutations in the ATF/CRE sequence reduces serum-, PDGF- and Src-stimulated transcription of COX-2 in mice (99, 126). This suggests a role for cAMP in regulating COX-2 expression. In addition the COX-2 promotor also contains a peroxisome proliferator-activated response element (PPRE) consensus site suggesting that peroxisome proliferators may modulate transcription of COX-2 (127, 128).

1.6.6. COX Knockout cells and mouse models

Cells lacking one of the COX genes have the ability to co-ordinately up-regulate cellular machinery to overcome the defects in prostanoid biosynthesis (129). It is observed that wild-type, COX-1 (-/-) and COX-2 (-/-) immortalised lung cells display differences in PGE_2 biosynthesis. Compared with wild-type cells, the COX-1 (-/-) or COX-2 (-/-) cells exhibit substantially elevated expression of the functional COX gene. In addition, basal and induced expression of the cytolsolic PLA2 is elevated in the COX-

deficient cells. The potential for cells to alter function and compensate for defects in the expression of certain genes has important clinical implications.

Mice that lack the gene for COX-1 appear to be perfectly healthy. Despite the cytoprotective role of the gastric mucosa postulated for COX-1, no gastric or intestinal ulceration, nor renal dysfunction is observed in COX-1 (-/-) knockout mice as a result of the disruption mutation (102). This result may not be surprising since the body uses parallel pathways to reinforce a common result and that use of NSAIDs does not alter kidney function. However, the prostaglandins produced by COX-1 are essential for foetal survival. Mortality rates are high for the majority of offspring born to COX-1 (-/-) knockout mice and it is proposed that the reduction in survival may be due to premature closure of the ductus arteriosus (102). By contrast, COX-2 (-/-) knockout mice show severe renal abnormalities and multiple reproductive failures. Female COX-2 (-/-) knockout mice display defective ovulation, blastocyst implantation and decidualisation and are infertile (130, 131). This implies a constitutive role for COX-2 in oocyte maturation and ovulation. In addition COX-2 plays an essential role in blastocyst implantation since the addition of selective COX-2 inhibitors to wild-type or COX-1 (-/-) mice reduces implantation of the blastocyst in a dose-dependant manner (131). The infertility in COX-2 (-/-) mice was not a result of reduced spermatogenesis, since male homozygous COX-2 knock-out mice produced normal spermatozoa (131). Moreover, the PGE_2 receptor isoforms are expressed correctly in COX-2 (-/-) mice suggesting that the reduction in implantation and decidualisation is not due to an alteration in the signal transduction pathways in the reproductive tract (131). Thus COX-2 plays an essential role in normal physiology of reproduction.

1.6.7. Prostaglandins and Prostaglandin Receptors

Prostanoids, collectively referred to as eicosanoids, are members of a group of compounds composed of oxygenated C_{18}, C_{20} and C_{22} derived from ω3 (n=3) and ω6 (n=6) fatty acids (99). Prostanoids can be categorised into 2 main groups: Either prostaglandins (PG), containing a cyclopentane ring or thromboxanes (TX) containing a cyclohexane ring. The exact metabolite synthesized is dependent on cell type and stimulus (132). Following synthesis, the prostanoid is transported out of the cell by means of a carrier-mediated process (133). Prostaglandins exert their biological function through receptor-mediated interaction. Separate receptors have been described for PGE_2, PGD_2, $PGF_{2\alpha}$, TxA_2 and PGI_2 (85, 134). There are eight types and subtypes of prostanoid receptors that are encoded by different genes. The receptors are G protein-coupled with seven transmembrane domains and form part of the superfamily of rhodopsin-type receptors. Each prostanoid receptor shows selective ligand binding specificity (134). More recently an intracrine function has been postulated for PGs, as PGs have been shown to interact with nuclear peroxisome-proliferator-activated receptors (PPARs) (105).

1.6.7.1. Prostaglandin E2 (PGE_2) and PGE_2 receptors.

PGE_2 exhibits a broad spectrum of physiological and pathological actions in the body. PGE_2 is biosynthesized in cells from PGH_2 by prostaglandin E synthase (PGES), a terminal enzyme in the COX-mediated PGE_2 biosynthetic pathway (85, 87, 88). Two glutathione-dependent forms of PGES, a cytosolic (cPGES) and membrane-bound isoform (mPGES) have been identified thus far. The cytosolic PGES preferentially converts COX-1-derived PGH_2 to PGE_2 and is associated with immediate prostaglandin biosynthesis (135). The inducible membrane-associated form is preferentially associated with COX-2 under conditions of limited arachidonic acid supply (but can couple to COX-1 under conditions where arachidonic acid is available) and is associated with delayed PGE_2 biosynthesis (136).

In vitro studies have shown that COX-2 inhibitors reduce PGE_2 production more profoundly than other prostaglandins (137). In macrophages, COX-2 preferentially metabolises arachidonic acid to PGE_2 (118). COX-2 and PGES expression and consequently PGE_2 biosynthesis is rapidly induced in response to administration of interleukin (IL)-1β (138) or lipopolysaccharide (139, 140). Moreover mPGES co-transfected with COX-2 has been associated with increased cellular proliferation and aberrant morphology (136). These data suggest that mPGES may act synergistically with COX-2 in promoting cellular transformation, presumably by augmented and sustained production of PGE_2.

PGE_2 elicits its autocrine/paracrine effects on target cells through interaction with transmembrane G protein coupled receptors (GPCRs) as mentioned in section 1.6.3 (141). To date four main sub-types of PGE_2 receptors have been identified based on responses to agonists and antagonists and are pharmacologically divided into EP1, EP2, EP3 and EP4 which utilise alternate and in some cases opposing intracellular signalling pathways. This diversity of receptors with opposing action may confer a homeostatic control on the action of an autocoid that is released in high concentrations close to its site of synthesis (142). PGE_2 interaction with the EP1 receptor increases intracellular calcium via G_q. EP2 and EP4 increase cAMP levels via $G_{\alpha s}$. Several splice variants exist for EP3 receptor and are coupled to different signalling pathways resulting in either a positive or negative cAMP response to PGE_2 administration depending on the splice variant and cell type. PGE_2 interaction with EP3 either decreases cAMP via $G_{\alpha i}$ or increases cAMP via $G_{\alpha s}$. (134, 143). It has generally been assumed that signal transduction cascades are initiated following ligand-receptor binding at the plasma membrane level. Recently however a nuclear location for EP receptors has been ascertained. In nuclear membrane extracts from cells, functional EP1, EP2, EP3 and EP4 receptors have been localised and their expression is associated with nuclear calcium flux and gene transcription. This suggests that PGE_2 may perform an intracrine function and directly regulate transcription of target genes (144, 145).

EP1 receptor expression is limited to organs such as the kidney, lung and stomach (146). EP1 receptors mediate neurotransmitter release as well as contraction of the smooth muscle of various tissues including the respiratory tract, gastrointestinal tract and myometrium (147). PGE_2 interaction with EP1, EP3 and EP4 is thought to be responsible for water resorption, ion transport and glomerular filtration in the kidney (134). EP1 knock-out mice display decreased aberrant foci formation to administration of carcinogens such as azoxymethane (148). EP2 is inducible in response to stimuli, and is the least abundant of the EP receptors. EP2 is induced in luminal epithelial cells in the uterus by gonadotropins. EP2 expression is induced coincident with COX-2 expression in the uterus at the time of blastocyst implantation, suggesting a role for EP2 in implantation (149, 150). The effects associated with EP2 receptors are considered to be inhibitory as they coincide with the increase in intracellular cAMP and activation of adenylate cyclase (147). EP2 null mutations in mouse models is associated with impaired ovulation and fertilisation, salt-sensitive hypertension, vasopressor or impaired vasodepressor response to intravenous PGE_2, loss of bronchodialation PGE_2 and impaired osteoclastogenesis *in vitro* (148). EP3 an EP4 receptors are widely distributed throughout the body (151, 152). Postulated roles for EP3 includes, regulation of smooth muscle contraction, acid secretion and modulation of central nervous system function (134). EP3 deficient mice display impaired febrile response to pyrogens, impaired duodenal bicarbonate secretion and mucosal integrity and enhanced vasodepressor response to intravenous infusion of PGE_2 (148). EP4 receptor expression increases when mice undergo pseudopregnancy (134). *In vitro* studies using rat colon epithelial cells have shown EP4 to be involved in mucin exocytosis suggesting a cytoprotective role performed by PGE_2 in normal gastrointestinal tissues (153). EP4 knock-out mice display patent ductus arteriosus, impaired vasodepressor response to intravenous infusion of PGE_2 and decreased inflammation bone resorption (148).

1.6.7.2. Other Prostaglandins And Their Receptors.

Prostaglandin D_2 binds to DP receptors. Very discrete expression of the DP receptor is detected in the human body. Localisation of DP receptor expression is confined to the retina and small intestine. Activation of the receptor leads to mobilisation of intracellular calcium, but not production of inositol phosphate (154). In mice, DP receptor expression is observed in the lung, ileum, stomach and uterus (155). PGD_2 is thought to play a role in sleep. PGD_2 perfused into the ventral surface of the rostral basal forebrain of rats induces bouts of slow-wave sleep suggesting that PGs mediate their role in sleep by interacting with the PGD_2-sensitive sleep-promoting zone in the brain (156, 157). DP receptor knock-out mice display decreased allergic responses towards ovalbumin-induced bronchial asthma suggesting that PGD_2 may act as a mediator of allergic asthma (148).

$PGF_{2\alpha}$ exerts its action via FP receptors, which are expressed most abundantly in the corpus luteum. $PGF_{2\alpha}$ is a potent inducer of luteolysis. Activation of FP receptors by $PGF_{2\alpha}$ results in tyrosine phosphorylation and subsequent increase in intracellular calcium flux, PLC activation and DNA synthesis, suggesting that stimulation of the pathway is associated with cellular proliferation (158). FP null mutations in mice are associated with loss of parturition (148).

PGI_2 or prostacyclin exerts its physiological functions via IP receptors. IP receptors are widely distributed throughout the dorsal root ganglia suggesting a role in mediation of pain processing. In the kidney, IP receptors are localised to the glomerulus suggesting a role in glomerular filtration (134). IP knock-out mice display sensitivity towards thrombosis as well as exhibiting decreased inflammatory swelling (148).

TXA_2 or thromboxane exerts its functions via TP receptors, which are expressed abundantly in the vasculature as well as the heart, lung and kidney (159). TXA_2 is necessary for platelet aggregation. TP null mutation in mice is associated with bleeding tendency and resistance to thromboembolism (148).

1.6.7.3. Peroxisome Proliferators And Peroxisome Proliferator-Activated Receptors (PPARS)

The traditional view of PGs are as effectors of biological action which mediate their autocrine/paracrine action by binding to GPCRs. However more recently nuclear PG receptors have been described (144, 145). In addition, PGJ_2 derivatives have been shown to interact with nuclear peroxisome proliferator-activated receptors (PPARs) (127). Thus another possible function of COX is to directly regulate gene transcription as a consequence of its perinuclear location (103). This regulation of cellular function may occur via PPARs (105).

PPAR ligands, which include fatty acids, prostaglandins and NSAIDs activate the transcription of genes involved in lipid metabolism. Activation of these genes occurs following binding of the PPAR ligands to the nuclear PPARs. PPARs are members of the nuclear steroid hormone receptor superfamily, including the steroid hormone, thyroid hormone, retinoid, PPAR subfamilies as well as a large number of orphan receptors, which act by altering the transcription of genes with which they associate by means of the peroxisome proliferator response element (PPRE) (128, 160). The PPAR subfamily comprises at least three subtypes found in vertebrate species: PPARα, PPARβ (also called PPARδ, NUC1 and FAAR) and PPARγ (160-162). PPARs are activated following interaction with heat-shock protein, phosphorylation and ligand binding. The activated form of PPAR interacts with the 9 cis-retanoic acid receptor alpha (RXRα) to form a complex, which then binds to the peroxisome proliferator responsive element (PPRE) in the target gene (160).

PPAR ligands interact with PPARs to enhance expression of COX-2 in colon cancer cells (128) by interacting with the PPRE region in the COX-2 promotor, causing enhanced transcription of COX-2 (99, 127). PPARs may thus play a role in cancer by activating the transcription of genes, such as COX-2, involved in carcinogenesis or by regulating gene expression as part of a chemopreventitive mechanism in response to NSAIDs. Activators of PPARα and PPARγ have

mechanism in response to NSAIDs. Activators of PPARα and PPARγ have demonstrated anti-tumor effects in various model systems (162, 163). PPARδ (also called PPARβ, NUC1 and FAAR) expression is associated with increased differentiation of various cell types including fibroblast, breast and colon epithelial cells and its expression has been associated with the development of colon cancer. Recent studies have shown that NSAIDs mediate their role in cancer prevention by inhibition of PPARδ. In addition PPARδ is a negative target of the APC gene, which is mutated in patients with FAP (an inherited predisposition to multiple colorectal polyps) (161). Thus PPARδ has been identified as another target of the APC pathway, which provides a link between NSAID-mediated chemoprevention and genetic alterations in colorectal tumors. Since the various PPARs appear to have either a tumor-promoting or tumor-attenuating effect, the biological role of PPARs in health and disease could be affected by the level of expression of the PPAR or its ligand in specific cells and may be cell type-specific. Since many COX inhibitors may act as PPAR agonists (128), our current understanding on the convergent roles of COX and PPARs in health and disease is limited.

1.7. Some General Physiological Functions of COX and Prostanoids

1.7.1. Stomach

COX-1 is responsible for producing cytoprotective prostaglandins in the stomach (86), although small quantities of COX-2 are also expressed (62). Activation of COX-1 leads to the production of prostacyclin (PGI_2) and PGE_2 which when released from the endothelium is antithrombogenic exerting a direct vasodilator action on the vessels of the gastric mucosa (164), and when released from the gastric mucosa is cytoprotective causing the release of bicarbonates and viscous mucous (165). PGs produced by COX-1 confer a cytoprotective role on the epithelial cells of the crypts of Lieberkuhn in the ileum. In irradiated mice, radiation injury results in a reduction in crypt stem cell number, which is further reduced by indomethacin but not the selective COX-2 inhibitor NS-398 (62). COX-2 is expressed in humans, mice and rats around the

periphery of gastric ulcers and may play a role in wound healing, since COX-2 inhibitors have a direct impact on angiogenesis and may delay ulcer healing (9)

1.7.2. Rheumatoid Arthritis

Inflammatory cytokines such as Interleukin (IL)-1 and IL-2 have been implicated in the inflammatory process resulting in rheumatoid arthritis (RA). The increase in inflammation is coincident with increase in COX-2 expression and PGE_2 synthesis in RA synovial tissues (166, 167). Inhibition of COX-2 and inflammatory prostanoids results in decreased inflammation (168). In addition anti-inflammatory cytokines such as IL-4 delivered adenovirally to RA-synovial tissue explants reduces the RA-associated inflammation by reducing levels of pro-inflammatory cytokines such as IL-1 as well as PGE_2 (169). Thus specific COX-2 inhibitors may be efficacious in the management of inflammatory disease such as RA (62).

1.7.3. Kidney

Maintenance of kidney function is dependent on vasodilator prostaglandins. COX-1 produces prostaglandins important for maintaining blood flow of the kidney (170). Glomerular filtration and cellular proliferation are regulated in the kidney by $PGF_{2\alpha}$ and PGE_2 produced and secreted by mesangial cells as well as PGI_2 and TXA_2 produced by renal capillaries (171). PGE_2 produced in the collecting tube modulates sodium ion and water resorption. Cultured rat mesangial cells increase their production of PGI_2 and PGE_2 after induction of COX-2. It is proposed that PGI_2 may directly stimulate renin secretion as a feedback control for inhibition of salt reabsorbtion (62). Under pathological conditions, increased PG synthesis in response to macrophage invasion of the kidney leads to impaired renal function (172).

1.7.4. Blood Platelets

COX-1 is the only isoform detectable in blood platelets and is responsible for the production of TXA_2 (62). TXA_2 is a potent stimulator of platelet aggregation (147). One of the more well-established side effects of NSAID therapy is loss of arachidonic acid-induced platelet aggregation. This effect of NSAIDs on platelet aggregation has been exploited as a therapy against thromboembolic disease (62). This therapy results in irreversible inhibition of COX-1, which in turn leads to inhibition of synthesis of TXA_2.

1.7.5. Bone

Prostaglandins regulate bone metabolism. PG biosynthesis in bone is stimulated by parathyroid hormone (PTH), cytokines, growth factors and mechanical stress (173). PGE_2 stimulates bone resorption and mineralisation (174, 175). In addition to inducing bone resorption, exogenous PGE_2 can induce bone formation (148).

1.7.6. CNS

COX-1 and COX-2 are found in neurones of the central nervous system (CNS) and may function in sensory processing via prostaglandins PGE_2 and PGD_2 (176). COX-1 expression is most abundant in the forebrain where it is postulated that prostaglandins are involved in integrative functions, including sensory processing. Low levels of COX-2 are reported in the forebrain (177). However, COX-2 is up-regulated by convulsive nerve activity, thus selective COX-2 inhibitors that can cross the blood brain barrier could modulate CNS function (176). It is postulated that PGE_2 modulates the febrile response by acting on the thermoregulatory area in the hypothalamus. PGE_2 production is stimulated by cytokines such as IL-1, which are released by the actions of pyrogens such as LPS and may act on the hypothalamus via the endothelial cells of the blood vessels perfusing the hypothalamus (62).

1.7.7. Ovulation

Prostaglandin synthesis is necessary for the release of mature oocytes from the ovary during ovulation. Lutenising hormone (LH) and follicle stimulating hormone (FSH) as well as phorbal esters stimulate ovulation *in vivo* by stimulating the production of PGE_2 and $PGF_{2\alpha}$ (86).

1.7.8. Luteolysis and Parturition

In ewes $PGF_{2\alpha}$ is an endogenous luteolysin, administered exogenously for synchronisation of oestrus. $PGF_{2\alpha}$ levels are observed to increase 10 fold on days 13 to 15, prior to the onset of luteolysis. COX activity is also observed to increase 3 fold in uterine tissue on days 13 to 15, indicating a role for COX and $PGF_{2\alpha}$ in luteolysis (86). PGE_2 and $PGF_{2\alpha}$ function to increase contraction of the uterine myometrium during pregnancy. Inhibitors of COX are seen to delay premature labour, whilst analogues of PGE_2 and $PGF_{2\alpha}$ stimulate labour.

1.8. Proposed Roles for COX in carcinogenesis

1.8.1. Angiogenesis

Angiogenesis is a multistep process characterised by; the activation of vascular endothelial cells by angiogenic factors; the migration of endothelial cells toward sources of chemotactic stimuli; degradation of the extracellular matrix to allow endothelial cell invasion of surrounding tissue and the differentiation of endothelial cells into microvessels. As the demand for nutrients and oxygen increases for tissue development, an increased vascularisation is necessary to supply nutrients to the tumor. Successful tumor establishment and metastasis depends on initiation of angiogenesis at the site of growth of the tumor cells. In order to increase vascularisation, cancer cells produce a wide variety of factors that contribute to angiogenesis, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), bFGF-binding protein,

platelet-derived growth factor (PDGF), endothelin-1, and iNOS (8, 178). COX-2 and PGE_2 are strongly associated with regulation of the angiogenic process during tumor development (179). In an *in vitro* model system, COX-2 overexpression in colon cells enhances the expression of angiogenic factors. Treatment of the COX-2 overexpressing cells with antibodies to combinations of angiogenic factors and by the NSAIDs, NS-398 and aspirin, inhibits the up-regulated angiogenic factor expression, suggesting that the observed increase in angiogenesis is mediated by COX-2 (8). Similarly, in nude mice implanted with sponges seeded with colon tumor cells, a rapid increase in tumor mass is observed coincident with an increase in vascular content. The increase in vascularisation and consequent tumor growth is reversed by treatment of mice with diclofenac, a dual COX-1 and COX-2 inhibitor (178).

Microvascular endothelial cells cover the entire inner surface of blood vessels in the body and differ from the large vessel endothelium. In addition to providing a selective barrier between the bloodstream and tissues, vascular endothelial cells are critical for angiogenesis (104). In an *in vitro* model system, overexpression of COX-2 in epithelial cells promotes cancer development and invasiveness by mediating the transcription of angiogenic factors that act on co-cultured endothelial cells to promote both migration of endothelial cells and their arrangements into tubular structures (7, 8). In carrageenin-induced granulation tissue in rats, PGE_2, as a consequence of induced COX-2, increases angiogenesis in a dose-dependent manner through VEGF formation. Administration of the selective COX-2 inhibitor NS-398 resulted in a coincident decrease in PGE_2 production, VEGF formation and reduction in angiogenesis (180). Moreover, COX-2 and PGE_2 may directly control the process of angiogenesis by acting on endothelial cells. Treatment of endothelial cells with selective COX-2 inhibitors has been shown to reduce microvascular tube formation and this effect is partially reversed by co-treatment with PGE_2 (9). Thus angiogenesis may be mediated in an autocrine/paracrine manner in tumor tissue by PGE_2 as a consequence of up-regulated expression of COX-2.

1.8.2. Apoptosis

Tissue homeostasis is a dynamic balance between cellular proliferation and cell death. Cell death is divided into two categories, necrosis and apoptosis (181). Apoptosis, or programmed cell death, is inhibited during tumorigenesis (1, 112, 182). Cell surface receptors which bind apoptotic ligands and intracellular sensors monitoring cell homeostasis are responsible for triggering apoptosis in response to abnormalities including DNA damage, hypoxia and oncogene action (183). Apoptosis is controlled by a variety of pro-apototic or anti-apoptotic genes. The anti-apoptotic bcl-2 gene is up-regulated in many tumors. Members of the bcl-2 family of proteins (Bax, Bak, Bid, Bim) which are pro-apoptotic or (bcl-2, bcl-Xl, bcl-w) which are anti-apoptotic, function by effecting cytochrome C release from mitochondria. Cytochrome C is a catalyst of apoptosis (184). These apoptotic proteins are differentially regulated in response to external stimuli. For example, the pro-apoptotic Bax is up-regulated in response to DNA damage. Bax in turn exerts its pro-apoptotic function by activating other pathways such as the p53 tumor suppressor gene as well stimulating cytochrome C release from mitochondria. Mutation or inactivation of other tumor suppressor genes also has severe implications for prolonging the cell cycle and facilitating cancer progression. Mutation of the p53 tumor suppressor gene, resulting in inactivation of the p53 protein is observed in 50 % of human cancers (185). In model systems, inactivation of the p53 tumor suppressor protein results in decreased apoptosis and increased cellular proliferation. Transfection of wild-type p53 tumor suppressor gene into certain cell lines, which lack the p53 gene, causes an increase in the apoptotic index (186). In mouse fibroblast cells, transfection of the p53 gene results in coincident decrease in the levels of COX-2. Overexpression of COX-2 and enhanced synthesis of PGE_2 may confer resistance to apoptosis by suppression of the transcription of target genes (such as *p53*) that may be involved in cellular growth/transformation (187). In various model systems tumorigenesis is reduced by administration of NSAIDs, presumably by stimulation of the apoptosis pathway by inhibition of COX (112, 182). In an *in vitro* model, rat intestinal epithelial (RIE) cells permanently transfected with COX-2 showed a reduction in the apoptosis index (69). Treatment of these RIE cells with NSAIDs resulted in

increased apoptosis (69). In addition, overexpression of COX-2 leads to prolongation of the G1 phase of the cell cycle through effects on cyclin D. This ultimately results in inhibition of apoptosis (69, 188). This suggests that COX-2 may mediate its role in cancer progression by inhibition of apoptosis.

1.8.3. Mitogenesis and proliferation

In normal physiology, cells require mitogenic growth signals in order to proliferate. Under pathological conditions such as cancer, cells acquire growth signal autonomy and many cancer cells acquire the ability to synthesise mitogenic growth factors to which they are responsive, forming an autocrine positive feedback loop to enhance mitogenesis and proliferation. Examples of this are demonstrated in glioblastomas and sarcomas, where cancer tissue produces PDGF and TGFα to enhance tumorigenesis in an autocrine manner (1). COX-2 mediates its role in tumorigenesis by increasing mitogenesis and cell proliferation in various model systems in response to stimulation with mitogens, cytokines and tumor promoters. In *in vitro* model systems of rat intestinal epithelial cells and mouse colon carcinoma cells, selective inhibition of COX-2 results in a decrease in serum-induced cell proliferation. In the same systems selective inhibition of COX-1 does not have the same anti-proliferative effect, suggesting that cellular proliferation in colon carcinomas is mediated by COX-2 and not COX-1 (189). Similar model systems have demonstrated that epidermal growth factor stimulation of COX-2 expression enhances mitogenesis of colon cancer cells. Blockade of COX-2 up-regulation by epidermal growth factor receptor antagonists or selective inhibition of COX-2 causes a dose-dependant decrease in mitogenesis (190). In COX-2 overexpressing colon cancer cell lines, proliferation is inhibited by administration of selective COX-2 inhibitors. In addition, the anti-proliferative effects following COX-2 inhibition were more exaggerated in cell lines highly expressing COX-2 compared with those that expressed COX-2 in lesser amounts (71). Since COX-2 is overexpressed in colon carcinomas, it is thus an attractive therapeutic target for treatment of colorectal cancer by NSAID therapy by down regulating mitogenesis and reducing cell proliferation. One of the main metabolites of COX-2 is PGE_2 (118) and COX-enzyme products such

as PGE_2 may thus act in a paracrine manner on adjacent cells to induce proliferation. In human bone cells, PGE_2 increases cell proliferation through activation of a verapamil-sensitive calcium channels by enhancing PLC activation and increasing intracellular calcium influx (191). The same mechanisms may be at work under pathological condition to enhance proliferation of cancer tissue. In non-small cell lung cancer, inhibition of PGE_2 synthesis as a result of NSAID treatment caused a reduction in proliferation and mitogenesis *in vitro* as well as *in vivo* in xenografts in nude mice (192). Thus, in carcinoma tissues, the process of tumorigenesis may be facilitated in an autocrine/paracrine manner by enhanced synthesis of PGE_2, as a result of COX-2 overexpression, which in turn would enhance mitogenesis and cell proliferation.

1.8.4. Tissue Invasion And Metastasis

Tissue invasion and metastasis are the terms used to describe the proliferation and movement of primary tumor cells from their site of origin to distant sites where new colonies are formed. Metastasis is the cause of 90 % of cancer related death (193).

An essential feature of tumor formation is local invasion of surrounding tissues. This is accomplished via the degradation of the extracellular matrix by enzymes called matrix metalloproteinases (MMPs), a family of proteolytic enzymes produced by both stromal and tumor cells. An increase in invasive and metastatic ability is mediated via up-regulation of protease genes, resulting in an increase in protease and matrix-degrading proteases – resulting in decreased cell surface interactions between adjacent cells and increased metastasis and invasion (1, 194). Matrix metalloproteinases such as MMP-2 and MMP-9 protein are up-regulated in cervical carcinomas compared with normal cervix suggesting that matrix and basement membrane degradation facilitates the spread of cervical cancers (195-198). In human cervical adenocarcinoma cells, epidermal growth factor (EGF) and transforming growth factor (TGF)-alpha stimulation increased MMP-2 levels concomitant with tumor cell migration (199). In addition, MMP-2 and MMP-9 are induced in cervical epithelial cells by seminal plasma (50). Thus in sexually active

women, repeated exposure to neoplastic epithelial cells to seminal plasma may may enhance cervical cancer progression by enhancing the expression of MMPs.

In order for tumor cells to become mobile to invade distant parts of the body where space and nutrients are not limiting, changes in cell-surface adhesion molecules (CAMs) – members of the immunoglobulin and calcium-dependent cadherin families are needed. The most widely observed alteration in CAMs is that involving E-cadherin, ubiquitously expressed in epithelial cells (200). Anti-growth signals are triggered when adjacent cells are coupled via E-cadherin. E-cadherin function is lost in many epithelial cancers – either by mutational inactivation or transcriptional repression or down regulation (201). More than 80 % of poorly differentiated tumors lack expression of E-cadherin. Expression of E-cadherin is down regulated in a number of solid tumors and is closely and inversely related to enhanced invasion of neoplastically transformed cells (202, 203). In addition, *in vitro* models overexpressing E-cadherin show reduced cell invasion and metastasis, further supporting the idea that E-cadherin acts as a suppressor of invasion and metastasis in epithelial cancers and that down-regulation of E-cadherin expression is associated with local invasion of tumor cells (1, 201, 204). In an *in vitro* model system, rat intestinal epithelial cells overexpressing COX-2 showed down regulation of E-cadherin compared with wild-type and COX-2 antisense cells suggesting that COX-2 up-regulation in tumors promotes cell metastases by down regulating cell adhesion molecules (69).

1.8.5. Malondialdehyde (MDA)

Several chemical carcinogens belonging to the class of polycyclic aromatic hydrocarbons and their dihydrodiol derivatives, as well as heterocyclic amines and aromatic amines are activated to mutagenic derivatives by COX, suggesting that multiple mechanisms exist in COX-mediated predisposition to carcinogenesis (205). The cyclooxygenase pathway generates malondialdehyde (MDA) by enzymatic and non-enzymatic breakdown of PGH_2. MDA is a direct acting mutagen in bacterial and mammalian test systems introducing frame-shift mutations and base-pair substitution

mutations (85). COX, by virtue of its peroxidase activity generates a range of xenobiotics and chemical carcinogens. Thus MDA as a result of co-expression of COX-2 and P450s may contribute to genetic instability and cancer progression (206).

1.9. Aims And Objectives Of The Thesis

As mentioned in section 1.1, cervical cancer is an important clinical problem in South African women. Gynaecological malignancies of this nature have an enormous socio-economic impact on health care, resources and morale of a country. Research into the molecular mechanisms governing cervical cancer and its precursors could lead to improved therapies for women suffering form this condition. Several investigators have reported on the efficacy of NSAIDs and selective COX inhibitors as possible therapies in the management of colorectal cancer. COX enzymes are up-regulated in numerous solid tumors and are considered to act either directly or indirectly in the process of tumorigenesis. The function of COX and relative contributions of its products towards the development of cervical neoplasia has yet to be elucidated fully. The aim of this research was to determine a possible functional role for COX and PGE_2 in cervical cell neoplasias. The specific aims of the research were to:

1. Determine the expression and localisation of cyclooxygenase (COX) and prostaglandin E_2 in cervical carcinomas and normal cervix. This was conducted on cervical cancer and normal tissues obtained from South African women as outlined in chapter 3.

2. Identify the prostaglandin receptors that may be involved in the role of prostaglandin E_2 in cervical carcinoma and possible signalling mechanisms involved. This was investigated by using cervical biopsy tissues and normal cervix obtained from South African women as described in chapter 3.

3. *Establish an inducible expression system to promote controlled overexpression of COX-1 in neoplastic cervical epithelial (HeLa) cells. This was achieved by stable transfection of the COX-1 gene into HeLa cells using a doxycycline-regulated gene expression system as described in chapter 4.*

4. Determine a possible autocrine/paracrine regulation of neoplastic cell function by COX-enzyme products by promoting inducible overexpression of COX-1 in HeLa cells. This was conducted by investigating possible genes, receptors and signal transduction pathways regulated by inducible expression of COX-1 in HeLa cells as described in chapter 5.

CHAPTER 2
GENERAL METHODS

2. MATERIALS AND METHODS

2.1. Chemicals and suppliers

All chemicals used were molecular biology grade, and were obtained from Sigma Chemical Company (Dorset, UK or Cape Town, RSA) and IBI (Cambridge, UK) unless otherwise stated. Enzymes were purchased from Boehringer Mannheim (Buckinghamshire, UK) or Promega (Southampton, UK or Cape Town, RSA). Phenol/Chloroform, pre-buffered with Tris pH 8.0, was purchased from Camlab (Cambridge, UK) and all photographic film was purchased from Eastman Kodak (Rochester, NY, USA) and supplied by Sigma. The following antibodies used for Western blotting were purchased from Santa Cruz Biotechnology, inc. (Autogenbioclear, Wiltshire, UK): COX-1 goat polyclonal (sc-1752); COX-2 goat polyclonal (sc-1745); β-actin goat polyclonal (sc-1616); VEGF rabbit polyclonal (sc-152); angiopoietin (Ang)-1 goat polyclonal (sc-6319); angiopoietin (Ang)-2 goat polyclonal (sc-7016); bFGF goat polyclonal (sc-1360) as well as the COX-1 and COX-2 blocking peptides (sc-1752p and sc-1745p respectively). The mouse anti-human CD34 primary antibody was purchased from Serotec (mca-547; Serotec, Oxford, UK). The polyclonal rabbit anti-PGES antibody was purchased from Caymen Chemical Co. (Caymen Chemical Co., Chesire, UK); anti-goat-alkaline phosphatase (AP), anti-rabbit-AP, cloning cylinders, G418, hygromycin (Hyg), doxycycline (DOX) and indomethacin were purchased from Sigma (Sigma Chemical Company, Dorset, UK). Samples and synthetic standards for the PGE_2 ELISA were purchased from Applied Therapeutics (Applied Therapeutics, Paisly, UK). NS-398 was purchased from Calbiochem (Calbiochem, Beeston, Nottingham, UK). HeLa-S3 cells were purchased from BioWhittaker (BioWhittaker, Berkshire, UK). HeLa Tet-Off cells and Tet-system-approved foetal calf serum were purchased from Clontech (Clontech, Hampshire, UK). Dulbecco's modified Eagle's medium nutrient mixture F-12 was purchased from Life Technologies (Gibco, Life Technologies, Paisly, UK), penicillan-streptomycin was purchased from PAA (PAA Laboratories Ltd., Middlesex, UK). ECF and ECLplus chemiluminescence systems were purchased from Amersham (Amersham, Little Chalfont, Bucks, UK).

2.2. Tissue collection and processing

Cervical specimens were obtained at the time of surgery/biopsy from patients that were attending the Gynaecologic Oncology Clinic at Groote Schuur Hospital, Cape Town and that had been previously diagnosed with invasive carcinoma of the cervix. Punch biopsies were taken from the lesion by an experienced Gynaecologist with a special interest in oncology. A portion of the biopsy was excised and fixed in formalin. The tissue was placed in disposable embedding moulds (Polysciences) followed by paraffin wax-embedding. Glass slides to be used for immunohistochemistry were washed in a 0.25 % solution of 3-aminopropyl triethoxysilane (TESPA, Sigma) in acetone, followed by a wash in acetone and finally a rinse in filtered double-distilled water and dried. Paraffin wax-embedded tissue was sectioned to a thickness of 5 μM using a hand operated "820" Spencer Microtome (American Optical Corporation) and a D-profile knife. Sections were floated on water, transferred to coated slides and dried overnight before use. The remaining portion of the biopsy was snap frozen in either dry ice or liquid nitrogen and stored at –70°C for quantitative RT-PCR or Western blot analysis or transported on ice for *in vitro* culture, PGE_2 stimulation and cAMP measurement. Histologically normal cervical samples were obtained from patients undergoing hysterectomy for non-malignant conditions. Informed consent was obtained from all patients before tissue collection. The study was approved by the University of Cape Town Research Ethics Committee.

2.3. RNA

2.3.1. Total RNA extraction.

Total RNA was isolated from fresh cervical tissue using a commercially available guanidinium thiocyanate-based extraction reagent Tri-Reagent (207) according to the manufacturer's protocol. Volumes were adjusted appropriately, allowing approximately 1 ml Tri-Reagent per 100 mg of tissue. Tissue, which was snap frozen after excision and stored at -70°C was homogenised for approximately 1 min using an Ultra-Turrak T8

homogeniser (IKA Labortechnik) in Tri-Reagent until completely dissociated and then supplemented with either 0.2 volumes of chloroform or 0.1 volume of 1-bromo-3-chloropropane (BCP) per volume of Tri-Reagent used. After vigorous shaking for 15 seconds, the mixture was allowed to stand at room temperature for 15 min before centrifuging at 12000g for 20 min at 4°C. Thereafter the upper aqueous layer, containing the RNA was transferred to a fresh RNase-free tube and the RNA precipitated with 1 volume of isopropanol. The RNA was pelleted by centrifugation at 12000g for 20 min at 4°C. Following removal of the supernatant, the RNA pellet was washed in 75 % ethanol and then dissolved in RNase-free water at 65°C for 10 min. RNA was quantified spectrophotometrically.

2.3.2. Determination of nucleic acid concentration

The concentration and quality of nucleic acids was determined by spectrophotometry at 260 nm and 280 nm. The concentration of the DNA or RNA was calculated from the 260 nm value obtained, given that an optical density of 1.0 is equal to 50 µg/ml for double stranded DNA, 33 µg/ml for single stranded DNA or 40 µg/ml for RNA. DNA and RNA quality was determined by dividing the 260 nm reading by the 280 nm reading; a ratio of 1.6 to 1.9 was taken to be of sufficient quality containing minimal protein contamination. Purified RNA was stored at –70°C.

2.3.3. Agarose gel electrophoresis

Plasmid DNA, restriction endonuclease digest products and RNA samples (Figure 2.1) were analysed by agarose gel electrophoresis. A 1.0% agarose gel was prepared by using 1 g Seakem agarose dissolved in 100 ml of 1.0X TBE buffer (appendix I). The agarose was melted and 200 µg/ml ethidium bromide added for visualisation of the DNA. The gel was poured to a depth of 1 cm, into a 7 cm by 10 cm gel tray containing an 8 cm comb and submerged in 1.0X TBE buffer in a midigel cell (Hybaid) once set. Plasmid DNA (10 µl) was run in a sample of solution containing 2 µl loading buffer (30 % w/v glycerol, 0.25 % w/v bromophenol blue, 0.25 % w/v xylene cyanol FF

and 0.5 M EDTA pH 7.0) and 8 µl water. Samples were electrophoresed at 100V for 1-2 hrs and compared to kb Ladder DNA markers (range 72 to 23130 bp), which were loaded in adjacent wells and run in parallel. The gel was viewed under UV light and documented by photography.

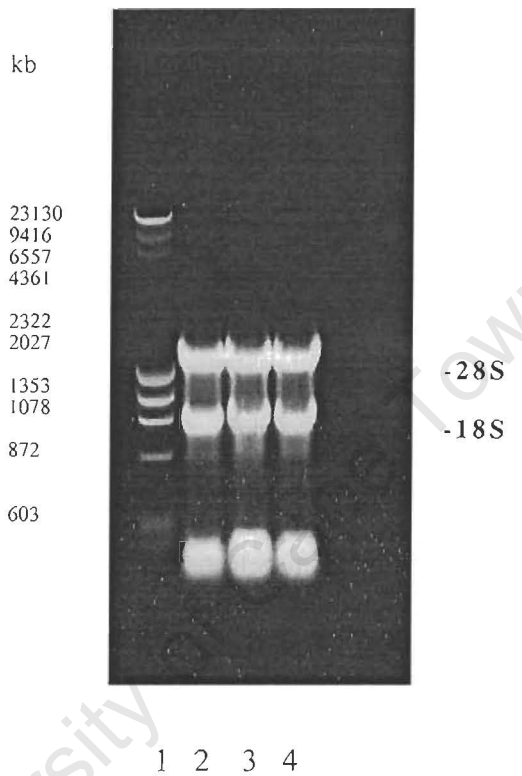


Figure 2.1. Photograph of EtBr-stained agarose gel showing RNA extracted according to section 2.3.1. Lane 1 shows kb Ladder DNA markers, Lanes 2- 4 are representative RNA samples extracted using Tri-Reagent.

2.3.4. Reverse-Transcription (RT) Reaction

Total RNA was extracted from cervical tissue using Tri-Reagent (Sigma) following the manufacturer's instructions as described in section 2.3.1. Aliquots of 3 µg of RNA were treated with DNase I (Gibco) following the manufactures protocol. Briefly, 3 µg total RNA was added to a reaction mixture containing 3 µl DNase 1 reaction buffer, 3 U DNase 1 to a final volume of 30 µl. The samples were incubated at room temperature for 15 min and then inactivated by addition of 3 µl of 25mM EDTA

at 65°C for 10 min. For reverse-transcription (RT) reactions, 1 µg of the DNase-treated RNA was reverse transcribed using a TaqMan GeneAMP RNA PCR kit (Perkin Elmer, PE Biosystems, Warrington, UK). After incubating the tubes for 10 min at room temperature, polymerase chain reaction (PCR) was performed for 1 cycle (1 hr 42°C, 5 min 99°C and 5 min 5°C) and contained 5 mM $MgCl_2$, PCR buffer, 1 mM of each deoxynucleoside triphosphate, 1 U/µl RNase inhibitor, 2.5 U/µl MuLV reverse transcriptase, random hexamers (1.25 µM), oligo-dT (1.25 µM) each (all from PE Biosystems) and RNase-free water to a final reaction volume of 10 µl. cDNA was stored at –20°C.

2.3.5. Real-time quantitative PCR

Real-time quantitative RT-PCR was performed to assess the relative expression of COX-1, COX-2 and prostaglandin E_2 receptor subtypes EP1-EP4 in HeLa cells and/or cervical carcinoma and normal tissues. RNA samples were extracted from cervical tissue and HeLa cells using Tri-Reagent as described in section 2.3.1 and were reverse transcribed as set out in section 2.3.4. A reaction mix was made containing Taqman buffer (5.5 mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP), ribosomal 18S forward and reverse primers and probe (all at 50 nM), forward and reverse primers for COX or EP receptor (300 nM), COX or EP receptor probe (200 nM), AmpErase UNG (0.01 U/µl) and AmpliTaq Gold DNA Polymerase (0.025 U/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 were 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 reaction run on an ABI Prism 7700 Quantitative PCR machine. COX and EP receptor primers and probe for quantitative PCR were designed using the PRIMER express program (PE Biosystems). Relative gene expression was determined manually from the imported PCR data by subtracting the average cycle

threshold value derived for the 18s ribosomal RNA from the average cycle threshold value obtained for the COX enzyme or EP receptor.

2.4. Protein

2.4.1. Protein extraction from cervical tissue

COX-1 and COX-2 protein expression in cervical carcinomas and normal cervix was assessed by Western blotting. Tissues were excised and transported as described in section 2.2 and protein extracted by homogenisation in protein lysis buffer (1% Triton X-100, 150mM NaCl, 10mM Tris/HCl pH7.4, 1mM EDTA, 0.1% SDS containing 2mM PMSF). Thereafter insoluble material was pelleted by centrifugation at 14000g for 20 mins at 4°C. 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 as described in section 2.4.3. A total of 50 µg of protein was resuspended in 20 µl of sample buffer (125 mM Tris-HCl pH6.8, 4% SDS, 5% 2-mercaptoethanol, 20% glycerol and 0.05% bromophenol blue) boiled for 5 mins at 95°C and run on a 10% SDS-polyacrylamide gel prior to Western blotting. All proteins were routinely stored at –70°C.

2.4.2. Protein extraction from cells

HeLa cells were grown until the desired confluency was reached in 10 cm dishes. Cells were lysed by addition of 400 µl of protein lysis buffer (1 % Triton X-100, 150 mM NaCl, 10 mM Tris/HCl pH7.4, 1 mM EDTA, 0.1 % SDS containing 2 mM PMSF). Proteins were extracted by freezing at –70°C or dry ice/ethanol and thawing at 37°C for 3 cycles. Thereafter insoluble material was pelleted by centrifugation at 14000g for 20 min at 4°C. The clarified lysate was removed to a new tube for protein quantification and SDS-PAGE.

2.4.3. Protein Quantification

All proteins were quantified according to the method of Bradford (208) using either the standard BIO-RAD assay (BIO-RAD) or DC Protein microassay (BIO-RAD) as per the manufacturer's instructions. Samples (in duplicate) were diluted in protein extraction buffer to a ratio of 1:50 to 1:100. A concentration range of bovine serum albumin (BSA, supplied with the assay) ranging from 0 µg/ml to 1000 µg/ml (in duplicate) was constructed in protein extraction buffer to achieve an OD_{595} response from 0.1 to 1.0 OD units. For the standard assay 0.1 ml of standard or sample was added together with 5.0 ml of dye reagent, diluted 1:4 according to the assay instructions, to a test-tube and incubated at room temperature for 5 min. Thereafter the colour reaction was assayed using the absorbance at 595 nm and compared to the reagent blank containing only sample extraction buffer and assay reagent. A standard curve was constructed from the standards and the concentration of proteins in the samples extrapolated from the standard curve. For the DC Microassay, 25 µl standards or sample were added to each well (in duplicate) of a 96 well plate. To this, 20 µl reagent A followed by 100 µl reagent B was added and the plate incubated at room temperature for 15 min. Thereafter the colour reaction was assayed using the absorbance at 690 nm using a Multiscan® MCC/340 plate reader. A standard curve was produced using the Assay Zap computer programme (Biosoft) and used to determine the average protein concentration of each sample.

2.4.4. SDS-PAGE

SDS-PAGE was performed using either pre-cast 4 % to 20 % Tris-Glycine gels (NOVEX, Invitrogen) or 10% Tris-Glycine gels prepared according to the method of Laemmli (209). For Laemmli gels, a 10 % w/v resolving gel and a 6.5 % w/v stacking gel was used. The resolving gel was prepared from 8.3 ml of 1.125 M Tris-HCl pH 8.8, 6.25 ml of acrylamide (Anachem, 40 % w/v acrylamide/bisacrylamide stock solution 19:1), 9.90 ml of ddH_2O, 300 µl of 10 % ammonium persulphate, 250 µl 10 % SDS and 20 µl of TEMED. The resolving gel was poured between the glass plates of a vertical protein gel apparatus (Bio-Rad), overlaid with water and left for 30 min to polymerise.

Thereafter, the water was removed and discarded and replaced with the stacking gel which was prepared from 4 ml of 0.375 M Tris-HCl pH 6.8, 2 mls of acrylamide (Anachem, 40 % w/v acrylamide/bisacrylamide stock solution 19:1), 5.575 mls of ddH_2O, 300 µl of 10 % ammonium persulphate, 125 µl 10 % SDS and 20 µl of TEMED. A comb was inserted for well formation. The gel was left to polymerise for 1 hr and then placed in the gel running tank with running buffer (25 mM Tris-HCl, 0.2 M glycine, 0.1 % SDS). A total of 100 µg of protein was resuspended in 38 µl of sample buffer (125 mM Tris-HCl pH 6.8, 4 % SDS, 20 % glycerol, 5 % 2-mercaptoethanol and 0.05 % bromophenol blue), boiled for 5 min at 95°C and loaded into separate wells of the gel. 10 µl of Seeblue™ (Novex) pre-stained protein markers were loaded into a separate well. Gels were run at 160V constant voltage prior to immunoblotting.

2.4.5. Western Blotting

Western blots were carried out according to the method of Harlow and Lane (210). After electrophoresis, the gel was transferred to a protein-free tray and equilibrated with transfer buffer (25 mM Tris/HCl, 0.192 M glycine, 20% methanol) for 15 min. Whatman no.3 paper (Whatman) and polyvinylidene difluoride membrane (PVDF) membrane (Millipore, Watford, UK) were cut to the dimensions of the gel and equilibrated in transfer buffer. The PVDF membrane was pre-soaked in methanol for 15 min followed by water prior to equilibration with transfer buffer. The blot was assembled by overlaying three pieces of pre-soaked Whatman paper with the PVDF membrane followed by the gel and three layers of Whatman paper to form a sandwich. Air bubbles were removed from each layer by rolling a clean glass pipette over each surface. Protein was transferred to the membrane for 1 hr at 12 V constant voltage or 390 mA constant current using a semi-dry blotter (BIO-RAD) assembled as per the manufacturer's instructions. Prestained molecular weight markers run in parallel on SDS-PAGE were used to determine whether transfer was successful. Following transfer, membranes were dried by soaking in methanol for 15 min. Membranes were blocked for 1 hr at 25°C in 5 % skimmed milk powder diluted in TBST (50 mM Tris-HCl, 150 mM NaCl and 0.05 % v/v Tween-20). Thereafter, membranes were incubated with the

relevant primary antibody at 4°C for 18 hrs. After washing three times with TBST, membranes were subsequently incubated for 1 hr respectively with the relevant secondary antibody. Proteins were either revealed on photographic film (Kodak) by chemiluminescence (ECLplus kit, Amersham) following the manufacturers instructions and quantified by scanning densitometry or by the ECF chemiluminescence system (Amersham) following the manufacturers instructions. Proteins developed by the ECF system were revealed and quantified by PhosphorImager analysis using the STORM 860 system (Molecular Dynamics, UK). The molecular weights of the proteins were determined relative to the mobility of the pre-stained markers on SDS-PAGE.

2.5. Control of antibody specificity

When synthetic antigen (blocking peptide) was not available, non-immune serum of the same species in which the primary antibody was raised was used, at equivalent dilutions, in TBS to control for antibody specificity. Where available, a blocking peptide specific for the antibody was purchased from a commercial supplier. To control for antibody specificity, the primary antibody was preadsorbed to a blocking peptide to compete the antibody from the antigen immobilised on PVDF or present in tissue sections mounted on glass slides. The preadsorbtion was carried out according to the manufacturer's protocol. Briefly, the highest antibody dilution at which a consistent positive result was achieved was determined. For example, for neutralisation of COX-1 and COX-2, this antibody dilution was determined to be 1:2000. Undiluted antibody was incubated with a 5 fold (by weight) excess of synthetic antigen (COX-1 and COX-2; Autogenbioclear) at 37°C for 2 hrs and then diluted in 5 % serum or 5 % fat free milk for immunohistochemistry or Western blot analysis respectively.

Control tissue for PGE_2 was incubated with rabbit anti-PGE_2 antibody preadsorbed to excess exogenous PGE_2. Briefly, the PGE_2 antibody was incubated together with 10 fold excess exogenous PGE_2 (Sigma) at 37°C for 2 hrs. Thereafter the antibody-ligand mixture was diluted and immunohistochemistry was performed as

described above. The rabbit antisera that were raised against PGE_2–complexed keyhole limpet hemocyanin have been previously characterised (211).

2.6. Histological staining of sections.

Histopathological diagnosis of cervical sections was carried out on haemotoxylin- and eosin-stained (H&E-stained) tissue sections (212). Sections were dewaxed in xylene, rehydrated in graded ethanol (100 %, 96 % and 70 % respectively) and washed in water Thereafter the tissue sections were sequentially stained with haemotoxylin and eosin, differentiated in acid-alcohol and blued in Scott's tap water. Finally sections were dehydrated, cleared in xylene and coverslipped with pertex (Cellpath, Hemel Hampstead, UK). Tissue sections used for IHC alone were counterstained with haemotoxylin only after immunohistochemistry, differentiated in acid-alcohol and blued in Scott's tap water. Finally sections were dehydrated and coverslipped as above. Histopathological typing was defined according to the International Federation of Obstetricians and Gynaecologists (30) staging upon physical examination.

2.7. Statistical analysis

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

CHAPTER 3

EXPRESSION, LOCALISATION AND SIGNALLING OF CYCLOOXYGENASE ENZYMES AND PROSTAGLANDIN E_2 IN CERVICAL TISSUES.

3.1. INTRODUCTION

Cervical cancer is considered an important clinical problem in sub-Saharan Africa and has a major impact on morbidity and health care costs. There is now much evidence indicating HPV as the initiator of cervical neoplastic transformation, however numerous studies have suggested that epithelial tumors may be regulated by COX-enzyme products. Two isoforms of the COX enzyme, COX-1 and COX-2 have been reported (90, 92). COX-1 expression is considered to be constitutive, generating prostaglandins for normal physiological functions (10, 67, 92). By contrast, the expression of COX-2, a product of an immediate early gene, is rapidly induced following stimulation of quiescent cells by growth factors, oncogenes, carcinogens and tumor-promoting phorbal esters (90, 92, 98). Transcription of COX-2 is up-regulated in numerous carcinomas (3, 73-75, 77, 79) and its expression has been localised to the neoplastically transformed epithelial cells in these tumors. A relationship between COX-2, its synthesized product PGE_2, and neoplastic transformation of epithelial cells has been established (187, 213). COX-2 overexpression and consequent enhanced and sustained PGE_2 biosynthesis play a potential role in neoplastic transformation of epithelial cells by increasing their proliferation rate, resistance to apoptosis and invasiveness. These effects are mediated by suppressing the transcription of target genes that may be involved in cellular growth/transformation (eg. p53) and adhesion (eg. E-Cadherin) (69, 187). Moreover, COX-2 and PGE_2 may potentiate cancer development and invasiveness by mediating the transcription of angiogenic factors that promote both migration of endothelial cells and their arrangement into tubular structures (8, 9) for neovascularization. More recently, elevated COX-1 levels have been reported in mouse lung tumors (82), human breast cancer (83), human prostate carcinoma (78) and human ovarian adenocarcinomas (81). A similar pattern of expression for COX-1 has been observed in these carcinomas as has been demonstrated for COX-2. These data suggest that both COX enzymes and/or their products including PGE_2 may function in promoting and maintaining the neoplastic state.

PGE_2 biosynthesis is regulated by three steps in the COX biosynthetic pathway (87). The pathway is initiated by arachidonic acid release by phospholipase A2 from

membrane glycerophospholipids. Cyclooxygenase isoenzymes catalyse the conversion of arachidonic acid to prostaglandin H_2. Prostaglandin E synthase (PGES) then catalyses the conversion of COX-derived PGH_2 to PGE_2 (87). Functionally distinct forms of PGES have been identified thus far. A cytosolic PGES, which preferentially converts COX-1-derived PGH_2 (135) and a membrane associated form, which is preferentially coupled to COX-2 (136). The biological actions of PGE_2 have been attributed to its interaction with G-protein-coupled seven-transmembrane-domain receptors (GPCRs), which belong to the rhodopsin superfamily of serpentine receptors (141). Four main sub-types of PGE_2 receptors have been identified (EP1, EP2, EP3, EP4) which utilise alternate and in some cases opposing intracellular pathways (142). EP1 receptor increases intracellular calcium via G_q. EP2 and EP4 increase adenosine 3', 5''-cyclic monophosphate (cAMP) levels via $G_{\alpha s}$ and EP3 either increases cAMP via $G_{\alpha s}$ or decreases cAMP via $G_{\alpha i}$. To date, the role of the different PGE_2 receptors, their divergent intracellular signalling pathways as well as their target genes involved in mediating the effects of PGE_2 on normal or neoplastically transformed cervical epithelium remains to be elucidated. Recent evidence suggests that COX enzyme products may potentiate the process of tumorigenesis via ligand binding to target prostanoid receptors and activation of signal transduction pathways such as the cAMP pathway. In endometrial adenocarcinomas, enhanced EP2 and EP4 receptor expression is associated with enhanced expression of COX-2 and signalling of cAMP (214). Moreover, a direct role for EP4 and PGE_2 in tumorigenesis has been established. PGE_2 treatment of colorectal carcinoma cells leads to increased motility and altered phenotype via ligand binding to the EP4 receptor and initiation of intracellular signal transduction pathways (6) and the consequent transcription of target genes.

The initial goal of this study was to determine the expression and localisation of COX-1, COX-2 and PGE_2 and signalling of PGE_2 in cervical carcinomas and normal cervix. The strategy adopted was to extract RNA and protein from cervical biopsy specimens and perform real-time quantitative RT-PCR and Western blot analysis to determine the expression of COX-1 and COX-2. In addition, the site of expression of COX-1 and COX-2 and synthesis of PGE_2 were localised in cervical carcinomas by immunohistochemistry by probing thin sections of paraffin wax-embedded cervical tissue with specific antibodies. Finally, a possible autocrine/paracrine role for PGE_2 in cervical

cancer was investigated by determining: (a) the expression of two PGE_2 membrane-bound receptors, namely EP2 and EP4 by real-time quantitative RT-PCR analysis, and (b) the possible intracellular signalling of the EP2/EP4 receptor by measuring the effect of PGE_2 treatment of carcinoma tissue on cAMP turnover.

3.2. MATERIALS AND METHODS

3.2.1. Tissue collection and processing

Cervical specimens were obtained and processed as described in section 2.2. Histopathological staining and classification was performed by an experienced pathologist as described in section 2.5. The ages of the patients ranged from 29 years to 81 years with a median age of 50.5 years. The extent of invasiveness of the carcinoma biopsies is represented in Table 1.

Table 1. Extent of invasiveness of cervical carcinoma biopsy samples.

Sample no.	Histological typing	FIGO stage
C59, C60, C62, C79, C65, C68-C70, C73-C76	Squamous carcinoma	1B; poorly differentiated
C10-C14, C28-C32	Squamous carcinoma	1B; well differentiated
C61, C71	Squamous carcinoma	1B; moderately differentiated
C5-C9, C24-C27, C37-C47 C63, C64, C67, C77	Squamous carcinoma	2B; well differentiated
C1-C4, C19-C23, C72, C78	Squamous carcinoma	3B; well differentiated
C36	Adenocarcinoma	1B; moderately differentiated
C15-C18, C33-C35, C48-C58, C66, C79	Adenocarcinoma	2B; well differentiated

3.2.2. Real-time quantitative PCR

Real-time quantitative RT-PCR was performed to assess COX-1 and COX-2 expression (n=14 squamous cell carcinomas, C1-14; n=4 adenocarcinomas, C15-C18 and n=8 normal cervices, N1-N8) and EP2 and EP4 expression (n=7 squamous cell carcinomas, C59-C65; n=1 adenocarcinoma, C66 and n=5 normal cervices, N22-N26). RNA samples were extracted from cervical tissue using Tri-Reagent (Sigma) as described in section 2.3.1. Approximately 1 μg of total RNA was reverse transcribed after DNAse-

treatment as described in section 2.3.4. A total of 200 ng of cDNA template was included for each sample in the PCR reaction and real-time RT-PCR was carried out as outlined in section 2.3.5 using sequence specific primers and probes. The sequence of the COX-1 primers and probe were as follows: Forward: 5'- TGT TCG GTG TCC AGT TCC AAT A-3'; Reverse: 5'- ACC TTG AAG GAG TCA GGC ATG AG –3'; Probe (FAM labelled): 5'- CGC AAC CGC ATT GCC ATG GAG T-3'. The sequence of the COX-2 primers and probe were as follows: Forward: 5'-CCT TCC TCC TGT GCC TGA TG-3'; Reverse: 5'-ACA ATC TCA TTT GAA TCA GGA AGC T-3'; Probe (FAM labelled): 5'- TGC CCG ACT CCC TTG GGT GTC A -3'. The sequence of the EP2 receptor primers and probe were as follows; Forward: 5'-GAC CGC TTA CCT GCA GCT GTA C-3'; Reverse: 5'–TGA AGT TGC AGG CGA GCA-3'; Probe (FAM labelled): 5'–CCA CCC TGC TGC TGC TTC TCA TTG TCT-3'. The sequence of the EP4 receptor primers and probe were as follows; Forward: 5'–ACG CCG CCT ACT CCT ACA TG-3'; Reverse: 5'–AGA GGA CGG TGG CGA GAA T-3'; Probe (FAM labelled): 5'–ACG CGG GCT TCA GCT CCT TCC T-3'. The ribosomal 18S primers and probe sequences were as follows; Forward: 5' –CGG CTA CCA CAT CCA AGG AA-3'; Reverse: 5'–GCT GGA ATT ACC GCG GCT-3'; Probe (VIC labelled): 5'–TGC TGG CAC CAG ACT TGC CCT C-3'. Adjustment for RNA loading was performed by using the 18s RNA as an internal standard. Relative gene expression in the carcinoma tissue compared with normal cervix was calculated by dividing the expression in carcinoma tissue by the expression in normal cervix. The data are presented as mean ± SEM for 3 independent experiments.

3.2.3. Western Blotting

COX-1 and COX-2 protein expression in cervical carcinomas and normal cervix was assessed by Western blotting. Proteins were extracted from cervical tissue (n=14 squamous cell carcinomas, C19-C32; n=4 adenocarcinomas, C33-C36 and n=8 normal cervices, N9-N16) by homogenisation in protein lysis buffer as described in section 2.4.1 Western blotting was performed after electrophoresis of 50 µg of total protein as described in

section 2.4.5. Control samples were incubated with goat anti-COX-1/-2 antibody pre-adsorbed to blocking peptide as described in section 2.5.

3.2.4. Immunohistochemistry (IHC)

3.2.4.1. Tissue pretreatment and primary antibody

The site of COX-1 and COX-2 expression and PGE_2 synthesis was localised in cervical tissues by immunohistochemistry using archival cervical blocks (n= 10 squamous cell carcinomas, C37-C47; n=10 adenocarcinomas, C48-C58 and n=5 normal cervix, N16-N21) obtained from the Department of Anatomical Pathology, University of Cape Town, South Africa. Tissue samples were prepared as described in section 2.2 and mounted onto coated slides. Sections were dewaxed in xylene for approximately 15 min and rehydrated in graded ethanol and water as described in section 2.6, followed by Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl pH 7.4). Thereafter the tissue sections were blocked for endogenous endoperoxidase for 30 min in 1 % hydrogen peroxide (H_2O_2) in methanol to reduce endogenous peroxidase activity as a peroxidase detection method was used for immunodetection. The slides were then washed in TBS for 5 min. Antigen retrieval was performed by pressure cooking for 2 min in 0.01 M sodium citrate pH 6 (for COX-1, COX-2 and PGE_2). No antigen retrieval was performed for CD34 immunohistochemistry. Sections were blocked by incubation with normal serum from the species in which the secondary antibody was raised (using either 5 % normal rabbit serum (for COX-1 and COX-2), 5 % swine serum (for PGE_2) or 5 % goat serum (for CD34) diluted in TBS. The sections were blocked for 30 min at 25°C. After careful removal of the normal serum, the tissue sections were incubated with either polyclonal goat anti-COX-1 antibody at a dilution of 1:400; polyclonal goat anti-COX-2 antibody at a dilution of 1:400; rabbit anti-PGE_2 antibody (kindly supplied by Professor RW Kelly, MRC Human Reproductive Sciences Unit, Edinburgh, UK) at a dilution of 1:100 or monoclonal mouse anti-human CD34 primary antibody at a dilution of 1:25. Control tissue was incubated with either 5 % antisera (for CD34) or goat anti-COX-1/COX-2 antibody pre-adsorbed to blocking peptide or rabbit anti-PGE_2 antibody pre-incubated with exogenous ligand as described in section 2.5. The slides were covered with Gelbond film (Flowgen,

Rockland, ME, USA), hydrophilic side down and incubated in a humidified chamber at 4°C for 18 hrs. The following day, the tissue sections were washed three times for 5 min each with TBS to remove excess primary antibody.

3.2.4.2. Secondary antibody and Horseradish Peroxidase detection system

The tissue sections probed with the goat anti-human COX-1/COX-2 and rabbit anti-PGE_2 primary antibodies were incubated with biotinylated rabbit anti-goat secondary IgG antibody (for COX-1/COX-2; Dako) or swine anti-rabbit secondary IgG antibody (for PGE_2; Dako) at a dilution of 1:500 at 25°C for 40 min. Excess secondary antibody was washed from the slides by washing the tissue sections three times for 5 min each in TBS. Thereafter the tissue sections were incubated with streptavadin-peroxidase complex (Dako) at 25°C for 20 min and then washed thoroughly (three washes for 5 min each with fresh TBS between washes). The horseradish peroxidase complex solution was prepared according to the supplier's protocol (Dako) in 0.05M Tris/HCl pH 7.6 at least 20 min before use. Tissue sections probed with the mouse anti-human CD34 antibody were developed using a Mouse EnVision Kit (Dako) as per the manufacturers instruction. Briefly, tissue sections were washed with TBST and then incubated with the supplied peroxidase-labelled polymer conjugated to goat-anti-mouse IgG. Bound antibody was visualised by incubating the tissue sections with a solution of 225 μM 3.3'-diaminobenzidine in 0.05 M Tris/HCl, pH 7.6 containing 0.01 % hydrogen peroxide (Dako). After the colour reaction had developed, the reaction was stopped by washing the tissue sections in water. The tissue sections were counterstained and mounted as described in section 2.6.

3.2.5. PGE_2 stimulation and cAMP measurement.

3.2.5.1. Determination of Basal cAMP levels in cervical tissues:

Initially, basal cAMP levels were measured in cervical tissue (n=6 squamous cell carcinomas, C67-C72 and n=5 normal cervix, N27-N31). Carcinoma and normal cervical tissues were obtained on the day of surgery/biopsy, sectioned finely and divided equally into three aliquots. The tissue was transported at 4°C and then incubated in 35mm tissue culture dishes containing 2 ml of Dulbecco's Modified Eagle Medium (DMEM) (Sigma), containing, 0.3 mg/ml L-glutamine, 100 IU penicillin and 100 µg streptomycin for 1.5 hrs. One aliquot of tissue was snap frozen to determine basal cAMP concentration in the tissue at the time of collection. The other two aliquots were incubated overnight at 37°C in humidified 5 % CO_2 in the presence or absence of 3 µg/ml indomethacin (a dual COX enzyme inhibitor). Subsequently, tissue sections were harvested by centrifugation at 2000g. The supernatant was discarded and the tissue homogenised in 0.1 M HCl. cAMP concentration was quantified by ELISA using a cAMP kit (Biomol; Affiniti, Exeter, UK) as per the manufacturer's protocol and normalised to protein concentration of the homogenate. Briefly, the ELISA was performed using the 96 well microtiter plate provided. The wells of the plate are pre-coated with goat anti-rabbit IgG. 100 µl of cAMP standards, to produce a standard curve ranging from 200 pmol/ml to 0.78 pmol/ml, and samples were added to the plate. Plates were then coated with the alkaline phosphatase-cAMP conjugate provided together with the polyclonal rabbit anti-cAMP antibody using 50 µl/well of each solution. The plate was incubated at room temperature for 2 hrs on a plate shaker at ~500 rpm. Thereafter the wells were aspirated and washed three times with the wash buffer provided (TBST containing sodium azide). The assay was developed by addition of 200 µl/well of p-nitrophenyl phosphate (provided with the kit). Development of the colour reaction was stopped by addition of 50 µl of stop solution provided (trisodium phosphate in water). Colour reaction was measured at 405 nm by spectrophotometry. The concentration of cAMP per sample was calculated by extrapolation from the standard curve using the Assay Zap computor programme (Biosoft). Protein concentrations were determined using protein assay kits (Bio-Rad, Hemel Hempstead, UK) as described in section 2.4.3.

3.2.5.2. cAMP production in cervical tissues in response to exogenous PGE_2

Cervical tissues (n=6 squamous carcinoma, C73-C78; n=1 adenocarcinoma, C79 and n=5 normal cervix, N32-N36) were sectioned finely, divided equally into three aliquots and incubated overnight in Dulbecco's Modified Eagle Medium (DMEM) (Sigma), 0.3 mg/ml L-glutamine, 100 IU penicillin and 100 μg streptomycin and 3 μg/ml indomethacin. Following overnight incubation, samples were incubated in the same medium containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Sigma) to a final concentration of 1 mM for 30 min at 37°C and then stimulated with 0 nM PGE_2, 300 nM PGE_2 or 50 μM forskolin (forskolin treatment in sample C74 was excluded due to the small size of the biopsy) for 5 min. Tissue sections were harvested by centrifugation at 2000 g. The supernatant was discarded and the tissue homogenised in 0.1 M HCl. cAMP concentration and protein concentrations were determined as mentioned above

3.3. RESULTS

3.3.1. Expression Of COX-1 And COX-2 In Cervical Carcinoma And Normal Cervix By Quantitative RT-PCR And Western Blot Analysis.

Expression of COX-1 and COX-2 in cervical carcinomas was investigated using real-time quantitative RT-PCR (Fig. 3.1A) and Western blot analysis (Fig. 3.1B).

Expression of COX-1 and COX-2 RNA were significantly increased in 78 % and 100 % of cases of squamous cell carcinoma respectively and 100 % of cases of adenocarcinoma investigated. By contrast, minimal COX-1 and COX-2 transcript were detected in normal cervical tissue by quantitative RT-PCR. COX-1 and COX-2 expression, as assessed by quantitative RT-PCR, was 19.9 ± 5.9 and 118 ± 32 fold greater in cervical carcinoma tissues than that observed in normal cervical tissue ($P<0.01$). Western blot analysis confirmed enhanced expression of COX-1 and COX-2 in cervical squamous cell carcinoma (85 % and 100 % of cases respectively; Fig. 1B, panel I) and adenocarcinoma (100 % of cases respectively; Fig 1B, panel II). Basal expression of COX-1 protein was detected in 87 % of cases of normal cervix. No COX-2 expression was detected in normal cervical tissue by Western blot analysis (Fig. B, panel III). Specificity of detection of the 72 kDa COX-1 and COX-2 protein was performed by competition studies using a specific immunogen (blocking) peptide as described in section 2.5 (a representative sample is shown).

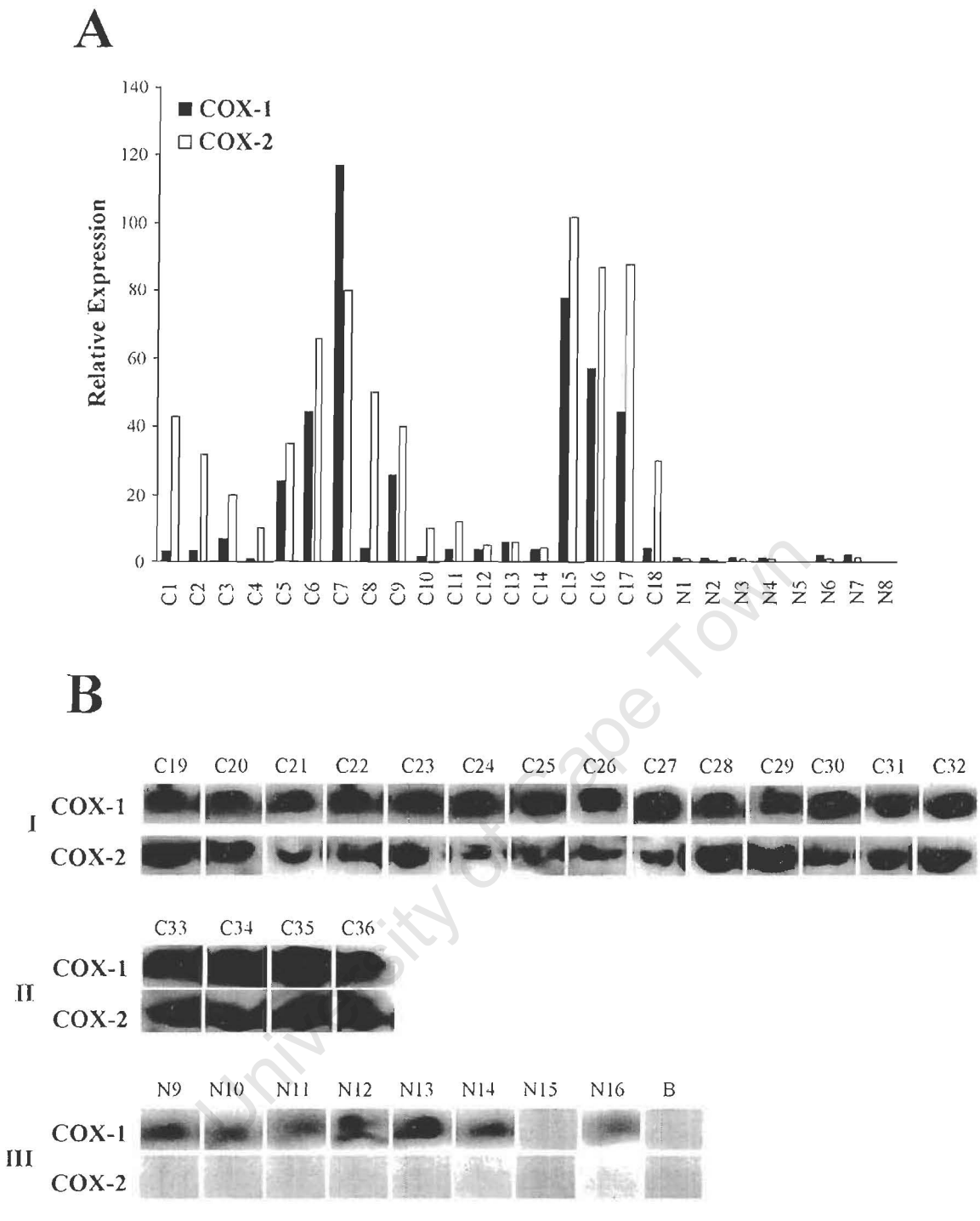


Figure 3.1. (**A**) Relative expression of COX-1 and COX-2 RNA in cervical squamous cell carcinoma (C1-C14), adenocarcinoma (C15-C18) and normal cervix (N1-N8) as determined by real-time quantitative RT-PCR. (**B**) Western blot analysis of total protein isolated from human cervical carcinoma tissue. A specific COX-1 and COX-2 band of approximately 72 kDa was detected in all squamous cell carcinomas (panel I; C19-C32) and adenocarcinomas (panel II; C33-C36). COX-1 expression was detected in 7/8 normal cervices. No COX-2 expression was detected in normal cervical tissue (panel III; N9-N16). Preadsorbing the antibody with the blocking peptide (panel III; B) abolished the COX-1 and COX-2 immunoreactivity (a representative sample is shown using sample C21).

3.3.2. Localisation Of The Site Of Expression Of COX-1 And COX-2 and Synthesis of PGE_2 In Cervical Tissues.

The site of COX-1 and COX-2 expression and PGE_2 synthesis in the carcinoma tissue was investigated by immunohistochemistry. Immunoreactive COX-1, COX-2 and PGE_2 were up-regulated in all carcinoma samples investigated.

Immunoreactive COX-1 was localised to neoplastically transformed squamous epithelium lining the exo-cervix in all squamous cell carcinomas investigated (n=10; Figures 3.2A and 3.2C) as well as the neoplastically transformed columnar epithelium lining the endocervical canal and glands in all adenocarcinomas (n=10; Figures 3.2E and 3.2G). Immunoreactive sections from two separate cases of squamous carcinoma and adenocarcinomas are shown. In addition, some diffuse stromal staining was observed in cells adjacent to the neoplastic epithelial cells in all carcinoma tissues. Preadsorbing the COX-1 antibody with the blocking peptide (COX-1 negative control) abolished the COX-1 immunoreactivity. Representative sections are shown for the respective squamous cell carcinomas (Figures 3.2B and 3.2D) and adenocarcinomas (Figures 3.2F and 3.2H).

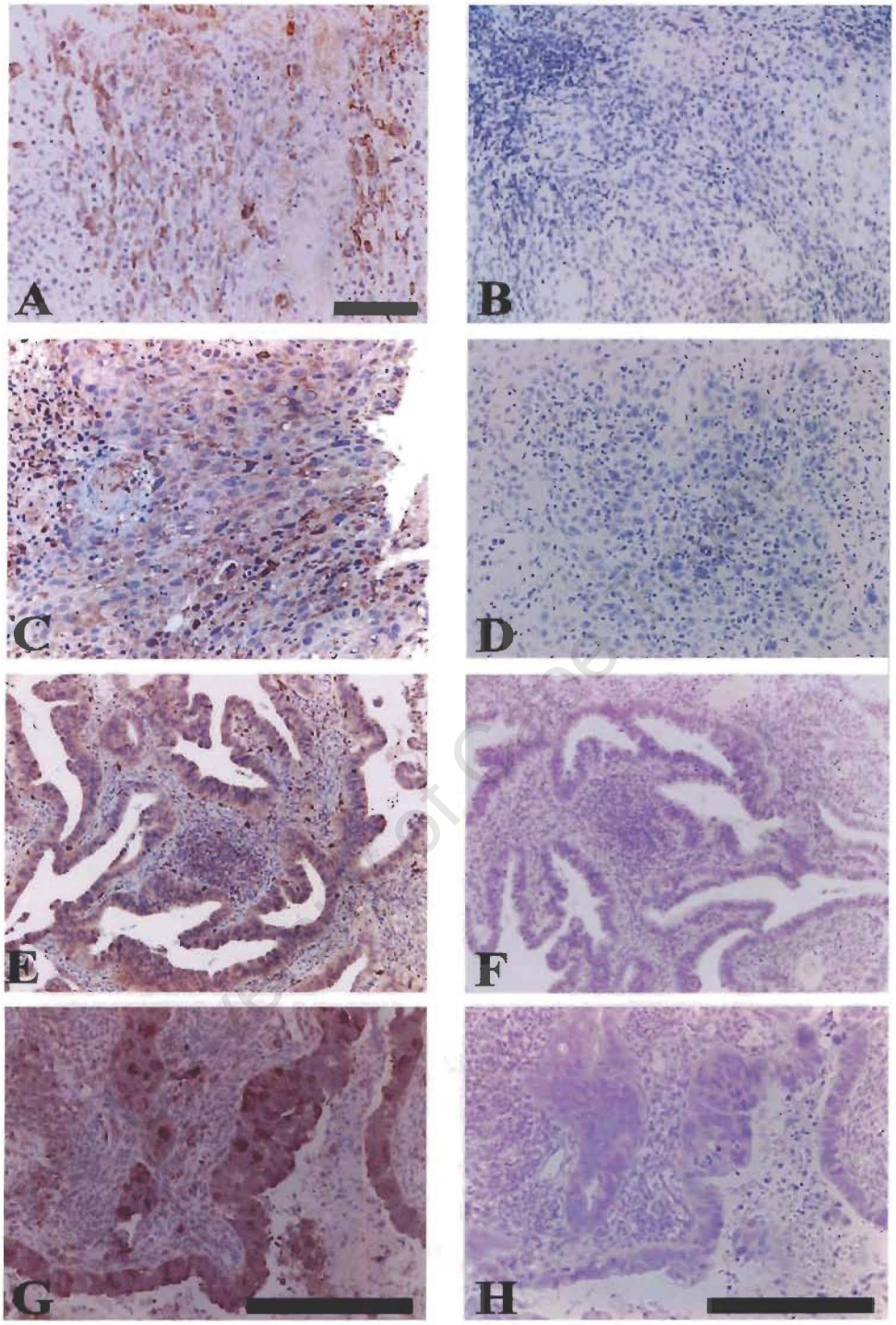


Figure 3.2. COX-1 expression is detected in neoplastically transformed epithelial cells of all squamous cell carcinomas investigated. (Figures A and C) as well as all adenocarcinomas investigated (Figures E and G). In this figure, two cases of squamous cell carcinoma and adenocarcinoma are shown. Sections that were stained with COX-1 antibody pre-adsorbed with the neutralising peptide are shown in Figures B, D, F and H respectively. Scale bar is 100 μm.

The site of COX-2 expression was localised to the neoplastically transformed squamous epithelium in squamous cell carcinoma (n=10; Figures 3.3A and 3.3C respectively), and to neoplastically transformed columnar epithelium lining the endocervical canal and the glandular epithelium of the endocervical glands in adenocarcinomas (n=10; Figure 3.3E and 3.3G respectively). Immunoreactive sections from two separate cases of squamous carcinoma and adenocarcinoma are shown respectively. Negligible COX-2 staining was observed in the stromal compartment in all carcinoma tissue investigated. Preadsorbing the antibody with the blocking peptide (COX-2 negative control) abolished the COX-2 immuno-staining indicating specificity of the COX-2 antibody. Representative sections are shown for squamous cell carcinoma (Figures 3.3B and 3.3D) and adenocarinomas (Figures 3.3F and 3.3H).

The site of PGE_2 synthesis was localised to the neoplastically transformed squamous epithelium in squamous cell carcinoma (n=10; Figures 3.4A and 3.4C respectively), and to neoplastically transformed columnar epithelium lining the endocervical canal and the glandular epithelium of the endocervical glands in adenocarcinomas (n=10; Figure 3.4E and 3.4G respectively). Immunoreactive sections from two separate cases of squamous carcinoma and adenocarcinomas are shown. Pre-incubating the PGE_2-antibody with exogenous PGE_2 ligand (PGE_2 negative control) prior to performing the immunostaining abolished the PGE_2 immunoreactivity in all carcinoma samples. Representative sections are shown for squamous cell carcinoma (Figures 3.4B and 3.4D) and adenocarcinomas (Figures 3.4F and 3.4H).

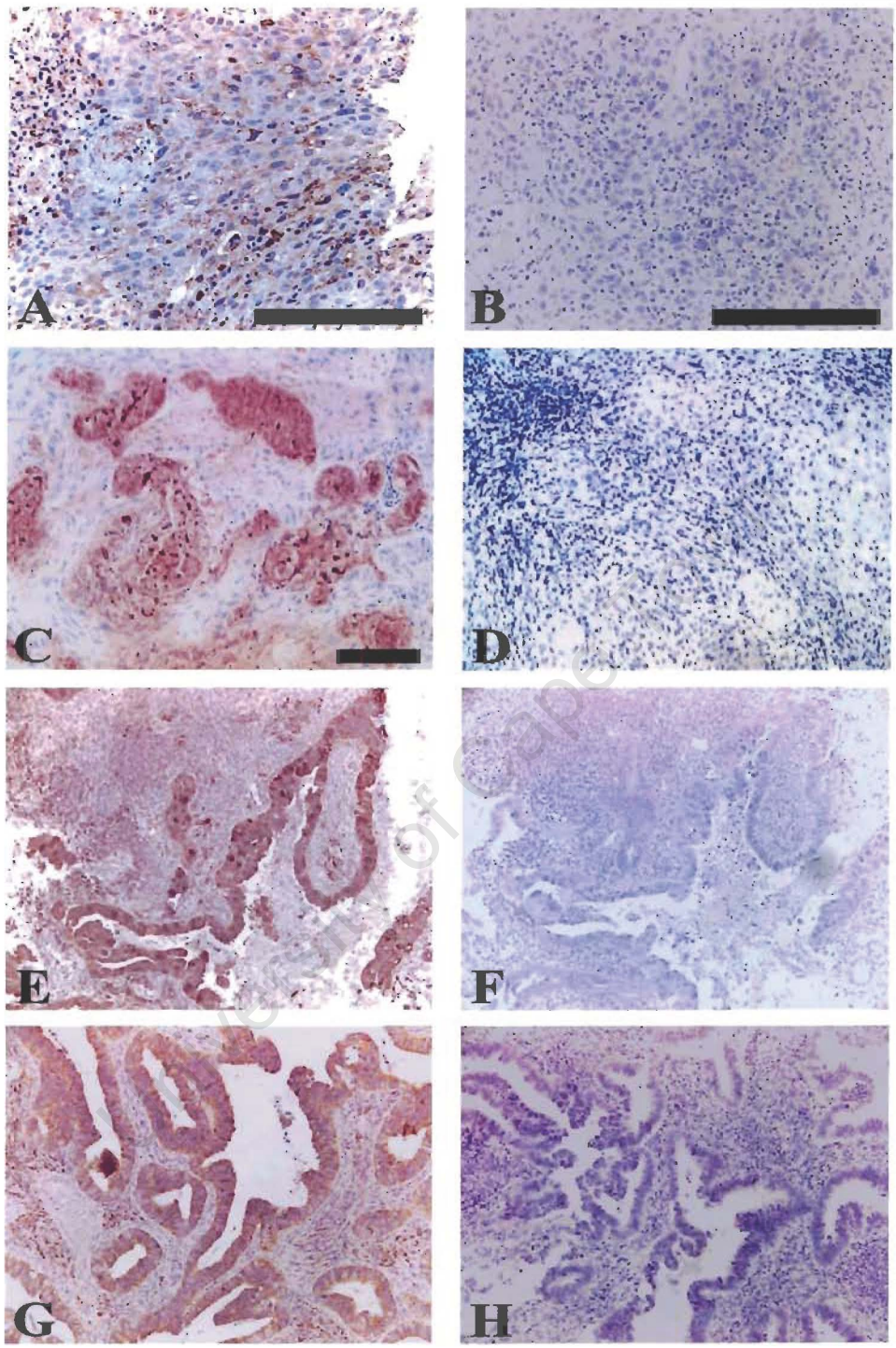


Figure 3.3. COX-2 expression is detected in epithelial cells of squamous cell carcinoma in all cases of squamous cell carcinoma (Figures A and C) and adenocarcinoma (Figures E and G) investigated. In this figure, two cases of squamous cell carcinoma and adenocarcinoma are shown. Sections that were stained with COX-2 antibody pre-adsorbed with the neutralising peptide are shown in Figures B, D, F and H respectively. Scale bar is 100 μm.

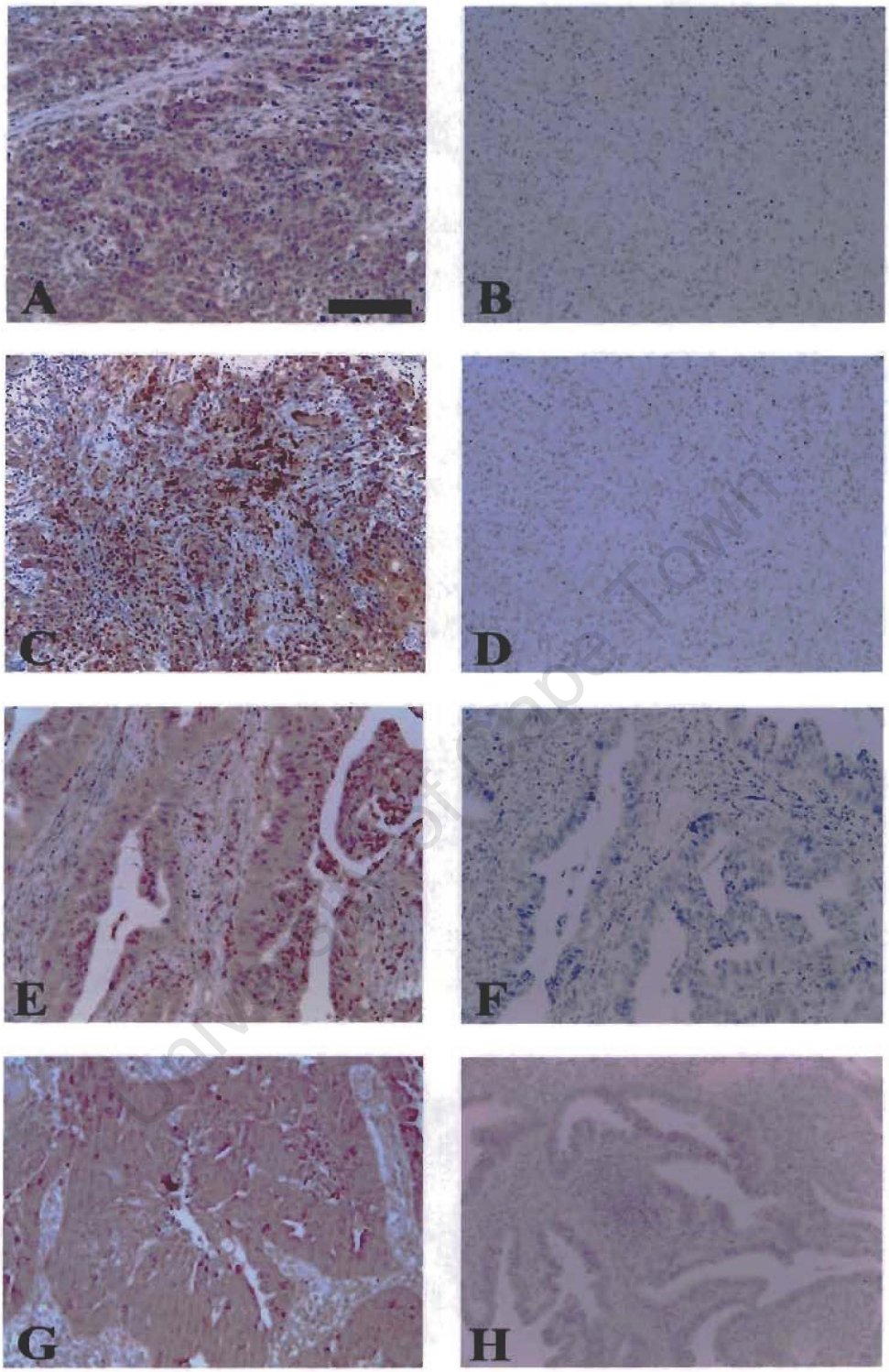


Figure 3.4. Localisation of the site of PGE_2 synthesis in epithelial cells of squamous cell carcinoma (A and C) and adenocarcinomas (E and G). Sections of tissue stained with PGE_2 pre-incubated with excess exogenous PGE_2 (negative controls) are shown for squamous cell carcinoma (B and D) and adenocarcinoma (F and H). Scale bar is 100 μm.

In addition, COX-2 (n=10, Figure 3.5A and 3.5B; arrowed) and PGE_2 (n=10; Figures 3.5C and 3.5D; arrowed) immunostaining was observed in endothelial cells lining the microvasculature in all squamous cell carcinoma and adenocarcinoma sections investigated. Two representative sections of cervical adenocarcinomas are shown respectively. To confirm that COX-2 expression and PGE_2 synthesis were localised to the endothelial cells of blood vessels, immunohistochemistry was performed on tissue sections using antibodies raised against the CD34 endothelial cell marker (Figure 3.5E; arrowed). The pattern of expression with CD34 was similar to that observed with COX-2 and PGE_2 thus confirming that COX-2 expression and PGE_2 synthesis is localised to the endothelial cell layer of blood vessels in human cervical carcinoma. No CD34 staining was observed in sections incubated with non-immune serum in place of primary antibody (Figure 3.5F). No immunoreactive staining for COX-1 was observed in endothelial cells of any of the squamous cell carcinomas or adenocarcinomas investigated.

Little or no staining for COX-1 (Figure 3.6A and B), COX-2 (Figure 3.6 C and D) and PGE_2 (Figure 3.6E and F) was observed in the normal cervical tissues.

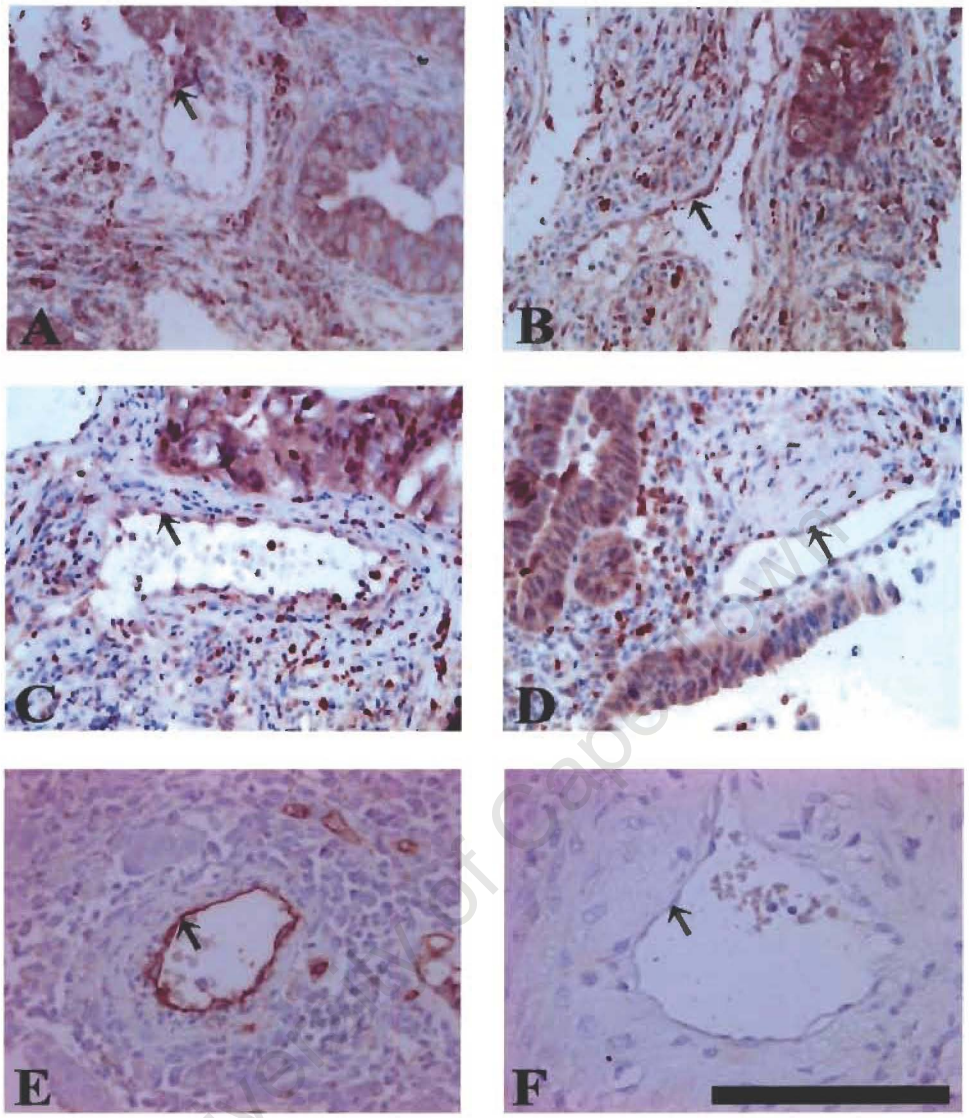


Figure 3.5. COX-2 expression (A and B; representing two different cases of adenocarinoma) and PGE_2 synthesis (C and D; representing two different cases of adenocarcinoma) are detected in endothelial cells (arrowed) of all carcinoma tissues. Vascular endothelial cells in cervical cancer tissues were localised using antibodies raised against the human CD34 endothelial cell marker (E). Figure F is a representative section incubated with non-immune goat serum in place of the primary antibody (CD34 negative control). Scale bar is 50 μm.

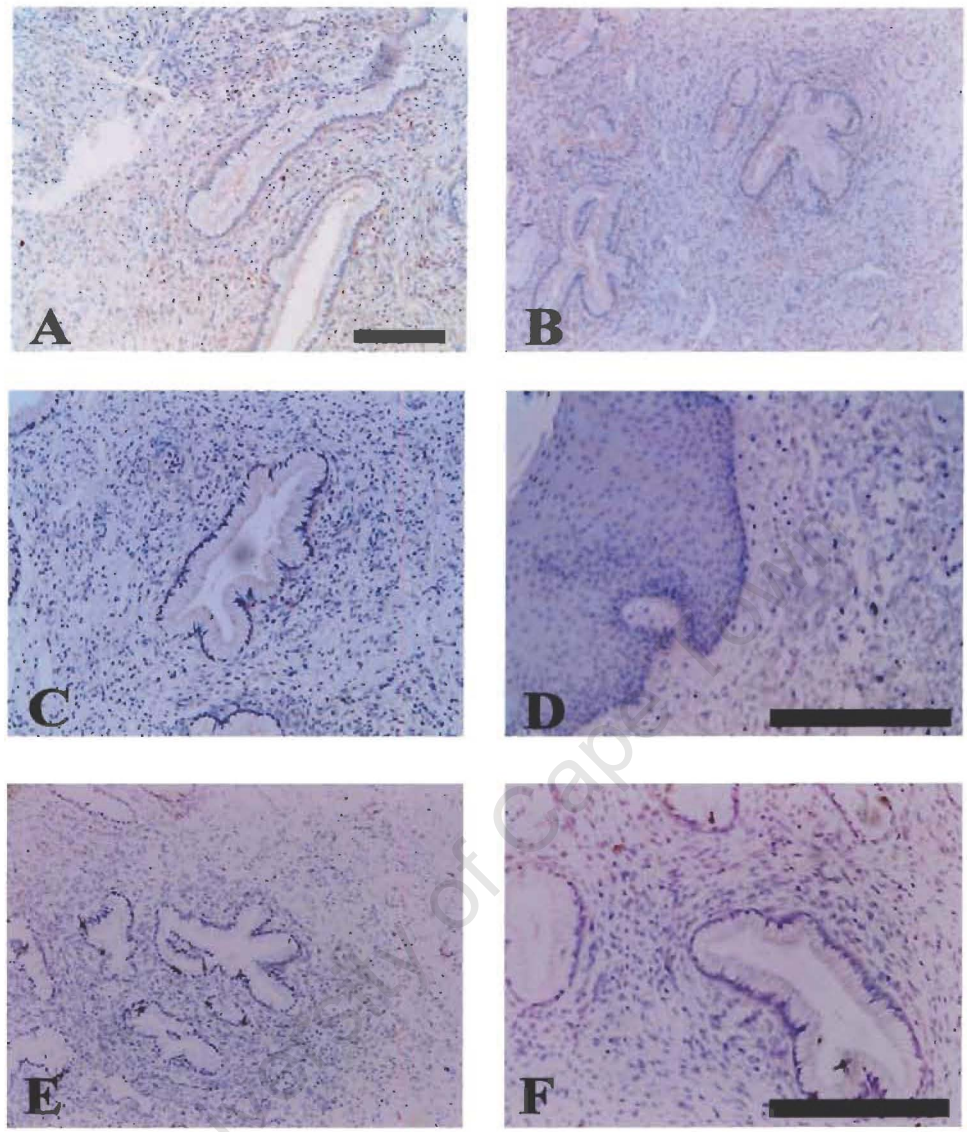


Figure 3.6. Localisation of COX-1 (A and B) and COX-2 (C an D) expression and PGE_2 synthesis (E and F) respectively in normal cervical tissues. Figures show staining in two different cases of normal cervix respectively. Minimal COX-1, COX-2 and PGE_2 signal was detected in normal cervical tissue. Scale bar is 100 μm.

3.3.3. Expression Of EP2 And EP4 Receptors In Cervical Tissues.

The expression of two subtypes of PGE_2 receptors, namely EP2 and EP4, was investigated by real-time quantitative RT-PCR in cervical carcinoma and normal cervix (Figure 3.7). Expression of both receptors was significantly up-regulated in all carcinoma tissues compared with normal cervix ($P<0.01$). The relative expression of EP2 and EP4 receptor in carcinoma tissue was 14.5 ± 3.2 and 106 ± 25.8 (respectively) greater than that detected in normal cervix.

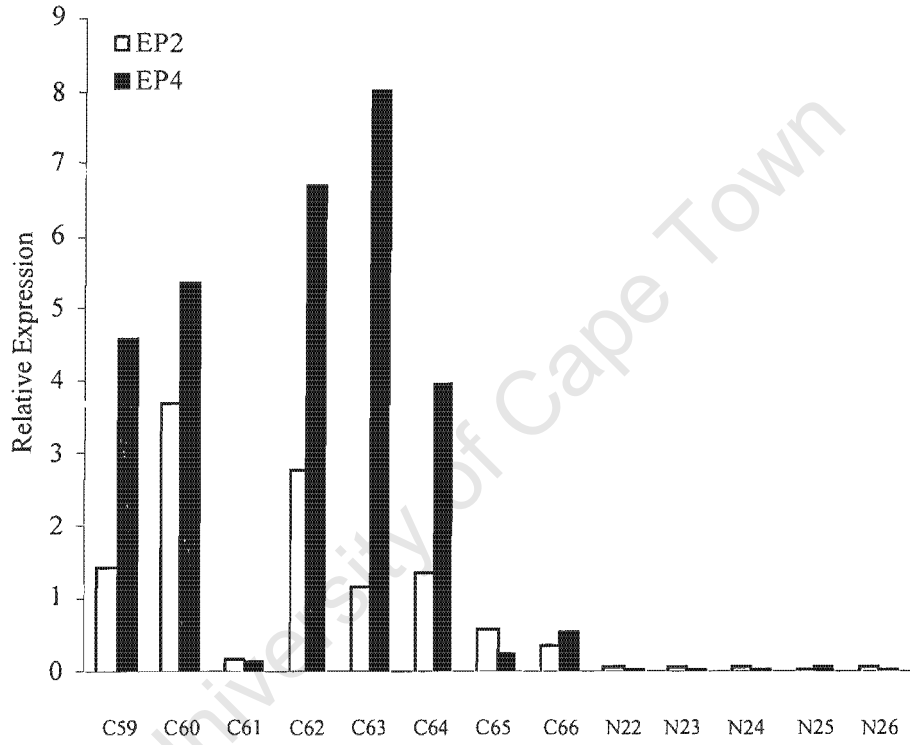
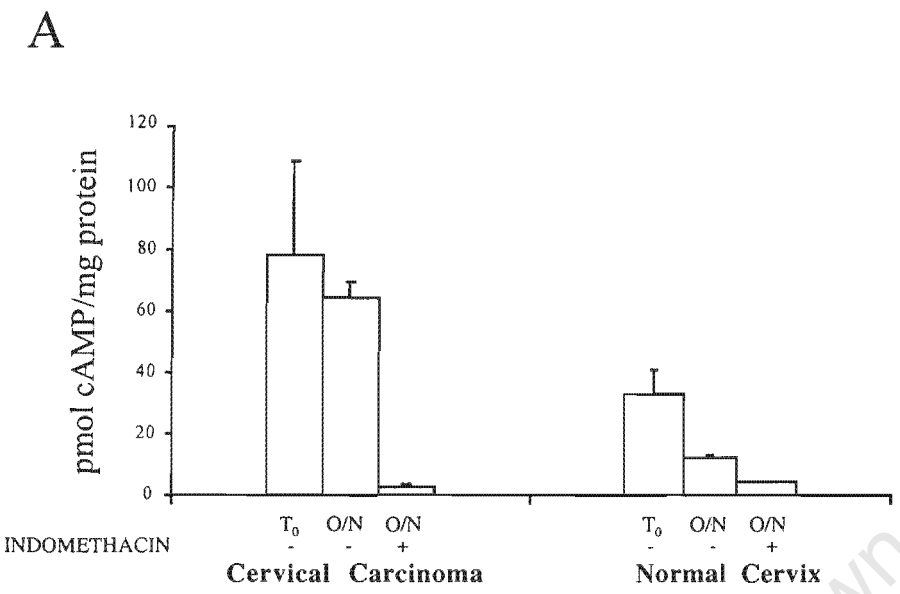


Figure 3.7. Relative expression of EP2 (empty bars) and EP4 (solid bars) receptors in cervical squamous cell carcinoma (C59-C65), adenocarcinoma (C66) and normal cervix (N22-N26) as determined by real-time quantitative RT-PCR.

3.3.4. cAMP Accumulation In Cervical Tissues In Response To Exogenous PGE_2.

In order to assess the activity of the EP2/EP4 receptors in the cervical tissue, basal levels of cAMP were determined at the time of tissue collection and after overnight incubation in the absence or presence of 3 μg/ml indomethacin (Figure 3.8A). cAMP concentration immediately after tissue excision was significantly higher in carcinoma compared with normal cervix (77.9 ± 30.9 vs 32.5 ± 8.7 pmol cAMP/mg protein; $P<0.05$). cAMP concentrations in carcinoma tissue following overnight incubation in the absence of indomethacin was similar to that detected in the tissue at the time of excision (64.2 ± 5.1 pmol cAMP/mg protein) but was significantly reduced when the tissue was cultured in the presence of indomethacin (2.59 ± 0.64 pmol cAMP/mg protein). In normal cervical tissue, levels of cAMP were reduced following overnight incubation in the absence of indomethacin (11.96 ± 1.35 pmol cAMP/mg protein respectively; $P<0.05$) and even more reduced following overnight incubation in the presence of indomethacin (4.0 ± 0.7 pmol cAMP/mg protein respectively; $P<0.01$) compared with cAMP levels at the time of tissue collection (32.5 ± 8.7 pmol cAMP/mg protein; $P<0.05$).

Subsequently, we determined the effect of exogenous PGE_2 and forskolin treatment on EP2/EP4 signalling in cervical tissues by measuring cAMP production in carcinoma and normal cervical tissues (Figure 3.8B). Stimulation of cervical carcinoma tissue with 300 nM PGE_2 or 50 μM forskolin (positive control) yielded a greater cAMP response than in normal cervical tissue treated in the same manner. Overall, the fold induction of cAMP generation after PGE_2 and forskolin stimulation was 51.1 ± 12.3 and 55.3 ± 15.84 respectively in cancer tissue and 5.8 ± 1.68 and 9.18 ± 1.59 respectively in normal cervix ($P<0.01$).



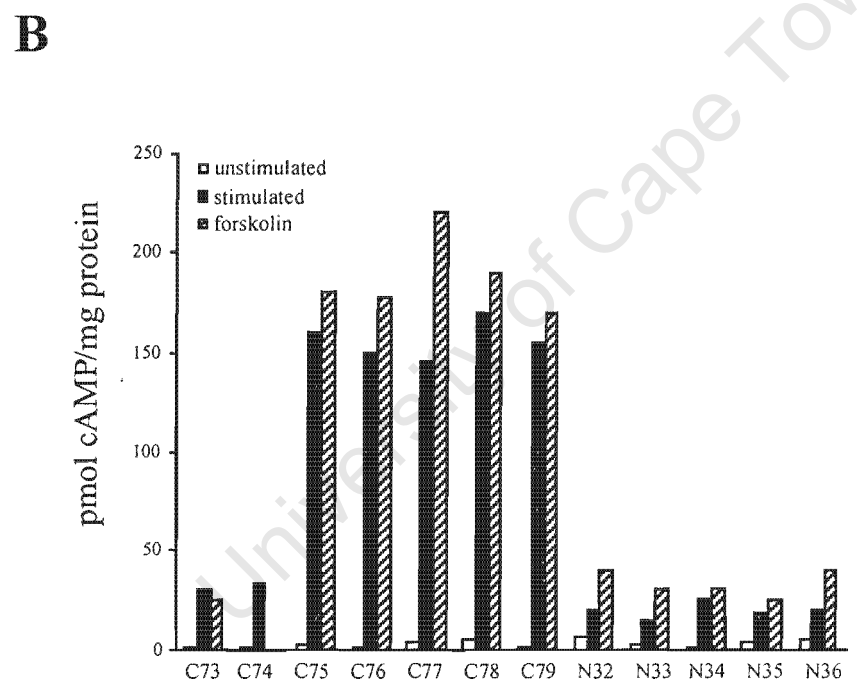


Figure 3.8. (A) Basal cAMP levels (pmol cAMP/mg protein) in cervical tissues (mean ± SEM of squamous cell carcinomas C67-C72 and normal cervix, N27-N31). Basal cAMP levels were determined shortly after biopsy (T0) and after overnight (O/N) culture in the absence (-) or presence (+) of indomethacin. (**B**) cAMP response (pmol cAMP/mg protein) in squamous cell carcinoma (C73-C78), adenocarcinoma (C79) and normal cervix (N32-N36). Cervical tissues were treated with indomethacin overnight and either stimulated with 300 nM PGE_2 (solid bars) or 50 µM forskolin (striped bars; positive control) or left unstimulated (open bars).

3.4. DISCUSSION

This study confirms enhanced expression of COX-1 and COX-2 in cervical squamous cell carcinomas and adenocarcinomas of various grades and stages of differentiation (described in table 1) as well as elevated synthesis of PGE_2 in squamous cell carcinoma and adenocarcinoma of the cervix in South African women. These data suggest a similar pattern of expression of COX-1 and COX-2 in cancer of the cervix as has been demonstrated in other carcinomas (3, 73-75, 77-79, 81-83). In this study increased COX expression is associated with increased synthesis of PGE_2 as COX-1, COX-2 and PGE_2 co-localised in neoplastic epithelial cells of all cervical carcinomas investigated. Although the synthesis of other prostanoids may also be enhanced by up-regulation of expression of COX enzymes, previous studies have demonstrated that PGE_2 is the predominant prostaglandin synthesized by COX-2 (118). Little is known of the product profile for COX-1 and mechanisms by which COX-1 may enhance or sustain tumorigenesis. A potential role for COX-1 in tumorigenesis has been established recently by the observation that immortalised endothelial cells transfected with COX-1 become tumorigenic when implanted into nude mice (80), implying that COX-1 does not function merely as a housekeeping gene. In cervical carcinomas, the expression of both COX-1 and COX-2 are elevated suggesting that both isoforms may play a role in cervical tumorigenesis. Since both COX enzymes catalyse the conversion of arachidonic acid to eicosanoids such as PGE_2 (85), a similar role may be postulated for COX-1 as has been described for COX-2. The exact role for COX-1, COX-2 and PGE_2 in carcinomas is still unclear. In model systems, enhanced synthesis of PGE_2 resulting from up-regulated COX-2 appears to have a role in potentiating malignant change in epithelial cells through immunosuppression by inhibiting T-cell and B-cell proliferation and differentiation and accessory monocyte/macrophage function (86), inhibiting apoptosis (69), increasing the metastatic potential of epithelial cells (7) and promoting angiogenesis (8). COX-1, COX-2 and PGE_2 may facilitate the process of cervical neoplasia in a similar manner.

In addition, in our study COX-2 and PGE_2 co-localised in the endothelial cells lining the microvasculature of the cervical carcinomas. COX and PGE_2 could control the

process of angiogenesis in cervical tumors either directly or indirectly in and autocrine/paracrine manner. In an *in vitro* model, overexpression of COX-2 and subsequent enhanced synthesis of PGE_2 in colon epithelial cells enhances the expression of angiogenic factors that act on endothelial cells resulting in enhanced endothelial cell migration and microvascular tube formation (8) for blood vessel proliferation. More recently, it was suggested that COX-2 and PGE_2 produced by endothelial cells may also directly regulate the process of angiogenesis (9). The arrangement of rat aortic endothelial cells into tubular structures is reduced following treatment with selective COX-2 inhibitors and this effect is partially reversed by co-treatment with PGE_2 (9). Hence, it is feasible to suggest that in cervical carcinomas the process of angiogenesis is regulated in an autocrine/paracrine manner by COX-1, COX-2 and PGE_2 through an epithelial-endothelial and/or endothelial-endothelial cell interaction. This is supported by our data demonstrating COX-1 expression in neoplastic epithelial cells and COX-2 expression and PGE_2 synthesis in neoplastic epithelial cells as well as endothelial cells.

PGE_2 acts on target cells via interaction with G-protein coupled receptors. To-date several of these receptors, which utilise alternate intracellular signalling pathways, have been cloned. In this study we investigated the expression of two PGE_2 membrane-bound receptors, namely EP2 and EP4, which mediate their effect on target cells via the PKA pathway by activating adenylate cyclase and increasing intracellular cAMP (141). In cervical carcinomas, expression of EP2 and EP4 receptors are up-regulated compared with normal cervix. Recent evidence has suggested a role for EP receptors in tumorigenesis. In endometrial adenocarcinomas, enhanced EP2 and EP4 receptor expression is associated with enhanced expression of COX-2 and signalling of cAMP (214), suggesting a role for EP2/EP4 receptor in reproductive tract tumorigenesis. Furthermore, a mitogenic role for PGE_2 and EP4 receptor has been reported recently in colon carcinoma cells. Enhanced proliferation and motility of colorectal carcinoma cells is associated with PGE_2/EP4 interaction and activation of signal transduction pathways (6). Thus, in cervical carcinomas, the process of tumorigenesis may be enhanced by increased expression of COX enzymes and synthesis of COX-enzyme products, such as PGE_2, in an autocrine/paracrine manner via the PGE_2/EP2/EP4 signal transduction pathway.

Although we have not localised the site of expression of the EP2/EP4 receptors in the cervical carcinomas, we can postulate that PGE_2 may be synthesized in cells and released close to its site of action. Therefore we can propose that in cervical carcinomas, EP2/EP4 receptor expression may be localised in neoplastic epithelial cells and vascular endothelial cells since we localised enhanced expression of PGE_2 in these cells in cervical carcinomas. Evidence in support of this can be drawn from endometrial models. Since the epithelium of the cervix, via the endocervical canal, is continuous from the vagina to the uterus, the spacial arrangement of receptors may be similar to that observed in the endometrium. In nonpregnant endometrium, EP2 and EP4 receptor expression is localised to the endometrial glandular epithelium and vascular cells and is associated with enhanced cAMP signalling (215). The augmented ligand-receptor binding brought about by up-regulated receptor expression and enhanced prostanoid biosynthesis in neoplastic epithelial and/or endothelial cells, could then amplify intracellular signalling and transcription of target genes to enhance vascularisation, invasiveness, cell migration and tumor mass.

Recently, a link between the neoplastic effect of carcinogen treatment and prostanoid signalling was made by the observation that EP1 receptor knock-out mice develope fewer aberrant crypt foci, which are thought to be preneoplastic lesions of the colon, following carcinogen treatment than wild-type mice. No alteration in the formation of aberrant crypt foci was observed when EP3 knock-out mice were administered carcinogen compared with wild-type mice (216). In addition, administration of the selective EP1 antagonist, ONO-8713, to carcinogen-treated wild-type mice showed a dramatic dose-dependent reduction in aberrant crypt foci compared with untreated animals (217). EP1 receptor antagonists could thus be of therapeutic benefit towards the treatment of colon cancer. However, it is envisaged that in addition to EP1, other receptor subtypes and intracellular signalling pathways may be associated with PGE_2 function in tumorigenesis. Due to limitations in the sizes and numbers of the biopsies obtained at surgery, it was not possible to investigate all intracellular signalling pathways that may be associated with PGE_2 function in cervical cancers (142). Future studies to elucidate the divergent intracellular signal transduction pathways associated with eicosanoids such as

PGE_2 may be efficacious towards implementing improved therapies for women with cervical carcinoma.

One of the signal transduction pathways associated with EP receptor signalling is the cAMP pathway. In cervical carcinoma tissue the basal cAMP concentration is elevated compared with normal cervix. Treatment of the cervical tissue with the COX enzyme inhibitor indomethacin significantly reduced the cAMP concentration. This suggests that the elevated basal cAMP concentration in the carcinoma tissue is mediated by COX-enzyme products. Moreover, treatment of cervical carcinoma tissue with exogenous PGE_2 following overnight incubation with the COX-enzyme inhibitor indomethacin, results in a rapid cAMP response, which is greater in the carcinoma tissue than in the normal cervical tissue. Interestingly, a greater cAMP response was observed in cervical carcinoma tissue, compared with normal cervical tissue, following treatment with forskolin further suggesting up-regulation of adenylate cyclase in cervical carcinomas. The cAMP pathway may thus be relevant in cervical carcinomas as both an index of activity as well as cancer progression. A potential role for the EP receptor-cAMP pathway in reproductive tract carcinomas has been reported recently. In uterine adenocarcinomas, cAMP signalling is elevated in response to administration of PGE_2 compared with normal tissue (214). Taken together these data confirm that PGE_2 synthesized in cervical carcinoma tissue mediates an autocrine/paracrine effect via the EP2/EP4 cAMP pathway.

In conclusion, these data confirm enhanced expression of COX-1 and COX-2 enzymes in cervical carcinomas of various grades and stages of differentiation. In cervical carcinomas elevated expression of COX-1 and COX-2 is associated with enhanced synthesis of PGE_2. The site of expression of COX-1, COX-2 and synthesis of PGE_2 was localised to the neoplastic epithelial cells lining the exo-cervix of all squamous cell carcinomas and the neoplastic columnar and glandular epithelial cells lining the endocervical canal and mucous glands of all adenocarcinomas. COX-2 and PGE_2 also localised in endothelial cells of the microvasculature. In addition in cervical carcinomas the expression of the EP2 and EP4 receptors are up-regulated compared with normal cervix and this is associated with enhanced cAMP accumulation in cervical carcinomas. Taken

together, these findings suggest that PGE_2, as a consequence of enhanced expression of COX enzymes, may exert an autocrine/paracrine effect in cervical carcinomas via the EP2/EP4 adenylate cyclase pathway to initiate transcription of target genes, which could enhance or sustain cervical tumorigenesis.

CHAPTER 4

CONSTRUCTION OF A HeLa COX-1 Tet-Off MODEL SYSTEM TO PROMOTE INDUCIBLE OVEREXPRESSION OF COX-1

4.1. INTRODUCTION

To date most *in vitro* studies have focussed on neoplastic events associated with COX-enzyme products as a consequence of COX-2 overexpression in epithelial cells. Until recently, COX-1 activity has been considered to be constitutive in cells and tissues, generating prostaglandins for normal physiological functions (62). However, COX-1 expression is elevated in several solid epithelial tumors (78, 81-83). In addition, COX-1 overexpressing cells implanted into nude mice is associated with enhanced tumorigenic effects (80), suggesting that under certain conditions COX-1 may play a role in tumorigenesis. We have demonstrated enhanced expression of COX-1 in cervical carcinomas (as discussed in Chapter 3), and localised this expression to neoplastic cervical epithelial cells. COX-1 originates from a 22 kb gene, located on chromosome 9. Gene products give rise to mRNAs of roughly 2.8 kb (62). Post-translational processing gives rise to glycosylated, integral membrane proteins localised to the endoplasmic reticulum and nuclear envelope with apparent molecular masses of 66-72 kDa. COX enzymes exist as homodimers, which bind 1 mole of high-spin ferric heme per mole monomer. The COX-1 enzyme has been crystalised and its crystal structure has revealed three domains: an N-terminal epidermal growth factor (EGF)-like module; a membrane-binding domain consisting of four amphipathic helices; and a globular catalytic domain containing the COX and peroxidase active sites (218).

The aim of this study was to create a COX-1 expression system in HeLa cells to investigate the molecular events associated with COX-1 and its synthesized products in cervical carcinoma cells. The HeLa cell line was chosen for this study, because of its cervical carcinoma origin, immortality and homogeneity. We believe that the HeLa cell model system provides a useful tool in studying the *in vitro* biological, genetic and ultrastructural properties of cervical carcinoma. HeLa cells were the first aneuploid epithelial-like cell line to be derived from human tissue and maintained continuously in serial cell culture. HeLa was derived from cervical adenocarcinoma origin, from the carcinoma of a 31 year old female by GO Gey, WD Coffman and MT Kubicek in

February 1951 (Cancer Research 12:124, 1952). Since its origin, the HeLa cell has been one of the most extensively studied cell lines. For this study a tetracycline (Tet)-regulated system (Tet-Off) system was chosen to promote controlled reversible inducibility of COX-1 in HeLa cells, thereby replicating *in vitro* the processes associated with COX-1 expression *in vivo*. The tetracycline-regulated system allows the expression of the gene of interest in the absence of tetracycline or its analogue doxycycline (DOX) and the decrease in expression of the enzyme in the presence of antibiotic (219).

4.2. MATERIALS AND METHODS

The Tet-Off™ system (Clontech; represented schematically in Figure 4.1) is a high-level gene expression system (219). In the Tet-Off system, gene expression is turned on whenever tetracycline (Tc) or doxycycline (DOX; a Tc derivative) is removed from the culture medium. The Tet repressor protein (TetR) negatively regulates genes of the tetracycline-resistance operon on the Tn 10 transposon in *E.coli*. The TetR prevents transcription of these genes by binding to the tet operator sequences (tetO) in the absence of Tc. The Tet-Off system consists of two critical components namely; the regulatory protein and the response plasmid containing the gene of interest. The regulatory protein of the Tet-Off system is a fusion of amino acids 1-207 of the TetR and the C-terminal 127 amino acids of the virion protein 16 (VP16) action domain of the herpes simplex virus. The addition of the VP16 domain to the TetR results in conversion of the TetR from a transcriptional repressor to a transcriptional transactivator (tTA). The hybrid transactivator stimulates minimal promotors fused to tetracycline operator (tetO) sequences.

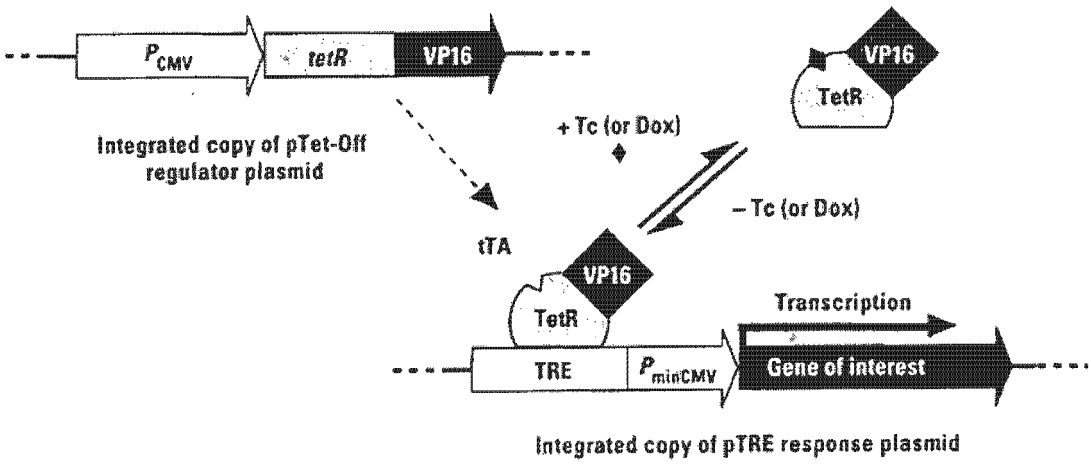


Figure 4.1. Schematic Representation of the Tet-Off system. The Gene of interest cloned into the pTRE2 vector is induced when tetracycline (Tc) or its analogue doxycycline (DOX) is removed from the culture medium.

The tTA is encoded by the pTet-Off regulator plasmid (Appendix II), which has the gene for neomycin resistance to permit selection of stably transfected cells with G418. The second component of the Tet-Off system is the pTRE2 response plasmid (Appendix III). The response plasmid expresses the gene of interest (Gene X; in our study this gene is COX-1) under control of the tetracycline response element or TRE. The TRE consists of seven direct repeats of a 42 bp sequence containing the tetO, and is located upstream of the minimal cytomegalovirus (CMV) promotor (P_{minCMV}), which lacks the strong enhancer elements associated with the CMV immediate early promotor. Lack of the enhancer elements results in tight regulation of the response, and no leakage of expression of Gene X. The pTRE2-Gene X plasmid is co-transfected with the pTK-Hyg vector, which contains the hygromycin (Hyg) gene under the control of the minimal thymidine kinase (TK) promotor (Appendix IV) to permit selection of stable transfectants using hygromycin.

4.2.1. Cell Culture

HeLa Tet-Off cells (Clontech) were grown under sterile conditions in culture flasks with a surface area of 170 cm^2 (Corning Science Products). Cells were routinely

cultured at 37°C and 5 % CO_2 (v/v) in 50 mls of complete DMEM medium (Dulbecco's modified Eagle's medium nutrient F-12 (Gibco) with glutamax-1 and pyridoxine), supplemented with 10 % Tet-system approved foetal calf serum (Clontech), and 1 % antibiotics (stock 500 IU/ml penicillin and 500 µg/ml streptomycin). Cells grew as a monolayer on the surface of the flask. Cell growth was observed daily, using an inverted light microscope, to determine the level of confluence. When confluent the cells were passaged. Generally, the method of passaging used was to take 50 % of the cells from one large flask and split the cells between 5 new large flasks, thus reducing the cell density to 1/10. To facilitate passaging, the growth medium was removed by vacuum suction and sequentially washed with 10ml of sterile PBS and 3 ml trypsin-EDTA (0.1 % trypsin and 0.04 % EDTA in PBS). The trypsin was removed immediately by vacuum suction and the flasks incubated for 5 min at 37°C. Subsequently, trypsin was inhibited by the addition of 10 mls of complete medium. Cells were resuspended by pipette action and 1 ml transferred to each new 50 ml flask (Corning Science Products). Cells were then transferred to the incubator and incubated at 37°C in humidified 5 % CO_2 (v/v). HelaTet-Off cells were maintained in medium containing 100 µg/ml G418.

4.2.2. Construction Of Plasmids

4.2.2.1. Preparation Of cDNA And Vector For Ligation.

The vector containing the full length COX-1 gene (kindly supplied by Dr Stephen Prescott, University of Utah, Salt Lake City, UT) was used as the template plasmid and was transformed into competent cells and grown on Luria Bertani (LB) agar (Appendix I) plates containing 100 µg/ml ampicillan at 37°C overnight. Single colonies were picked and grown in LB broth (Appendix I) containing 100 µg/ml ampicillan at 37°C under constant agitation overnight and plasmid DNA was isolated as described in section 4.2.3 and purified as described in section 4.2.4 for transfection.

The COX-1 cDNA was enzymatically digested from the template plasmid using Bam HI restriction endonuclease (Promega) at 37°C. The pTRE2 response plasmid (Clontech) was purchased from a commercial supplier to give ready access to a

tetracycline-regulated expression (Tet-Off) system (219). The pTRE2 plasmid and template plasmid (containing the COX-1 cDNA) were digested using 50U of Bam HI restriction endonuclease in Bam HI reaction buffer (6 mM Tris-HCl pH 7.9, 150 mM NaCl, 6 mM $MgCl_2$, 1 mM DTT and 0.05 mg/ml BSA), made up to 100 µl with ddH_2O. Restriction digests were electrophoresed on 1.0 % ethidium bromide agarose gels as described in section 2.2.3 and sized using kb markers (Gibco). The appropriately sized COX-1 cDNA and digested pTRE2 vector were excised and purified using the QIAquick Gel extraction kit (Qiagen). DNA fragments were excised from the gel using a scalpel and placed separately in clean tubes. Thereafter, the gel slices were weighed, 3 volumes (w/v) of QG buffer (Qiagen) was then added and samples heated at 50°C for 10 min. Subsequently, the samples were added to the supplied columns, centrifuged for 1 min at 13,000 rpm, 0.5 mls of QC buffer (Qiagen) was then added to the columns and centrifuged as before. The DNA was then washed by the addition of PE buffer (Qiagen), followed by centrifugation as before. DNA was eluted using 30 µl of EB buffer (10 mM Tris-HCl pH 8.5). The DNA concentration was determined by spectrophotometry (section 2.3.2). The digested pTRE2 plasmid was treated with shrimp alkaline phosphatase (Boehringer Mannheim) to prevent self-ligation.

4.2.2.2. Ligation Reaction.

For ligation, the cDNA fragment was incubated with the pTRE2 vector at a 3:1 molar ratio of insert to vector (18.9 ng to 50 ng) and ddH_2O was added to a final volume of 20 µl. Ligation was then achieved by incubating the DNA at room temperature for 5 min with Ready-To-Go™ T4 DNA Ligase (6 Weiss units T4 DNA ligase, 66 mM Tris-HCl pH 7.6, 6.6 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM spermidine, 10 mM DTT and stabilisers, Amersham Pharmacia Biotech). The ligation reaction was mixed by pipetting and then incubated at 16°C overnight. The ligated vector (2 µl) was then used to transform competent TOP10 cells as described in section 4.2.2.3. The clone containing the correctly sized insert was then used to propagate 100 mls of LB broth (Appendix I) and the plasmid was recovered as described in section 4.2.3 and purified as described in

section 4.2.4. The plasmid was digested as described in section 4.2.5 and sequenced as outlined in section 4.2.6 using sequence specific primers to confirm the orientation of the cDNA insert.

4.2.2.3. Transformation into Competent Cells.

Ligated plasmid containing insert was transformed into competent TOP10 cells using TOP10 One Shot kit (Invitrogen). Briefly 2 µl of 0.5 M ß-mercaptoethanol was added to a vial of competent cells together with 2 µl of TOPO cloning reaction and 100 µg DNA and mixed prior to incubation on ice for 30 min. Cells were heat-shocked at 42°C for 30 seconds and transferred to ice for 2 min. To this mixture, 250 µl SOC medium (supplied with the kit) was added and the tube incubated under vigorous shaking at 37°C for 30 min. Thereafter, 50 µl and 100 µl aliquots were streaked out and grown on LBagar plates containing 10 mg/ml ampicillan at 37°C overnight. Single colonies were picked, and inoculated into 10ml of LB broth containing 10mg/ml ampicillan and grown at 37°C under constant agitation overnight. Plasmid DNA was recovered as described previously (Section 4.2.3) using Qiagen endofree plasmid isolation kit (Qiagen). The orientation of the insert was determined by restriction digest and automated DNA sequencing using sequence specific primers as described in section 4.2.6.

4.2.3. Large Scale Plasmid DNA Recovery – Maxiprep

Large-scale plasmid recovery was carried out using the endofree™ plasmid maxi kit (Qiagen), an alkaline lysis plasmid recovery system, according to manufacturers instructions. Briefly, 100 mls of an overnight bacterial culture was pelleted by centrifugation at 4°C for 15 min at 6000g. The pelleted cells were resuspended in 10mls of buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 100 µg/ml RNase A). The cells were then incubated for 5 min at room temperature with 10ml of buffer P2 (200 mM NaOH and 1 % SDS), which facilitates cell lysis. The cell lysate was neutralised by the addition of 10 ml of 3 M potassium acetate pH 5.5, transferred to a QIAfilter,

incubated for 10 min and then filtered. Thereafter, 2.5 ml of buffer ER (Qiagen) was added and the filtered cell lysate was incubated for 30 min on ice. Subsequently the cell lysate was applied to the QIAGEN-tip and then washed twice with 30 ml of buffer QC (1 mM NaCl, 50 mM MOPS pH 7.0 and 15 % isopropanol). The plasmid DNA was then eluted with 15 ml of buffer QN (1.6 mM NaCl, 50 mM MOPS pH 7.0 and 15 % isopropanol) and precipitated with 10.5 ml of isopropanol. The sample was then centrifuged at 4°C for 30 min at 15000g. The supernatant was removed, the DNA pellet was washed with 15 ml 70 % ethanol and then centrifuged at 4°C for 15 min at 15000g. Thereafter, the pellet was air dried and resuspended in 1.5 ml of TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). The concentration of plasmid DNA was then determined by spectrophotometry (section 2.3.2).

4.2.4. Purification of Plasmid DNA

Purification of plasmid DNA was performed by phenol-chloroform precipitation-extraction. Briefly, enzymatic digestion reactions were made up to a volume of 100 µl with ddH_2O and 100 µl of tris-buffered phenol:chloroform:IAA (Camlab) was added. Samples were then vortexed for 15 seconds and then centrifuged for 15 seconds at 13000 rpm. Following centrifugation, the upper layer, containing the DNA, was removed, placed in a clean tube and 100 µl of chloroform:IAA was added. Samples were vortexed for 15 seconds and then centrifuged for 15 seconds at 13000 rpm. Subsequently, the upper layer, containing the DNA was removed, placed in a clean tube and incubated at -20°C for 1 hr with 0.1 volume of 5 M ammonium acetate plus 2.5 volume of absolute ethanol. Thereafter, the DNA was pelleted by centrifugation at 4°C for 20 min at 13,000 rpm. The supernatant was removed and discarded and the DNA pellet was washed with 70 % ethanol followed by centrifugation at 4°C for 5 min at 13000 rpm. The pellet was air dried and resuspended in 20 µl of ddH_2O.

4.2.5. Identification Of Clones With cDNA Insert Of Correct Size

The plasmid vector (pTRE2, Clontech) used in this study has a number of unique enzyme restriction sites (Appendix III). Digestion of the plasmid vector with restriction enzymes enables the size of the ligated cDNA insert to be confirmed. Identification of the vector containing the DNA insert of correct size as well as the direction of cDNA insert was carried out by restriction endonuclease digestion and further confirmed by automated DNA sequencing. DNA from competent cells transformed with vector containing the COX-1 cDNA was digested with *EcoRI*.

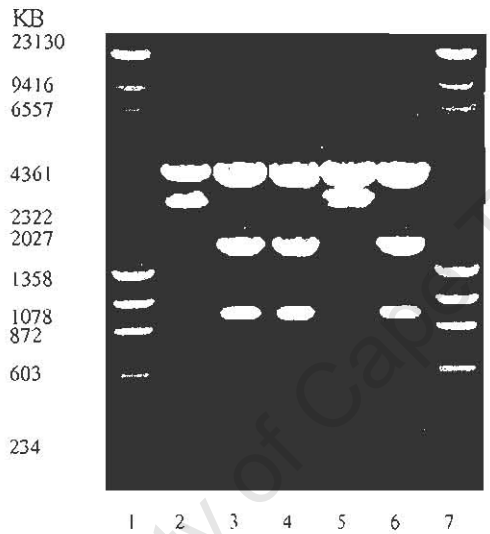


Figure 4.2. Agarose gel electrophoresis of *EcoRI* digested plasmid containing insert in the sense orientaion (Lanes 2 and 5) and anti-sense orientation (Lanes 3, 4, 6) The sizes of the fragments were determined relative to the mobility of the molecular weight standard (Lanes 1 and 7). The agarose gel was stained with ethidium bromide.

For endonuclease digestion, plasmid vector (0.5 µg to 1µg) was incubated for 1 to 4 hrs at 37°C in a reaction mix containing 10 U of *EcoRI* restriction enzyme in *EcoRI* reaction buffer (90 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$ and 0.05 mg/ml BSA) to a final volume 10 µl with ddH_2O. Digest products were subsequently analysed by agarose gel electrophoresis (section 2.3.3). Both sense and anti-sense clones were expected following ligation of the COX-1 cDNA and pTRE2 plasmid. Predicted sizes for fragments following *EcoRI* digestion of plasmid containing COX-1 cDNA in the sense orientation (5' to 3' orientation) were 255 kb, 2905 kb and 3032 kb respectively. Digestion of the plasmid containing COX-1 cDNA in the anti-sense orientation yielded

fragments of sizes 2222 kb, 3032 kb and 938 kb when compared with the molecular weight standard (kb ladder). The electrophoretic profile following *EcoR1* digestion is shown in Figure 4.2. Only clones containing the COX-1 cDNA in the sense (5'-3') direction were kept. Orientation of the COX-1 cDNA and success of the ligation into the pTRE2 vector was further confirmed by DNA sequencing as described in section 4.2.6.

4.2.6. Automatic DNA Sequencing

DNA sequencing was performed using the dideoxynucleotide chain termination technique (220). Sequencing reactions were prepared using the ABI Prism™ Terminator Cycle Sequencing ready reaction kit (PE Applied Biosystems) and the base pair sequence of samples obtained using the Applied Biosystems Model 373A DNA sequencing system.

4.2.6.1. Sequencing Reactions

DNA sequencing reactions were prepared by incubating 400-500 ng of plasmid DNA with 4 µl of terminator ready reaction mix, 4 µl of half term (400 mM Tris-HCl pH 9 and 1 mM $MgCl_2$) and 3.2 pmol of the required sequencing primer to a total volume of 20 µl with ddH_2O. The sequence-specific primers used for sequencing are as follows: 5'-CGCCTGGAGACGCCATCC-3' and 5'-CCACACCTCCCCCTGAACC-3' (Clontech). Sequencing reactions were placed in a PCR machine (Touchdown™ Temperature Cyclin System, Hybaid) and incubated for 25 cycles of 96°C for 15 seconds, 50°C for 25 seconds and 60°C for 4 min. Subsequently, 16 µl of ddH_2O and 64 µl of 95 % ethanol was added to the sequencing reactions and the samples were left to precipitate at room temperature for 15 min. Thereafter, samples were centrifuged at 4°C for 20 min at 13,000 rpm, air dried and resuspended in 4 µl of formamide/EDTA loading buffer (5 µl formamide: 1 µl 50 mM EDTA, pH 8.0).

4.2.6.2. Sequencing Gel

A gel mix was prepared using 50 g urea, 15 mls acrylamide (Anachem, 40% w/v acrylamide/bis-acrylamide stock solution 19:1), 2 g amberlite and ddH_2O to a volume of 90 mls. The gel mix was stirred and heated until the urea dissolved and subsequently filtered. Following filtration, 10 mls of 10x TBE (Appendix I), 45 µl of TEMED, and 500 µl of 10 % ammonium persulphate was added. The gel mix was then poured between the glass plates. Once polymerised, the gel was pre-run for 30 min at 30W and then 1X TBE was added (Appendix I) to the buffer tanks. Prior to loading, sequencing reactions were heated to 95°C for 5 min, loaded onto the gel, and the gel run overnight at 30W. Sequence data was collected using the automatic data collection and analysis programs (PE Applied Biosystems).

4.2.7. Transfection And Selection Procedure

HelaTet-Off (Clontech) a commercially available stable cell line transfected with the pTet-Off vector (221) was purchased (Clontech) for the purpose of creating a double stable HeLa (cervical epithelial) cell line for tetracycline-regulated expression of COX-1. Hela Tet-Off cells were maintained as described in section 4.2.1.

Approximately 50000 cells were seeded in a final volume of 1ml per well in each well of a 12 well dish in complete medium containing 100 µg/ml G418 per well. Cells were allowed to attach and grow overnight. The pTRE2 vector containing COX-1 cDNA (2 µg) in the sense direction together with pTKHyg (0.1 µg) was transfected into each well (done in quadruplicate) using lipid pfx-5 (Invitrogen) diluted in optimem (Gibco). In parallel, cells were transfected with pcDNA6/V5/His/LacZ (Invitrogen) cDNA to control for tranfection efficiency (section 4.2.8). Cells were also incubated with optimem only or optimem containing pfx-5. Transfected and control cells were incubated for 4 hrs at 37°C in 5% humidified CO_2. Thereafter the medium was replaced with fresh complete medium containing no G418. Cells were allowed to grow for 72 hrs. Transfected cells were then seeded together with wild-type cells using a ratio of transfected:wild-type of 1:10. Clones were selected against 200 µg/ml hygromycin B (Hyg; Sigma) in the presence of 1

μg/ml doxycycline (DOX; Sigma) to prevent transcription of the COX-1 cDNA cloned into pTRE2.

The culture medium was changed every 48 hrs with fresh DOX to ensure suppression of the COX-1 gene. Once control untransfected cells had died, at least 50 Hyg-resistant clones were picked using cloning cylinders (Sigma). Briefly, cloning cylinders were placed over isolated colonies and the cells trypsinised and transferred to a well in a 96 well plate. Clones were allowed to grow under continuous selection with Hyg in the presence of DOX and then screened for the ability to express the COX-1 gene in the presence and absence of DOX by real-time RT-PCR and Western blot analysis. All clones were maintained in 1 μg/ml DOX, 200 μg/ml Hyg and 100 μg/ml G418.

4.2.8. β-Galactosidase Assay

Hela Tet-Off cells transfected with the pcDNA6/V5/His/LacZ (Invitrogen) cDNA were assayed with β-galactosidase (X-gal) to measure transfection efficiency. Transfected cells would turn blue in the presence of β-galactosidase. 48 hrs after transfection, cells were washed three times with phosphate buffered saline (PBS) and then fixed with 0.2 % glutaraldehyde/2 % formaldehyde in PBS for 5 min. Thereafter the cells were washed three times with PBS and then incubated overnight at 37°C with the staining solution (5 mM $K_3Fe(CN)_6$ 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 1 mg/ml X-gal). The following day the blue cells were counted and expressed as a percentage total cells to give the transfection efficiency.

4.2.9. Quantitative RT-PCR

Quantitative RT-PCR was carried out on Hyg-resistant HeLa COX-1 Tet-Off clones as described in section 3.2.2. Briefly, $2x10^5$ cells were seeded in 6 well dishes and grown for 72 hrs in the presence or absence of DOX. Cells were harvested with Tri-

Reagent and the RNA isolated as described in section 2.3.1. RNA was reverse transcribed as described in section 2.3.4.

4.2.10. Western blot analysis

Single Hyg-resistant colonies were picked as described in section 4.2.7 and allowed to propagate on monolayer in flasks until confluent as described in section 4.2.1. Once confluent, cells were seeded into 5cm dishes at approximately $5x10^5$ cells/dish in duplicate. One dish was grown for 72 hrs in the presence of DOX and the other was grown in medium containing no DOX for 72 hrs. Cells were harvested as described in section 2.4.2 and protein quantified according to section 2.4.3. Approximately 20 μg of total clarified cell lysate was loaded onto a gel and electrophoresed as described in section 2.4.4. COX-1 inducibility was assessed by Western blot analysis as described in section 2.4.5 using a specific goat anti-COX-1 antibody (section 2.1) at a dilution of 1:500.

4.3. RESULTS

4.3.1. Selection Of Clone With The Greatest Inducible Overexpression Of COX-1 By Real-Time PCR.

Quantitative real time RT-PCR analysis was performed on isolated stably transfected colonies. A total of 3 clones (clones 3.1, 2.2 and 1.2) out of 50 were identified as having the greatest inducible overexpression of COX-1. Following the removal of DOX from the culture medium, the relative expression of COX-1 was observed to increase compared with cells maintained uninduced with DOX for 72 hrs. Real-time RT-PCR analysis revealed a variation in inducible COX-1 overexpression between the 3 clones selected for further investigation. As shown in figure 4.3, clone 1.2 produced the greatest overexpression of COX-1 when DOX was removed from the culture medium, with the lowest COX-1 expression in the uninduced state.

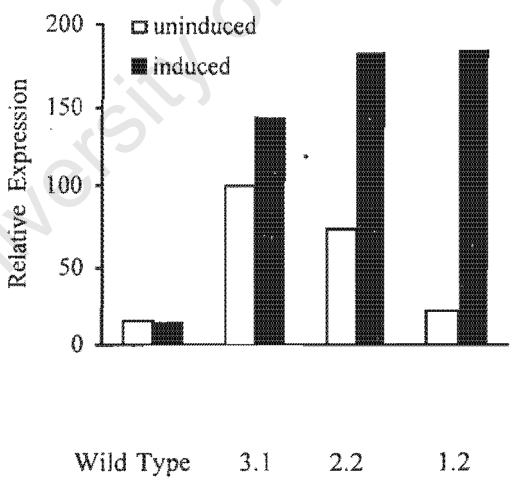


Figure 4.3. Real-time PCR expression of wild-type untransfected Hela Tet-Off cells, as well as HeLa COX-1 Tet-Off clones 3.1, 2.2 and 1.2. Open bars represent cells grown in the presence of DOX (uninduced) for 72hrs and solid bars represent cells grown in the absence of DOX (induced) for 72 hrs.

In order to determine the effect of DOX on COX-1 expression, untransfected wild-type HeLa Tet-Off cells were grown in the presence or absence of DOX for 72 hrs.

As shown in Figure 4.3, no difference in the relative expression of COX-1 was observed between wild-type cells grown in the absence or presence of DOX. In all experiments, uninduced cells were maintained in 1 μg/ml DOX, which was supplemented daily.

4.3.2. Western Blot Analysis Of Stable Transfectants.

Western blot analysis was performed on clones 1.2, 2.2 and 3.1, which produced the greatest inducible overexpression of COX-1 by real-time RT-PCR analysis. Following the removal of DOX from the culture medium, an immunoreactive band migrating at approximately 72 kDa was observed to increase in intensity when compared with cells maintained with DOX after 72 hrs (Figure 4.4). Although all three clones exhibited the same growth characteristics and phenotype and produced similar results, the clone with the greatest COX-1 overexpression (clone 1.2) was chosen for further studies.

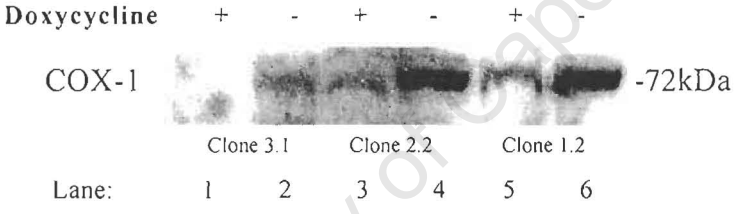


Figure 4.4. Western blot analysis of Hyg-resistant clones. HeLa Tet-Off cells were transfected with COX-1 cDNA and stable transfectants were selected against 200 μg/ml Hyg. Approximately 20 μg per lane of each clone, uninduced (maintained with DOX) and induced (grown without DOX) was electrophoresed and probed with a specific antibody raised against the C-terminus of Human COX-1. Lanes 1, 3, 5 shows clones 3.1, 2.2 and 1.2 uninduced (maintained with medium containing DOX). Lanes 2, 4, 6 shows the same clones 72hrs after the removal of DOX from the culture medium.

4.4. DISCUSSION

In chapter 3 we demonstrated elevated expression of COX-1 in cervical carcinomas. In order to ascertain a role for COX-1 in cervical carcinomas, we developed an inducible expression system (HeLa COX-1 Tet-Off system) in HeLa cells to promote inducible expression of COX-1. This system will allow for the investigation of the potential roles of the diverse COX-1 enzyme products in mediating events associated with cervical neoplasias. These factors may act in concert in an autocrine/paracrine/intracrine manner via their target receptors.

In this chapter, the successful establishment of a HeLa Tet-Off double stable cell line is described. The overexpressing system following removal of DOX from the culture medium is confirmed by real-time RT-PCR and Western blot analysis. Various gene expression systems have been used in the past to replicate various cellular and biochemical events, observed *in vivo*, in a cell line. These expression systems use eukaryotic promoters responsive to heavy metal ions, heat-shock or hormones and generally suffer from leakiness of the inactive state (219). The Tet-Off system allows for tight control of gene expression, thereby reducing the leakiness which is often associated with eukaryotic expression systems. The transactivator tTA produced in HeLa cells binds specifically to the tetO sequences *in vitro*. This association is prevented by DOX. When the tTA is bound to the tetOs placed upstream of minimal promoters, tTA efficiently activates transcription of the downstream target gene in a DOX dose-dependent manner (219). In the Tet-Off expression system each clonal cell line is used as its own control (cells cultured in the presence of DOX) and the overexpression of the integrated target gene is modulated solely by removing DOX from the culture medium. This eliminates the need for a control clonal cell line transfected with vector alone (as used with constitutive stable expression systems) thereby overcoming the inherent variation that arises from different sites of integration of DNA between different clones.

We picked 50 Hyg-resistant colonies, three of which produced inducible expression of COX-1 following the removal of DOX from the culture medium. Real time RT-PCR analysis revealed different levels of inducible expression of COX-1 between the three clones. This difference in inducibility may be due to the site of integration of the transfected COX-1 gene or leaky transgene expression. If the transfected COX-1 gene integrated into a highly active section of the genome, then transcription may result in the presence of DOX causing leakiness of the system. However, all three clonal lines (clones 1.2; 2.2 and 3.1) derived from these colonies exhibited similar growth, phenotypic and biochemical characteristics and yielded similar experimental results, although the COX-1 overexpression of clone 3.1 was reduced compared with that of clones 2.2 and 1.2. This was attributed to the level of inducibility, as clone 3.1 had a greater background level of expression of COX-1 and lower level of inducible expression than the other two clones as determined by real-time PCR. The clonal line which produced the greatest COX-1 overexpression and lowest background (clone 1.2) was chosen for further investigation. In order to establish whether DOX has any effect on basal COX-1 levels, wild-type HeLa Tet-Off cells were grown in the presence or absence of DOX for 72 hrs. No significant difference in COX-1 RNA expression was determined by quantitative real-time PCR.

The Tet-Off system has been extensively used to study the effect of various genes *in vitro* as well as *in vivo* using animal models. Several investigators have used the Tet-Off system to investigate the effect of conditional switching of angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF)-binding protein and FGF-2 expression on the growth of human tumor cells or cells injected into nude mice (222-224). In an *in vitro* model system, FGF-2 transfected into a human endometrial adenocarcinoma cell line, under the control of the TetR, caused an increase in the proliferation of co-cultured endothelial cells. The endothelial cell proliferation was inhibited by treating the FGF-2 overexpressing cells with tetracycline. The same FGF-2 overexpressing cells injected into mice was associated with increased tumorigenesis at the site of injection. Injection of the FGF-2 overexpressing cells into mice that had been administered tetracycline failed to induce the same tumor growth at

the site of injection (224). Similarly, in breast cancer cells transfected with the VEGF gene under control of the TetR, an increase in vascularisation was observed in nude mouse xenografts in the absence of tetracycline. The effect was reversed by switching the gene expression off by addition of tetracycline [Yoshiji, 1997 #852

In conclusion, we have generated an inducible COX-1 expression system in HeLa cells to promote inducible and reversable expression of COX-1. This system has provided us with a powerful tool to investigate the potential role of COX-1 and its synthesized eicosanoids in cervical carcinomas.

CHAPTER 5

Autocrine/Paracrine Regulation of Cyclooxygenase-2, Prostaglandin E (PGE) Synthase, Prostaglandin E_2 Receptors and Angiogenic Factors in HeLa Cells Following Inducible Overexpression Of Cyclooxygenase-1

5.1. INTRODUCTION

Numerous studies have now demonstrated that epithelial tumors may be regulated by COX-enzyme products (4, 7, 10, 80, 225). The relative contributions of COX-1 and/or COX-2-derived products in mediating events associated with neoplasia remain to be fully elucidated. Most studies have focussed on COX-enzyme products in model sytems overexpressing COX-2 (4, 8, 69, 188). COX-2 overexpression and subsequent enhanced synthesis of eicosanoids such as PGE_2 facilitates neoplastic transformation of epithelial cells by reducing cell surface adhesion molecules and inhibiting apoptosis (69), enhancing the metastatic potential of epithelial cells (7) and promoting angiogenesis (8). This has prompted the suggestion that the increased level of prostaglandins and other eicosanoids present in cancer tissue is a consequence of induced COX-2. More recently however, it has been demonstrated that both COX isoforms are inducible. In some cell types, including pulmonary artery endothelial cells, COX-1 levels are induced during differentiation (93, 94). Furthermore, COX-1 expression can be induced *in vitro* by vascular endothelial growth factor (VEGF), (96), arachidonic acid, forskolin, dibutryl-cAMP and PGE_2 (95). We have demonstrated elevated expression of COX-1 in human squamous cell carcinoma and adenomacarcinoma of the cervix by quantitative RT-PCR and Western blot analysis and localised expression to neoplastically transformed cervical epithelial cells by immunohistochemistry (discussed in Chapter 3). Similar elevated COX-1 expression has been reported in mouse lung tumors (82), human breast cancer (83), human prostate carcinoma (78) and human ovarian adenocarcinoma (81). These data suggest that under certain conditions, both COX enzymes and/or their products may function in promoting and maintaining the neoplastic state.

This study was designed to investigate the expression and molecular signalling of COX-1 in cervical carcinoma cells by using the inducible COX-1 expression system (HeLa COX-1 Tet-Off system) described in chapter 4.

5.2. MATERIALS AND METHODS

5.2.1. Treatments

In this study we investigated the effect of COX-1 overexpression on various parameters of molecular pathways. Initially we investigated the effect of COX-1 overexpression on the expression of COX-2, PGES and synthesis of PGE_2. This was done by growing HeLa COX-1 Tet-Off cells in the absence or presence of DOX and absence or presence of the dual COX enzyme inhibitor indomethacin (3μg/ml) or selective COX-2 inhibitor NS-398 (10μM) for a time-course of 24, 48 and 72 hrs. COX-2 and PGES expression was determined by Western blot analysis as described in section 5.2.3, and PGE_2 synthesis was determined by ELISA as described in section 5.2.4. The detailed experimental protocols are outlined in the relevant sections. An autocrine/paracrine role for COX enzyme products was determined by investigating the effect of COX-1 overexpression on the expression of the PGE_2 receptors (EP1-4). This was carried out by growing HeLa COX-1 Tet-Off cells in the the presence and absence of DOX and presence and absence of indomethacin. The effect of COX-1 overexpression on the EP receptors was anaylsed by real-time RT-PCR analysis as described in section 5.2.5, and the possible signalling pathways associated with them (cAMP and inositol phosphate pathways) by measuring accumulation of total inositol phosphates (section 5.2.6) and cAMP (section 5.2.7) in response to stimulation of uninduced and induced cells with PGE_2. Finally the effect of COX-1 overexpression on the expression of pro-angiogenic factors was determined, following overexpression of COX-1 for a time-course of 24, 48 and 72 hrs, in the presence or absence of the COX enzyme inhibitors NS-398 or indomethacin. The effect of COX-1 overexpression on the expression of the pro-angiogeneic factors was analysed by Western blot analysis as described in section 5.2.3.

5.2.2. Cell Culture

HeLa COX-1 Tet-Off cells were routinely maintained in Dulbecco's modified Eagle's medium as described in section 4.2.1. with the addition of 200 μg/ml Hyg and 1 μg/ml DOX. Wild-type Tet-Off cells were maintained as described in section 4.2.1. HeLa–S3 parental cells were maintained in the same medium without the addition of G418, DOX or Hyg. COX-1 expression was induced by removing DOX from the culture medium. COX inhibition studies were conducted following removal of DOX from the culture medium and by growing cells in medium containing 3 μg/ml indomethacin or 10 μM NS-398.

5.2.3. Western Blotting

Immunoblot analysis was performed on supernatant fractions of wild-type HeLa Tet-Off cells, HeLa COX-1 Tet-Off cells and HeLa S-3 cells. Briefly, cells were seeded in 5cm dishes and allowed to attach overnight. The following day, the cells were synchronised to cell cycle by incubating with serum-free medium for 24 hrs. Thereafter, the medium was replaced with fresh complete medium and the cells were grown in the presence or absence of DOX for 24, 48 and 72 hrs respectively. In parallel, cells were co-treated with indomethacin or NS-398. HeLa-S3 cells were grown in medium containing no DOX. Cells were harvested by lysing in protein lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 % Triton X-100, 0.1 % SDS). The protein content in the supernatant fraction was determined as outlined in section 2.4.3. The clarified cell lysates (20 μg) were denatured and electrophoresed on 4 % to 20 % Tris-Glycine gels (NOVEX, Invitrogen). The proteins were transferred onto polyvinylidene difluoride membrane (PVDF, Millipore, Watford, UK) and subjected to immunoblot analysis as described in section 2.4.5. Membranes were incubated overnight with either COX-1 (1:500), COX-2 (1:500), β-actin (1:500), PGES (1:250), VEGF (1:500), angiopoietin (Ang)-1 and -2 (1:250) or bFGF (1:500) specific primary antibodies. Thereafter, membranes were subsequently incubated for 1 hr with rabbit anti-goat secondary antibody (for

COX-1/2, β-actin, Ang-1/2, bFGF) at a dilution of 1:30000 or goat anti-rabbit secondary antibody (PGES, VEGF) at a dilution of 1:30000. Proteins were normalised for loading against β-actin on the same blot. Immunodetection was performed using the ECF chemiluminescence system following the manufacturer's instructions. Proteins were revealed and quantified by PhosphorImager analysis using the STORM 860 system (Molecular Dynamics, UK.). Protein size and fold induction was calculated as described in section 2.4.5. COX-1 negative control to determine specificity of the COX-1 antibody was performed according to standard protocols described in section 2.5. Data are presented as mean ± SEM from 4 independent experiments.

5.2.4. PGE_2 Assay

The effect of COX-1 overexpression on PGE_2 secretion was assessed using the HeLa COX-1 Tet-Off cells. Cells were seeded in 5cm dishes at a cell density of $5x10^5$ cells/dish and were allowed to grow and attach overnight. The following day, the cells were synchronised to cell cycle by incubating with serum-free medium for 24 hrs. Thereafter the cells were induced for 24, 48 and 72 hrs respectively, by DOX withdrawal from the culture medium, in the presence or absence of indomethacin or NS-398. Arachidonic acid to a final concentration of 5 μg/ml was added to the culture medium following COX-1 overexpression for 6 hrs. Thereafter, 1 ml of medium was removed and added to 1 ml of methyloximating solution. Control uninduced cells were treated similarly but maintained with 1 μg/ml DOX supplemented daily. PGE_2 secretion into the culture medium was assayed by ELISA (226). The ELISA was performed using 96 Well plates (Amine-binding plates; Costar, High Wycombe, UK) coated with donkey anti-rabbit antibody. Plates were then coated with rabbit immunoglobulin G (1 mg/ml diluted in PBS with 1 % carbonate buffer, pH 9.6) at 200 μl/well for 16 hrs at 4°C. The solution was aspirated and blocking solution (50 mM glycine, 10 mg/ml bovine serum albumin) added at 25 μl/well for 2 hrs at 23°C. The plates were then washed and donkey anti-rabbit serum (Scottish Antibody Production Unit, Carluke, UK) added to a final volume of 150 μl/well, before

washing, air-drying and storage with desiccant at 4°C. The link was prepared by ether extraction and reverse phase chromatography using 20 mg of synthetic PGE_2, 320 µl dry dimethylformamide, 3 µl butylchloroformate and 0.05 mM biocytin. Samples and synthetic standards were diluted in ELISA buffer (150 mM NaCl, 100 mM Tris-HCl, 0.05 % Tween-20, 50 mM phenol red, 1 mM 2-methylisothiazolone, 1 mM bromonitrodioxane, 2 mM EDTA, 2 mg/ml bovine serum albumin to a final pH 7.2), and 100 µl of each added in duplicate to the plate. The link was diluted $1:1.5x10^6$ in ELISA buffer and 50 µl added to each well. Antisera, diluted 1:50000 in ELISA buffer, was added to a final volume of 50 µl to all wells except those used for measuring non-specific binding. Plates were incubated at 4°C for 16 hrs, washed and 100 µl/well of 0.2 unit/ml streptavidin-peroxidase (Boehringer Mannheim) was added. Plates were then incubated at for 20 mins at 23°C on an orbital shaker, washed and substrate (0.3 g/L urea-hydrogen peroxide, 0.1 g/L tetramethyl benzine in 100 mM sodium acetate, pH 6.0) added to a final volume of 200 µl/well for 10 mins before quenching with 50 µl/well 1M sulphuric acid. Colour reaction was measured at 450 nm by spectrophotometry. The rabbit antiserum that was raised against PGE_2–complexed keyhole limpet hemocyanin has been previously characterised (211). Data are presented as mean ± SEM from 3 independent experiments.

5.2.5. Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed to assess the effect of COX-1 overexpression on prostaglandin E_2 receptor (EP1, EP2, EP3 and EP4) expression. Briefly, cells (2×10^5) were seeded in 6 well plates, allowed to attach and grow overnight in the presence of 1 µg/ml DOX. The following day, the cells were synchronised by incubating with serum-free medium for 24 hrs. Thereafter, the medium was replaced with fresh complete medium and COX-1 overexpression was induced by growing cells in medium containing no DOX. Control cells were maintained in 1 µg/ml DOX. Cells were harvested after 24, 48 and 72 hrs with 1 ml/well Tri-Reagent (Sigma) as per the manufacturers protocol described in section

2.3.1. RNA samples were reverse transcribed as outlined in section 2.3.4. Quantitative RT-PCR was performed as outlined in section 2.3.5 using sequence-specific primers and probes. The sequence of the EP1 receptor primers and probe were as follows: Forward: 5'- AGA TGG TGG GCC AGC TTG T-3'; Reverse: 5'- GCC ACC AAC ACC AGC ATT G -3'; Probe (FAM labelled): 5'- CAG CAG ATG CAC GAC ACC ACC ATG- 3'. The sequence of the EP2 receptor primers and probe were as follows; Forward: 5'-GAC CGC TTA CCT GCA GCT GTA C-3'; Reverse: 5'–TGA AGT TGC AGG CGA GCA-3'; Probe (FAM labelled): 5'–CCA CCC TGC TGC TGC TTC TCA TTG TCT-3'. The sequence of the EP3 receptor primers and probe were as follows; Forward: 5' –GAC GGC CAT TCA GCT TAT GG- 3'; Reverse: 5'- TTG AAG ATC ATT TTC AAC ATC ATT ATC A- 3'; Probe(FAM Labelled): 5' CTG TCG GTC TGC TGG TCT CCG CTC-3'. The sequence of the EP4 receptor primers and probe were as follows; Forward: 5'–ACG CCG CCT ACT CCT ACA TG-3'; Reverse: 5'–AGA GGA CGG TGG CGA GAA T-3'; Probe (FAM labelled): 5'–ACG CGG GCT TCA GCT CCT TCC T-3'. The ribosomal 18S primers and probe sequences were as follows; Forward: 5' –CGG CTA CCA CAT CCA AGG AA-3'; Reverse: 5'–GCT GGA ATT ACC GCG GCT-3'; Probe (VIC labelled): 5'–TGC TGG CAC CAG ACT TGC CCT C-3'. Expression of EP receptors was normalised to RNA loading for each sample using the 18s ribosomal RNA as an internal standard. Relative gene expression in induced HeLa Tet-Off cells compared with uninduced cells was calculated by dividing the expression in induced cells by expression in uninduced cells. The data are presented as mean ± SEM from 3 independent experiments.

5.2.6. Total Inositol Phosphate Assays

Functionality of the EP1 receptor was measured by PGE_2 stimulation of total inositol phosphate production as described (227). Briefly 2 x 10^5 HeLa COX-1 Tet-Off cells were plated in 6 well dishes containing 4 ml/well of complete medium containing 200 µg/ml Hyg and 1 µg/ml DOX as mentioned above. Cells were allowed to attach overnight. The following day, the cells were washed three times with sterile

phosphate buffered saline (PBS) and then synchronised by incubating overnight with fresh medium containing no foetal calf serum (FCS). COX-1 Tet-Off cells were induced, by DOX withdrawal from the culture medium for 48 hrs. In parallel, uninduced cells supplemented daily with DOX to a final concentration of 1 μg/ml were treated similarly and used as a control. Cells were incubated with inositol-free DMEM (Gibco) containing 1 % dialyzed heat activated FCS and 0.5μCi/well myo-^{3}H-inositol (Amersham) for 48 hrs. Medium was removed and the cells washed with 1 ml buffer (140 mM NaCl, 20 mM HEPES, 4 mM KCl, 8 mM glucose, 1 mM $MgCl_2$, 1mM $CaCl_2$, 1 mg/ml bovine serum albumin) containing 10 mM LiCl and then incubated for 1 hr at 37°C in 0.5 ml buffer containing 10 mM LiCl and PGE_2 at the required concentration. Reactions were terminated by the removal of PGE_2 and the addition of 1 ml ice cold 10 mM formic acid, which was incubated for 30 min at 4°C. Total ^{3}H-inositol phosphate was separated from the formic acid cell extracts on AG 1-X8 anion exchange resin (Bio-Rad) and eluted with a 1M ammonium formate/0.1 M formic acid solution. The associated radioactivity was determined by liquid scintillation counting. Data are presented as mean ± SEM.

5.2.7. PGE_2 stimulation and cAMP measurement.

Functionality of the up-regulated prostaglandin E_2 receptors was assessed by measuring cAMP accumulation following COX-1 induction in the presence or absence of COX-enzyme inhibitor. Cells (2 x 10^5) were plated in 6 well dishes containing 4ml/well of complete medium containing 200 μg/ml Hyg and 1 μg/ml DOX as mentioned above. Cells were allowed to attach overnight. The following day, the cells were washed three times with sterile phosphate buffered saline (PBS) and then synchronised by incubating overnight with fresh medium containing no foetal calf serum. COX-1 Tet-Off cells were induced, by DOX withdrawal from the culture medium for 48 hrs at 37°C in humidified 5 % CO_2 in the presence or absence of 3 μg/ml indomethacin. In parallel, uninduced cells supplemented daily with DOX to a final concentration of 1 μg/ml were treated similarly and used as a control. Thereafter the culture medium was removed and replaced with serum-free medium

containing IBMX (Sigma) to a final concentration of 1 mM for 40 mins at 37°C. Cells were then stimulated with 0 nM PGE_2 or 300 nM PGE_2 for 5, 10, 20 or 30 min respectively. Following stimulation, the medium was removed and the cells lysed in 0.1 M HCl. cAMP concentration was quantified by ELISA using a cAMP kit (Biomol) as described in section 3.2.3.1 and normalised to protein concentration of the lysate. Protein concentrations were determined using protein assay kits as described in section 2.4.3. The data are presented as mean ± SEM from 3 independent experiments.

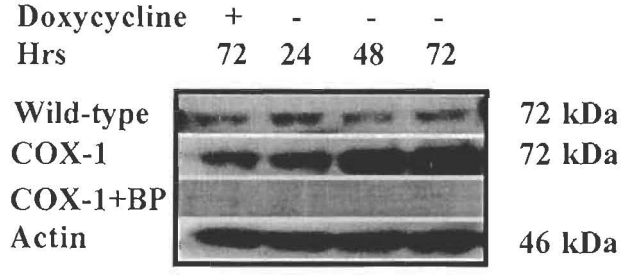
5.3. RESULTS

5.3.1. Inducible COX-1 Expression in HeLa Tet-Off Cells

To investigate the effect of COX-1 overexpression in neoplastic cervical epithelial cells, we established a doxycycline (DOX)-regulated expression system in HeLa cells. As shown in Figure 5.1A, following DOX withdrawal from the culture medium and subsequent induction of COX-1, an immunoreactive band migrating at approximately 72 kDa was observed to increase in intensity after 48 hrs, with maximal sustained induction after 72 hrs. Basal levels of COX-1 expression were determined from the COX-1 expression in cells maintained uninduced in 1 μg/ml DOX for 72 hrs. The fold induction for COX-1 overexpression above basal for 24, 48 and 72 hrs was determined to be 1.5 ± 0.34, 3.7 ± 0.45 and 4.7 ± 0.56 fold respectively. In parallel, wild-type HeLa Tet-Off cells were grown for 72 hrs in the presence or absence of DOX. No significant difference in COX-1 expression above basal was observed (Figure 5.1A). COX-1 was normalised for protein loading against β-actin on the same blot. Pre-adsorbing the COX-1 antibody with the blocking peptide (BP) abolished the COX-1 immunoreactivity indicating specificity of the COX-1 antibody.

PGE_2 biosynthesis and the functionality of the transfected COX-1 cDNA was assessed by measuring PGE_2 secretion into the culture medium following COX-1 induction for 24, 48 and 72 hrs respectively. A time-dependent increase in PGE_2 secretion into the culture medium accompanied the induction of COX-1 expression. PGE_2 production was significantly elevated after 48 hrs (272.2 ± 18.8 nM; $p<0.05$) and 72hrs (537 ± 22.5 nM; $p<0.01$) respectively when compared with PGE_2 levels in uninduced cells (118 ± 6.75 nM; Figure 5.1B). The addition of indomethacin reduced the PGE_2 levels to 62 ± 7 nM and 76 ± 0.7 nM after 48 hrs and 72 hrs respectively ($p<0.01$). Co-treatment of cells with NS-398 (selective COX-2 inhibitor) partially reduced PGE_2 levels to 132 ± 26.2 and 268 ± 17 nM after 48 hrs and 72 hrs respectively ($p<0.05$).

A



B

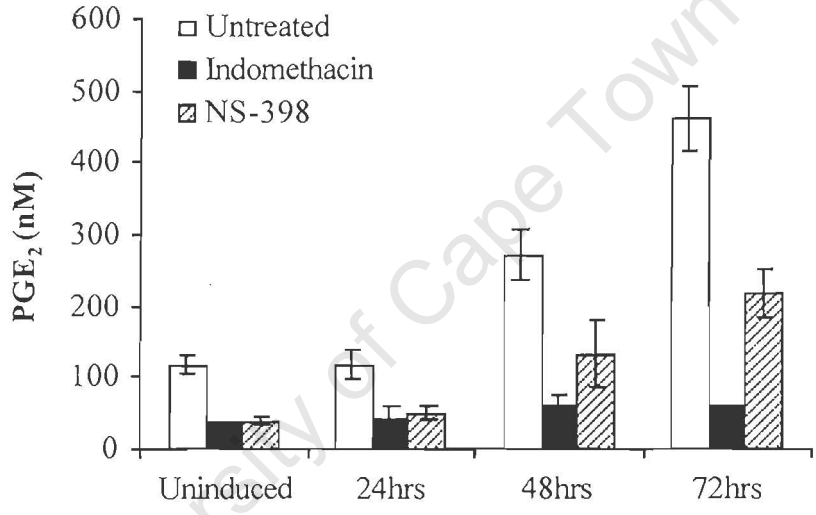


Figure 5.1. **(A)** Western blot analysis of 20 µg of total clarified cell lysate isolated from Wild-type HeLa Tet-Off and HeLa COX-1 Tet-Off cells grown for 72 hrs in the presence of DOX or 24, 48 and 72 hrs respectively in the absence of DOX. No immunoreactivity was detected by preadsorbing the antibody with the blocking peptide (BP). COX-1 was normalised for protein loading against β-actin on the same blot. **(B)** The functionality of the transfected COX-1 cDNA was assessed by ELISA, by measuring PGE_2 secretion into the culture medium following COX-1 induction in the presence or absence of COX-enzyme inhibitor, and treatment of HeLa COX-1 Tet-Off cells with 5 µg/ml arachidonic acid.

5.3.2. COX-1 Overexpression induces COX-2 and PGES

COX-enzyme products including PGE_2 are known to induce COX-2, as well as COX-1 expression (95). In order to investigate the effect of COX-1-enzyme products on expression of COX-2 and the microsomal glutathione-dependent inducible PGES, HeLa cells were grown in the presence or absence of the dual COX-enzyme inhibitor indomethacin or highly selective COX-2 inhibitor NS-398 for 24, 48 and 72 hrs. Following the removal of DOX from the culture medium, a time-dependent increase in COX-1 overexpression was observed in HeLa COX-1 Tet-Off cells after COX-1 induction for 48 hrs, with maximal sustained overexpression after 72 hrs (Figure 5.2A). Concomitant with this increase in COX-1 expression was a 3.2 ± 8.9 fold increase in COX-2 expression after 72 hrs and a 2.5 ± 0.45 and 1.3 ± 0.78 fold increase in PGES after 24 hrs and 48 hrs respectively (Figure 5.2A). After 72hrs PGES levels had returned to basal. Co-treatment of the HeLa COX-1 Tet-Off cells, induced for 24, 48 and 72 hrs respectively, with indomethacin or NS-398 showed no alteration in COX-1 overexpression (Figures. 5.2B and 5.2C), however indomethacin (3 μg/ml) treatment inhibited COX-2 as well as PGES induction (Figure 5.2B). No significant change in COX-2 expression was observed after treatment of HeLa COX-1 Tet-Off cells with NS-398 (Figure 5.2C). Induction of PGES by COX-1 overexpression was delayed by 24 hrs following treatment of HeLa COX-1 Tet-Off cells with NS-398 (Figure 5.2C).

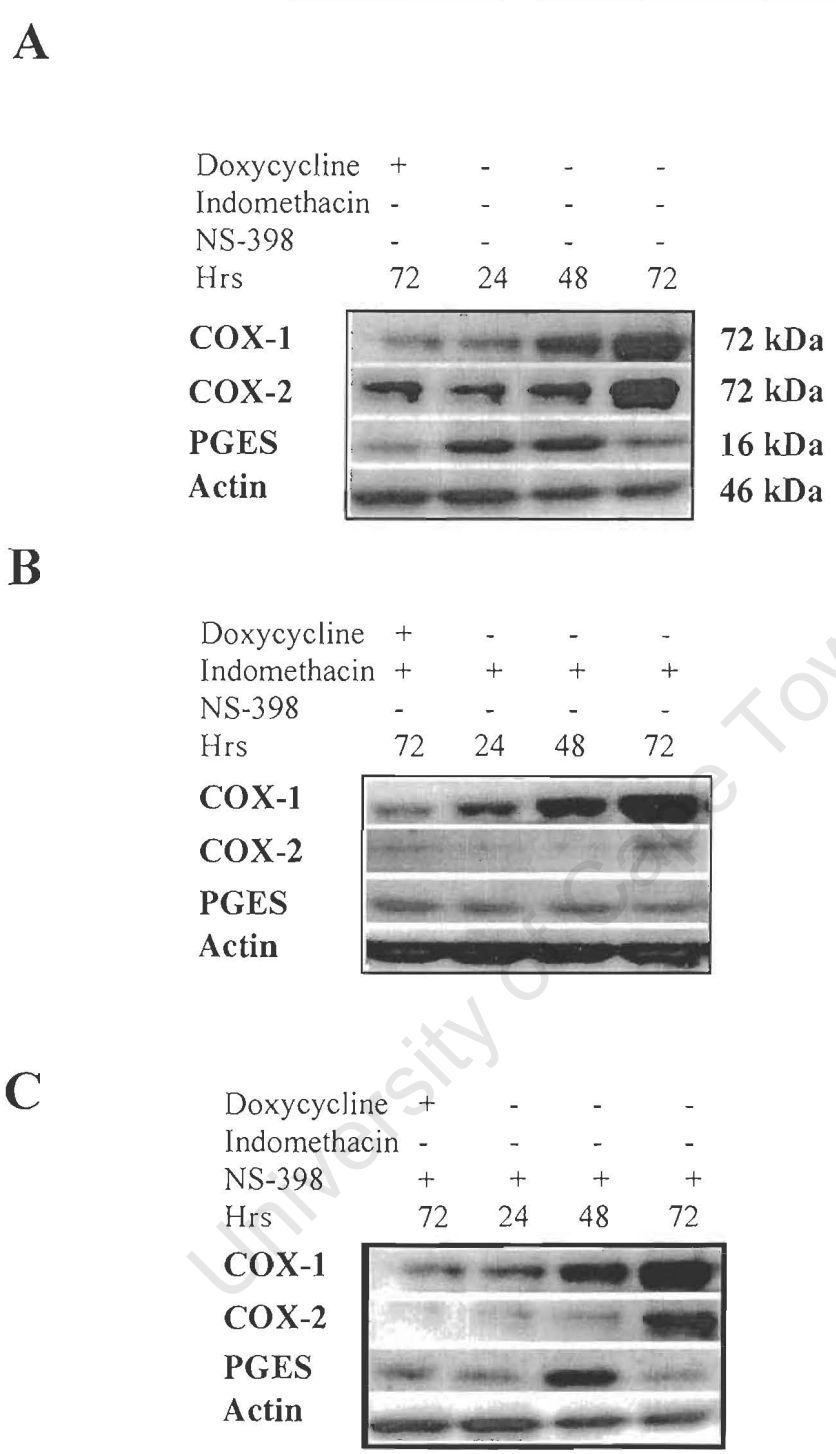


Figure 5.2. Western blot analysis of 20 µg of total clarified cell lysate isolated from HeLa COX-1 Tet-Off cells grown for 72 hrs in the presence of DOX (uninduced) or 24, 48 and 72 hrs respectively in the absence of DOX to induce COX-1 expression. (**A**) Expression of COX-2 and PGES are induced coincident with COX-1 overexpression in HeLa COX-1 Tet-Off cells. (**B**) Co-treatment of HeLa COX-1 Tet-Off cells with indomethacin abolishes the COX-1-mediated up-regulation of COX-2 and PGES. (**C**) Partial inhibition of the COX-1-mediated up-regulation of COX-2 and PGES expression in HeLa COX-1 Tet-Off cells by the selective COX-2 inhibitor NS-398. Proteins were normalised for loading against β-actin on the same blot.

5.3.3. PGE_2 induces COX-2 Expression in HeLa Cells

In order to demonstrate whether COX-2 up-regulation in HeLa cells is induced by COX-1-mediated PGE_2, HeLa S-3 cells were treated with 0 nM (control) or 300 nM PGE_2 for 24, 48 and 72 hrs respectively. After treatment of HeLa cells with 300 nM PGE_2 for 72 hrs, COX-2 levels were elevated by 3.7 ± 0.78 fold compared with control cells grown for 72 hrs in the absence of PGE_2 (Figure 5.3). No significant elevation in COX-1 expression was observed in HeLa S-3 cells after 72 hrs of stimulation with PGE_2. These data suggest that the up-regulation of COX-2 expression, following induced overexpression of COX-1, may be mediated by PGE_2.

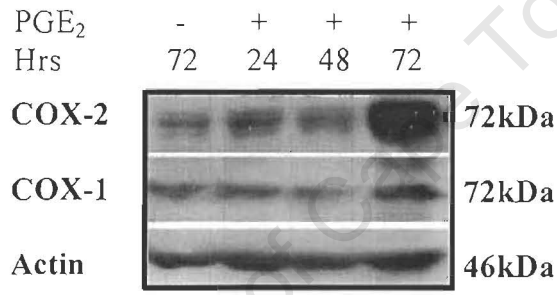


Figure 5.3. Immunoblot of HeLa S-3 cells grown in the presence or absence of 300 nM PGE_2, showing PGE_2 mediated induction of COX-2. No significant increase in COX-1 expression was observed in cells inbuated with PGE_2. Proteins were normalised for loading against β-actin.

5.3.4. COX-1 Overexpression in HeLa Cells induces Prostaglandin E_2 Receptor Expression

The effect of COX-1 over-expression on the four subtypes of PGE_2 receptors, namely EP1-EP4, was investigated by real-time quantitative RT-PCR, following DOX withdrawal from the culture medium and subsequent induction of COX-1. Induced overexpression of COX-1 for 24, 48 and 72 hrs had no significant effect on EP1 receptor expression when compared with cells co-treated with indomethacin. This suggested that although PGE_2 may be functioning via EP1 receptors coupled to inositol phosphate production and release of intracellular

calcium in these cells, its contribution to events associated with COX-1 up-regulation was minimal. COX-1 overexpression for 48 and 72 hrs respectively significantly induced levels of EP2 receptor transcript in HeLa COX-1 Tet-Off cells when compared with cells co-treated with indomethacin (Figure 5.4, $p<0.01$). Levels of EP3 receptor transcript were significantly induced after 48 hrs of COX-1 overexpression ($p<0.05$) compared with cells grown in the presence of indomethacin. EP4 receptor transcript was significantly up-regulated after COX-1 overexpression for 24, 48 and 72 hrs compared with cells co-treated with the COX-enzyme inhibitor (Figure 5.4; $p<0.05$).

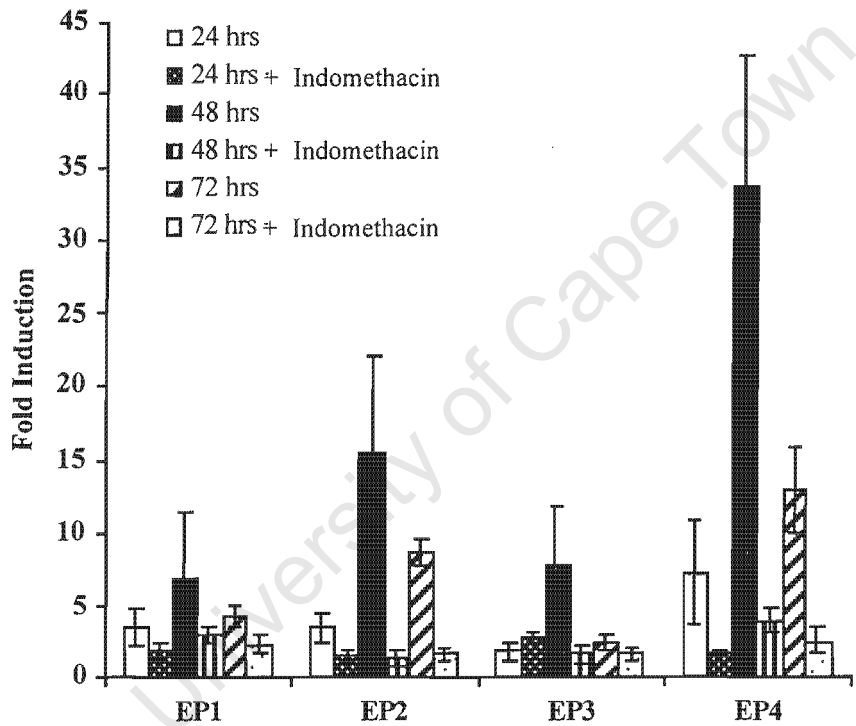


Figure 5.4. Fold induction of expression of prostaglandin E_2 receptors (EP1 - EP4) in HeLa COX-1 Tet-Off cells following overexpression of COX-1 for 24, 48 and 72 hrs respectively in the presence or absence of indomethacin as determined by real-time quantitative RT-PCR. Fold induction was determined by dividing the relative expression in induced cells by the relative expression in uninduced cells

5.3.5. Enhanced cAMP Production in COX-1 Overexpressing Cells in Response to PGE_2

The effect of COX-1-induced up-regulation of the cAMP-linked PGE_2 receptors on cAMP production in HeLa COX-1 Tet-Off cells was determined following overexpression of COX-1 and stimulation with exogenous PGE_2. Following induction of COX-1 for 48 hrs, basal cAMP concentration was determined in cells untreated with exogenous PGE_2. No significant difference in basal cAMP production was detected in uninduced (cells maintaintained with 1 μg/ml DOX) and induced cells (Figure 5.5).

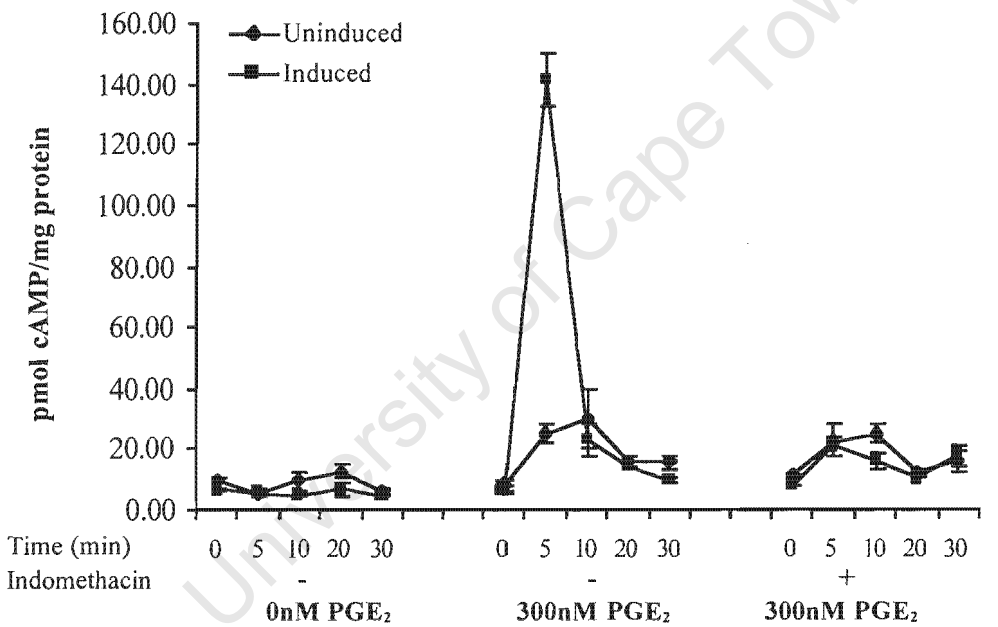


Figure 5.5. cAMP levels (pmol cAMP/mg protein) in HeLa COX-1 Tet-Off cells. Cells were either maintained with 1 μg/ml DOX (uninduced) or induced by incubation in culture medium without DOX for 48 hrs in the presence or absence of the COX-enzyme inhibitor indomethacin. Following induction, cells were stimulated with 0 nM PGE_2 or 300 nM PGE_2.

Treatment of uninduced cells with 300 nM PGE_2 resulted in a 2.43 ± 1.07 fold increase in cAMP production above basal after 5 min of PGE_2 administration ($p<0.05$). Cells in which COX-1 was induced for 48 hrs prior to stimulation with exogenous PGE_2 showed a rapid transient 12.67 ± 3.7 fold cAMP response above basal after 5 min of PGE_2 administration ($p<0.01$). This rapid cAMP accumulation

in COX-1 overexpressed cells in response to PGE_2 was abolished by co-treatment of HeLa cells with the COX-enzyme inhibitor indomethacin. The activity of EP1 receptor was investigated by measuring inositol phosphate accumulation (227) following PGE_2 stimulation. Following stimulation with PGE_2, no significant difference in inositol phosphate accumulation was observed between cells maintained uninduced for 48 hrs (9.96 ±0.28 cpm) and cells in which COX-1 was induced for 48 hrs in the presence or absence of indomethacin (9.46 ±1.1 cpm, 9.0 ±0.54 cpm respectively $p>0.05$).

5.3.6. Induction of Angiogenic Factors in Response to COX-1 Overexpression

The effect of COX-1 overexpression in HeLa Tet-Off cells on the angiogenic factors; vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) was assessed by Western blot analysis. Overexpression of COX-1 in HeLa Tet-Off cells for 72 hrs resulted in a 2.3 ± 0.45 and 4.5 ± 1.2 fold increase in bFGF and VEGF expression respectively (Figure 5.6A and 5.6B). In addition, overexpression of COX-1 for 48 hrs and 72 hrs respectively resulted in a 2.3 ± 0.78 and 2.1 ± 0.98 fold increase in Ang-1 and Ang-2 expression (Figure 5.6C and 5.6D). Moreover, co-treatment of HeLa COX-1 Tet-Off cells with indomethacin inhibited the COX-1 associated up-regulation of VEGF, bFGF, Ang-1 and Ang-2. Co-treatment of cells with NS-398 moderately reduced the up-regulation of bFGF (Figure 5.6A), VEGF (Figure 5.6, Ang-1 (Figure 5.6C) and Ang-2 (Figure 5.6D) expression suggesting that products from both COX enzymes were modulating expression of these factors.

5.4. DISCUSSION

COX-1 expression is elevated in human breast cancer (83), human ovarian adenocarcinomas (81), human prostate carcinoma (78) and murine models of lung tumorigenisis (82). In addition, COX-1 expression can be induced *in vitro* by tobacco carcinogen (228), vascular endothelial growth factor (VEGF), (96), arachidonic acid, forskolin and dibutryl-cAMP (95). We have established a doxycycline-regulated

expression system to investigate the effect of overexpression of COX-1 in HeLa cells.

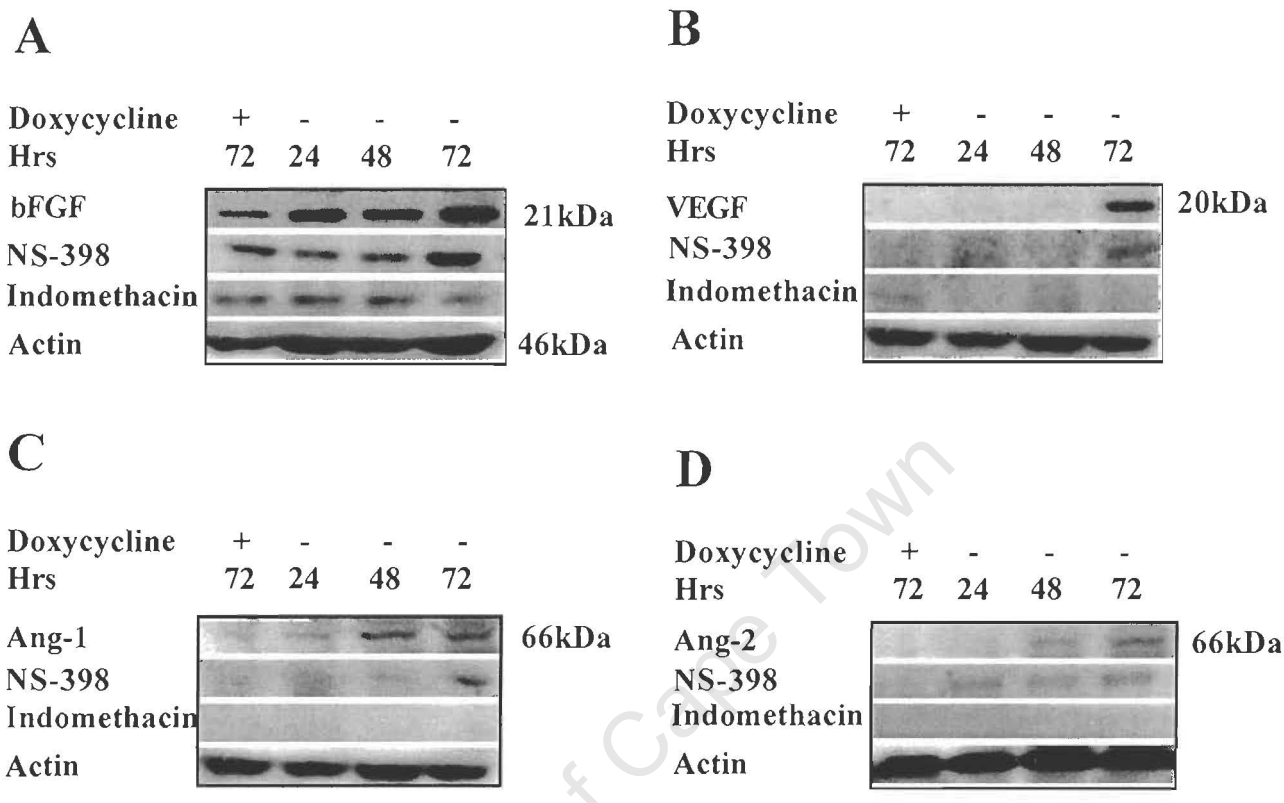


Figure 5.6. Western blot analysis of 20 µg of total clarified cell lysate isolated from HeLa COX-1 Tet-Off cells grown for 72 hrs in the presence of DOX (with or without indomethacin or NS-398) for 24, 48 and 72 hrs respectively in the absence of DOX (with or without indomethacin or NS-398) to induce COX-1 expression. Immunoblots of (**A**) bFGF; (**B**) VEGF; (**C**) Ang-1; (**D**) Ang-2 expression. Proteins were normalised for loading against β-actin.

Overexpression of COX-1 in HeLa cells up-regulates expression of COX-2 and the membrane-bound glutathione-dependent PGES concomitant with increased PGE_2 production. Co-treatment of HeLa COX-1 Tet-Off cells with indomethacin, but not NS-398, abolished the up-regulation of expression of COX-2 and PGES. This suggests that the observed up-regulation of COX-2 and PGES is mediated by prostanoids produced by overexpression of COX-1. In addition, NS-398 treatment significantly reduced the PGE_2 synthesis at 72 hrs but not 48 hrs. This is not surprising, since COX-2 expression in HeLa COX-1 Tet-Off cells was only maximally induced at 72 hrs, therefore the PGE_2 production after 72 hrs of COX-1

overexpression, is dependent on activity of both COX enzymes. Furthermore the reduction in inducible PGES expression to basal levels at 72 hrs, when PGE_2 levels are maximal, implies that PGES expression may be negatively regulated by PGE_2. These data suggest that the up-regulation of COX-2 and PGES is mediated by COX enzyme products, including PGE_2. Two glutathione-dependent forms of PGES, a cytosolic and membrane-bound isoform have been identified. The cytosolic PGES preferentially converts COX-1-derived PGH_2 to PGE_2 (135). The inducible membrane-associated form is preferentially associated with COX-2 (136). In an *in vitro* model system, interleukin (IL)-1β stimulation of cells rapidly induces the expression of COX-2 and PGES (138). Similarly, inducible PGES activity has been described in lipopolysaccharide-stimulated rat peritoneal macrophages, coincident with increased COX-2 expression and PGE_2 biosynthesis (139, 140). This suggests that COX-2 and PGES may be co-regulated. Thus it is feasible to suggest that in cervical carcinomas, up-regulated expression of COX-1 and enhanced synthesis of COX-1-enzyme products may potentiate tumorigenesis by regulating the expression of COX-2/PGES and synthesis of PGE_2. COX-2 is a known tumor promotor in rodent models of colorectal cancer (68). In addition, in an *in vitro* model, COX-1 overexpressing cells implanted in nude mice is associated with enhanced tumorigenicity (80). Since both COX isoforms catalyse the same reaction (99), a state of sustained tumorigenesis may be achieved in cervical carcinomas by both COX isoenzymes working in concert via convergent biosynthetic pathways such as the COX/PGES/PGE_2 biosynthetic pathway.

PGE_2 treatment of HeLa cells resulted in induced COX-2 expression. Previous studies have demonstrated that PGE_2 can up-regulate COX-2 expression in human adenocarcinoma cell lines (229) and other cell lines via the cAMP-dependent PGE_2-receptors (95). Since COX–2 expression is up-regulated by PGE_2, enhanced synthesis of PGE_2 as a consequence of COX-1 and COX-2 up-regulation could regulate cervical hyperplasias and carcinomas via a positive feed-forward system, which would further increase expression of COX-2 and subsequently lead to enhanced tumorigenesis. This may be further mediated by regulation of gene

transcription following interaction of PGE_2 with EP receptors located in the nuclear envelope. Not much is known about the functions of the nuclear EP receptors. Although we have demonstrated that PGE_2 induces COX-2 expression in HeLa cells, it is important to emphasise that several diverse eicosanoids, including PGE_2, produced as a consequence of COX-1 overexpression and up-regulation of COX-2 and may collectively be involved in the downstream events associated with induced COX-1 expression in our model system.

The biological actions of PGE_2 have been attributed to its interaction with G-protein-coupled receptors, of which 4 subtypes (EP1-4) have been identified (141). COX-1 overexpression in HeLa cells resulted in significant up-regulation of the cAMP-dependent PGE_2-receptors after 48 hrs of COX-1 overexpression. This up-regulation was inhibited by co-treatment with indomethacin, suggesting that the up-regulation was mediated by COX-enzyme products. In rat models of mesangioproliferative glomerulonephritis, induction of EP2 receptor parallels up-regulation of glomerular COX-1 suggesting a role for EP2 receptor in enhancement or resolution of disease (230). In reproductive tract carcinomas, little is known of the roles of EP receptors in tumorigenesis, however recent studies in endometrial adenocarcinomas, demonstrate elevated expression and signalling of EP2 and EP4 receptor compared with normal endometrium, suggesting a role for EP2 and EP4 receptor in endometrial carcinomas (214). In addition, a direct role for EP receptors in tumorigenesis has been reported recently in colon cancer cells. In this model, enhanced proliferative and tumorigenic effects were mediated by PGE_2 following interaction with the EP4 receptor and stimulation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt/PKB) pathway (6). It is likely that similar mechanisms may exist in cervical carcinomas to enhance growth and proliferation via EP receptors in a cAMP-dependent manner.

EP receptor signalling in our model system was assessed by measuring cAMP and inositol phosphate production in response to stimulation with exogenous PGE_2. cAMP activity was measured in HeLa cells following overexpression of COX-1 for

48 hrs and stimulation with exogenous PGE_2. A significant fold increase in cAMP production was observed after 5 min of PGE_2 stimulation in COX-1 induced compared with uninduced cells. This augmented cAMP response was abolished by growing cells in medium containing indomethacin. These data suggest that PGE_2, produced by COX-1 overexpression, may be acting in an autocrine/paracrine manner via the cAMP-linked PGE_2-receptors to mediate its effect on target genes. Since COX-1 overexpression had no significant affect on EP1 expression and stimulation of HeLa cells with PGE_2 resulted in no increase in inositol phosphate accumulation above basal, this suggested that although PGE_2 may be functioning via EP1 receptors coupled to inositol phosphate production and release of intracellular calcium in these cells, its contribution to events associated with COX-1 up-regulation was minimal. Since COX-1 overexpression in HeLa cells induces COX-2 and EP receptor expression, it is feasible to suggest that PGE_2 may facilitate the process of cervical tumorigenesis in an autocrine/paracrine manner following enhanced EP receptor expression and ligand-receptor interaction, and activation of intracellular signal transduction pathways such as the cAMP pathway. cAMP is known to enhance cellular proliferation in certain cell types. The exact mechanism by which cAMP stimulates cell-type-specific effects on proliferation is still unclear. However, in Wistar rat thyroid cells, cAMP mediates its role in proliferation via a PKA-dependent and PI3K-dependent mechanism, suggesting that signalling circuits exist that couple cAMP to the activation of PI3K-dependent signals (231). Since PGE_2/EP4 interaction induces changes in colon cell morphology and growth via downstream activation of the PI3K/Akt pathway (6), it is possible that similar divergent pathways may promote cervical cell growth and proliferation following PGE_2/EP receptor binding and activation of cAMP and other signal transduction pathways, such as PI3K/Akt.

Cancer cells produce a wide variety of factors that contribute to angiogenesis, including bFGF, VEGF, bFGF-binding protein and platelet-derived growth factor (PDGF) (8). Our data demonstrate that COX-1 overexpression in HeLa cells results in the up-regulation of expression of pro-angiogenic factors. Induced overexpression

of COX-1 resulted in an increase in bFGF, VEGF, Ang-1 and Ang-2 expression. Co-treatment of these cells with indomethacin abolished the up-regulation of these angiogenic factors. This suggests that the up-regulation of these factors is mediated by prostanoids produced by COX-1 overexpression. Moreover, since the effects of COX-1 overexpression can be reversed by COX inhibition with indomethacin, this implies that these affects are not an artefact of forced overproduction of the enzyme. Partial reduction in expression of these factors by treatment with NS-398 suggests that both enzymes (COX-1 and COX-2) converge to regulate expression of target genes possibly through common prostanoid synthetic pathways. In another model system, COX-2 overexpression and increase in PGE_2 synthesis in colon carcinoma cells results in the up-regulation of bFGF and VEGF and this is associated with arrangement of endothelial cells into tubular structures (8). PGE_2 is known to up-regulate VEGF expression and thereby regulate angiogenesis (232). The up-regulation of angiogenic factors by COX enzymes is important in regulating angiogenesis and maintenance of the neoplastic tissue. As the demand for nutrients and oxygen increases for tissue development, an increased vascularisation is necessary to supply nutrients to the tumor (178). In this study, we also observe the regulation of the angiogenic factors Ang-1 and Ang-2 by COX enzymes. Ang-1 is required for recruitment of perivascular cells leading to the formation and stabilisation of capillaries, vessel maturation and endothelial cell survival (233, 234). Endothelial cell sprouting, which is the first step in both angiogenesis and neovascularisation is mediated following interaction of Ang-1 with the endothelial cell-specific Tie-2 receptor (235). Ang-1 and other angiogenic factors such as VEGF may act synergistically to increase vascular sprouting and branching (236, 237). In addition Ang-1/Tie-2 interaction enhances the mitogenic effect of VEGF on endothelial cell growth (238). By contrast, Ang-2 is a natural Tie-2 receptor antagonist, destabilising cell contacts and thus allowing access to angiogenic factors such as VEGF (239). In our model system, enhanced synthesis of prostanoids as a consequence of up-regulated COX-1 may thus act in an autocrine/paracrine manner to up-regulate the expression of target receptors and intracellular signalling to a host of angiogenic

factors, which could act on endothelial cells and lead to the recruitment of new blood vessels to enhance tumor mass.

In conclusion, these data suggest that COX-1 overexpression in cervical carcinoma cells and consequent enhanced prostanoid biosynthesis modulates the expression of COX-2, PGES and prostaglandin E_2 receptors and suggests that COX-enzyme products may act in an autocrine/paracrine manner via the cAMP pathway to enhance tumorigenesis. In addition, COX-1-enzyme products may also directly modulate tumorigenesis by enhancing tumor mass by inducing the expression of factors involved in angiogenesis such as VEGF, bFGF, Ang-1 and Ang-2.

CHAPTER 6
DISCUSSION AND CONCLUSIONS

6.1. DISCUSSION

This thesis reports on the expression and localisation of COX-1 and COX-2 and synthesis of PGE_2, and a possible autocrine/paracrine function of COX-enzyme products in cervical carcinomas. These studies were designed to determine a possible functional role for COX enzymes in cancer of the cervix.

Initially, COX-1 and COX-2 expression was investigated by quantitative real-time RT-PCR and Western blot analysis, following RNA and protein isolation from cervical carcinoma biopsies and normal cervix. The data obtained demonstrate that expression of both COX isoforms are up-regulated in cervical squamous cell carcinomas and adenocarcinomas at various grades and stages of differentiation compared with normal cervix. The site of COX-1 and COX-2 expression and PGE_2 synthesis was localised by immunohistochemistry using paraffin wax-embedded cervical carcinoma and normal cervical tissue. COX-1 and COX-2 expression and PGE_2 synthesis were localised to the neoplastic epithelial cells of the cervical carcinoma tissue. In addition, COX-2 expression was localised in endothelial cells lining the microvasculature of the cervical carcinomas. A possible autocrine/paracrine signalling for PGE_2 in cervical carcinomas was investigated using cervical carcinoma and normal cervical tissue explants. In this study, we demonstrated up-regulated expression of two cAMP-mediated PGE_2 receptor isoforms, namely EP2 and EP4 in cervical carcinomas compared with normal cervix. This was coincident with enhanced cAMP-signalling of cervical carcinoma explants to administration of PGE_2.

In order to determine a possible role for COX-1 in cervical carcinomas, we constructed a doxycycline (DOX)-regulated expression system (Tet-Off system) in HeLa (cervical carcinoma) cells to promote inducible expression of COX-1. Overexpression of COX-1 in HeLa Tet-Off cells up-regulated expression of COX-2 and prostaglandin E synthase (PGES) concomitant with increased PGE_2 production. DOX and indomethacin treatment abolished the up-regulated expression of COX-2 and PGES and synthesis of PGE_2. Treatment of cells with the selective COX-2 inhibitor NS-398 partially abolished the up-regulated expression of COX-2, PGES

and PGE_2 suggesting that the observed up-regulation was mediated by enzyme products from both COX enzymes. In HeLa COX-1 Tet-Off cells, COX-1 overexpression significantly up-regulated expression of the cAMP-linked EP receptors (EP2/EP3/EP4). The up-regulation of receptor expression was associated with enhanced cAMP-signalling in response to administration of PGE_2. Incubation of cells with DOX or indomethacin abolished the up-regulated expression of the EP receptors and enhanced accumulation of cAMP. Finally, COX-1 overexpression in cervical carcinoma cells up-regulated the expression of the pro-angiogenic factors VEGF, bFGF, Ang-1 and Ang-2. Administration of indomethacin abolished the up-regulation of these factors. The selective COX-2 inhibitor NS-398 only partially abolished the up-regulated expression of the angiogenic factors, suggesting that in HeLa cells, COX-mediated up-regulation of angiogenic factor expression is mediated by COX-enzyme products from both COX enzymes. These studies confirm up-regulated expression of COX-enzymes, PGE_2 receptors (EP2 and EP4) and synthesis of PGE_2 in cervical carcinomas and suggest a possible autocrine/paracrine regulation of neoplastic cell function by COX-enzyme products, including PGE_2.

6.1.1. Factors That May Lead To Expression Of COX Enzymes In The Cervix

Cancer of the uterine cervix is an important clinical problem in developing countries. Although the evidence has been largely epidemiological, sexually transmitted infection of the metaplastic cervical epithelium by HPV appears to be the progenitor of the disease, as more than 80 % of all cervical carcinomas are diagnosed HPV positive (20, 39, 240). *In vitro* studies using HeLa cells, which are derived from cervical adenocarcinoma origin and which are infected with HPV 18, have shown that dormant tumor suppressor pathways such as the p53 and Rb can be reactivated and cells can be subsequently made senescent by inhibition of expression of the E6/E7 HPV oncogenes (40, 241). Following transcription of the E6/E7 oncogenes, expression of the E6/E7 oncoproteins promotes inflammation, tissue remodeling and epithelial hyperplasia via the release of IL-1α (43). In our studies we have determined up-regulation of expression of both COX-1 and COX-2 in cervical carcinomas, as has been demonstrated in other solid epithelial tumors (78,

82, 83), suggesting a role for COX-enzymes in cervical tumorigenesis. The initial trigger for up-regulation of COX-enzymes during the process of neoplastic transformation of cervical epithelial cells is currently unknown, however COX-1 and COX-2 expression are modulated by intracellular cytokines such as IL-1 and by tumor growth factor (TGF) β1 in various model systems (242-245). *In vitro* studies have shown that administration of IL-1α or IL-1β to cells causes a rapid increase in intracellular PLA2 expression, mobilisation of arachidonic acid (AA) and coincident increase in expression of COX-2, and synthesis and release of PGE_2, by activation of the MAPK cascade (47, 246-249). Although there is no evidence linking HPV infection, COX expression and cervical cell neoplasia, it is feasible to suggest that following infection of the cervix and transformation of the epithelium with virus, the subsequent release of cytokines such as IL-1 could be the trigger for up-regulation of expression of COX-1 and/or COX-2 in the cervix.

6.1.2. Factors Which May Regulate and Maintain COX Enzyme Expression in Cervical Carcinomas

As mentioned earlier, the exact trigger for up-regulation of COX enzymes in cervical carcinomas is unknown and may be multifactorial. However, following viral transformation of cervical epithelial cells by HPV and progression to neoplasia, several other factors may also play a role in modulating and maintaining the expression of COX enzymes in cervical carcinomas. These factors include: infection of the cervical epithelium with microbia; regulation of COX enzyme expression by prostaglandins present in seminal plasma in sexually active women and autocrine/paracrine/intracrine regulation of COX enzyme expression and neoplastic cell function by COX enzyme products. A model for regulation of COX enzyme expression and neoplastic cervical cell function is depicted schematically in Figure 6.1.

One of the factors, which may play a potential role in regulating COX enzyme expression in cervical carcinomas, is infection of the cervix by microbia. Infection of the cervical epithelium with chlamydia or other microbe could cause an

autoimmune response such as that observed in the endometrium under conditions of endometriosis (250), as many women presenting with cervical cell dysplasias also test positive for infections such as chlamydia (13). Consequently, during infection a variety of cytokines are secreted from these immune cells and macrophages, including IL-1 and IL-2 (250). These cytokines could then act on the neoplastic epithelial cells of the cervix to induce COX enzyme expression.

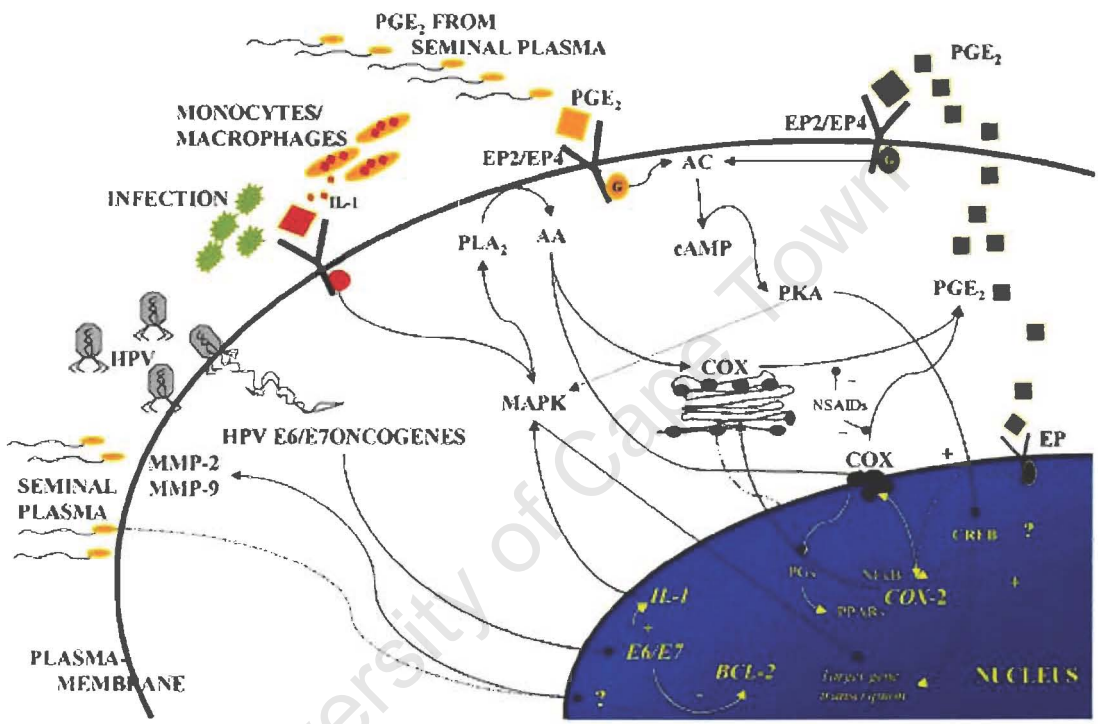


Figure 6.1. A model for regulation of COX enzyme expression in cervical carcinoma cells.

In sexually active women, neoplastic cervical epithelial cells may be directly regulated by PGE_2 present in seminal plasma. Prostaglandins are present 5000 to 10000 fold more in seminal plasma than at the site of inflammation and PGE_2 is one of the predominant prostanoids detected (251). PGE_2 is known to induce COX-2 expression in a number of model systems (95, 252, 253). In addition, we have demonstrated that PGE_2 up-regulates COX-2 expression in HeLa cells (as discussed in chapter 5). Thus, in sexually active women, COX enzyme expression in cervical carcinomas may be modulated by prostaglandins present in seminal plasma.

COX enzyme expression in cervical carcinomas may also be regulated and maintained in an autocrine/paracrine/intracrine manner by prostanoids, such as PGE_2,

acting on transmembrane or nuclear receptors. PGE_2 is known to induce expression of COX-1 and COX-2 in various model systems via ligand-receptor binding to EP-receptors and activation signalling pathways (95, 252, 253), and may also autoregulate COX enzyme expression via a positive feed forward system. This autoregulation of COX enzyme expression is illustrated in Figure 6.1 and may occur as follows:

Enhanced synthesis of PGE_2, as a consequence of up-regulated COX expression, acts in an autocrine/paracrine manner on target cells through interaction with G protein-coupled transmembrane receptors to activate intracellular signalling via various signal transduction pathways such as the cAMP pathway. Activation of the cAMP pathway and subsequent increase in cAMP accumulation could in turn increase transcription of COX-2 and consequently COX-2-enzyme products such as PGE_2, in a positive feed forward system, via the cAMP-response element in the COX-2 gene promotor region (99). This would consequently enhance the synthesis of PGE_2, PGE_2 receptors and enhance signal transduction and gene transcription. This model is supported by our data generated using cervical carcinoma explants (discussed in Chapter 3) and HeLa COX-1 Tet-Off model system (discussed in Chapter 5). In cervical carcinomas, the expression of PGE_2 receptors (EP2 and EP4) are up-regulated coincident with enhanced cAMP signalling compared with normal cervix. In addition, COX-1 overexpression in HeLa cells and subsequent enhanced synthesis of prostanoids, including PGE_2, up-regulates the expression of COX-2, cAMP-mediated EP receptors (EP2/3/4) and accumulation of cAMP. Thus in cervical carcinomas, enhanced biosynthesis of PGE_2 and other COX-enzyme products produced as a consequence of COX up-regulation, could act in an autocrine/paracrine manner to up-regulate target receptors such as the EP receptors and consequently initiate downstream intracellular signal transduction pathways, which would in turn cause the transcription of target genes.

Furthermore, COX-enzymes by virtue of their perinuclear location may release their products in close proximity to nuclear EP receptors or nuclear peroxisome proliferator-activated receptors (PPARs), which may in turn directly

regulate the transcription of target genes involved in tumorigenesis in an intracrine manner. Little is known of the function of the nuclear EP receptors, however PGJ_2 derivatives produced by COX enzymes have been shown to interact with PPARs (127). The activated form of the PPAR binds to the peroxisome proliferator responsive element (PPRE) in the target gene (160). Activation PPARs as a consequence of enhanced prostanoid biosynthesis, brought about by up-regulated expression of COX, would further enhance COX-2 transcription by binding to the PPRE in the promotor region of the COX-2 gene (99, 127, 128).

Although we have only reported on one of the prostanoid products biosynthesized by COX enzymes, namely PGE_2, it is important to emphasise that a variety of other eicosanoids, including prostanoids will be produced in cervical carcinomas and following overexpression of COX-1 in our model system. It is thus feasible that other eicosanoids may also regulate COX enzyme expression in neoplastic cervical epithelial cells. COX enzyme expression may thus be regulated and maintained in cervical carcinomas by several eicosanoids converging in an autocrine/paracrine/intracrine manner via their respective receptors and signal transduction pathways to fulfil a common purpose.

6.1.3. The Role Of COX In Cervical Carcinomas

The exact roles of COX enzymes and PGE_2 in cervical carcinomas are still poorly understood. Several investigators have reported on COX enzymes and their products, such as PGE_2, playing a potential role in tumorigenesis by inhibiting apoptosis (69), enhancing cellular proliferation (71) and mitogenesis (189), enhancing metastasis (7), and increasing angiogenesis (8). Although we have not demonstrated a role of COX enzymes in apoptosis, proliferation or metastasis, COX and PGE_2 may act similarly in cervical carcinoma cells as has been demonstrated for colorectal carcinoma cells using various *in vitro* model systems (7, 8, 69, 71, 188, 254). This may be achieved by down regulating cell surface adhesion molecules such as E-cadherin and inhibition of apoptosis in cervical cells overexpressing COX-1 and/or COX-2 as has been demonstrated for colon cancer cells overexpressing COX-2 (69).

In our study, we investigated the affect of COX-1 overexpression in HeLa cells on the expression of pro-angiogenic factors, which may be involved in new blood vessel formation at the site of tumorigenesis. We determined that COX-1 overexpression in HeLa cells promotes the expression of the pro-angiogenic factors VEGF, bFGF, Ang-1 and Ang-2. The process of neovascularization is an essential part of tumorigenesis, as constant blood supply is needed to provide the tumor with oxygen and nutrients. Endothelial cell sprouting is considered the first step in neovascularization and angiogenesis and is mediated in endothelial cells following interaction of Ang-1 with the endothelial cell-specific Tie-2 receptor (235). Ang-1/Tie-2 ligand-receptor binding may enhance the mitogenic effect of angiogenic factors such as VEGF, which may act synergistically to increase vascular sprouting and branching (236-238). Ang-2 is a natural Tie-2 receptor antagonist, which destabilises cell contacts thus allowing access to angiogenic factors such as VEGF (239), for further endothelial cell sprouting and vascularization.

In cervical carcinomas, the process of angiogenesis may be facilitated following up-regulation of COX-1 and COX-2 and angiogenic factors such as Ang-1 and Ang-2, which may act on the Tie-2 receptor on adjacent endothelial cells to

initiate neovascularization. Following initiation of angiogenesis by Ang-1/2, other angiogenic factors such as bFGF and VEGF may then act synergistically to potentiate the process.

In other model systems, COX-2 overexpression and increase in PGE_2 synthesis in colon cancer cells results in the up-regulation of expression of angiogenic factors such as bFGF and VEGF and this is associated with arrangement of co-cultured endothelial cells into tubular structures (8). More recently, it was suggested that COX-2 and PGE_2 produced by endothelial cells may also directly regulate the process of angiogenesis (9). The arrangement of rat aortic endothelial cells into tubular structures is reduced following treatment with selective COX-2 inhibitors and this effect is partially reversed by co-treatment with PGE_2 (9). Thus the process of angiogenesis in cervical carcinomas may be controlled directly following up-regulation of COX enzyme expression, enhanced synthesis of prostanoids and expression of pro-angiogenic factors which act on adjacent endothelial cells to cause endothelial cell sprouting, or indirectly in an autocrine/paracrine manner by COX-1 and COX-2 enzyme-products via an epithelial-epithelial interaction (where prostanoids such as PGE_2 produced in a paracrine manner by COX-1 overexpressing epithelial and stromal cells up-regulates COX-2 expression and PGE_2 biosynthesis in adjacent epithelial cells which then acts on endothelial cells), epithelial-endothelial interaction (where COX-1 and/or COX-2 overexpressing epithelial cells produce prostanoids which directly acts on target receptors on endothelial cells) or endothelial-endothelial cell interaction (where prostanoids such as PGE_2 produced by endothelial cells acts in an autocrine/paracrine manner on adjacent endothelial cells).

This is supported by our data demonstrating up-regulated expression of COX-1 in neoplastic epithelial cells and stromal cells and up-regulated COX-2 expression and PGE_2 synthesis in neoplastic cervical epithelial cells and endothelial cells in cervical carcinoma tissues as well as up-regulation of expression of pro-angiogenic factors in HeLa cells overexpressing COX-1. Thus COX enzymes and eicosanoids such as PGE_2 may play a role in cervical carcinomas by mediating the

transcription of pro-angiogenic factors to increase blood supply in an environment where nutrients and oxygen are limiting.

6.2. CONCLUSIONS

In conclusion, these data confirm the elevated expression of COX-1 and COX-2 and synthesis of PGE_2 in cervical carcinomas of various grades and differentiation. Both COX-1 and COX-2 and PGE_2 are localised to the neoplastic cervical epithelial cells. In addition, COX-2 and PGE_2 are localised to neoplastic endothelial cells of the microvasculature. PGE_2 may exert an autocrine/paracrine effect in cervical carcinomas through interaction with EP2/EP4 receptors and activation of the PKA signalling pathway. COX-1 overexpression in cervical epithelial cells results in up-regulation of expression of COX-2 and PGES and synthesis of PGE_2. In addition COX-1 overexpression in neoplastic cervical epithelial cells up-regulates the expression of the cAMP-linked EP receptors concomitant with rapid cAMP-responsiveness of COX-1 overexpressing cells to administration of exogenous PGE_2. Moreover COX-1 overexpression in neoplastic cervical epithelial cells up-regulated the expression of the pro-angiogenic factors VEGF, bFGF, Ang-1 and Ang-2. Taken together, these data suggest that COX enzymes may directly modulate cervical tumor growth in an autocrine/paracrine manner via enhanced ligand-receptor interaction and increased intracellular signal transduction, which would cause transcription of target genes and increase the expression of angiogenic factors, which could act on endothelial cells to enhance the supply of oxygen and nutrients to the tumor and enhance tumor mass and cervical tumorigenesis.

FUTURE STUDIES

Our data generated in chapter 3 demonstrate that both COX isoforms are up-regulated in cervical carcinomas . Thus the use of selective COX-2 inhibitors may be only of partial therapeutic benefit in treatment of women with cervical carcinoma. In addition, treatment of cervical carcinomas with aspirin may also be of limited therapeutic potential, since aspirin therapy is associated with side affects such as gastrointestinal ulceration, thromboembolism and platelet and renal dysfunction. It is therefore anticipated that the effects of COX-enzyme products on neoplastically transformed cervical epithelial cells can be more effectively targeted at the signalling level to disrupt or inhibit specific functions. With this in mind, future studies to elucidate the molecular pathways associated with COX enzymes in cervical carcinomas may provide a valuable contribution towards the biological and patho-physiological roles of COX enzymes in cervical carcinomas. The HeLa COX-1 Tet-Off system will provide a powerful tool to elucidate the diverse signal transduction pathways associated with COX-1 products and may lead to improved therapies for women with cervical carcinoma and reproductive tract carcinomas more generally.

The near completion of the Human Genome Project, and the identification of most of the genes in the human body has meant that genes which were previously unknown have been mapped out. This has introduced the possibility that cell-type specific genes may exist, which can be exploited as potential targets for gene therapy for a host of diseases, including cervical cancer. To-date, the target genes for the role of COX and action of PGE_2 in mediating cellular growth, proliferation and angiogenesis in cervical carcinomas remain largely unknown. Future studies to outline these genes should be conducted. Target genes for COX-1 can be identified using the HeLa COX-1 Tet-Off system in combination with microarray technology to profile many crucial cellular pathways and functions including oncogenes, tumor suppressors, cell cycle regulators, signal transduction pathways, apoptosis and angiogenesis. This technology may prove to be a useful tool towards the elucidation of the genes and molecular markers involved in cervical carcinomas and could result in potential gene therapy.

Although the human genome has been mapped out, almost to completion, many of the genes and gene-products are currently unknown. It would be interesting to use proteomics in conjuction with the microarray technology to determine the proteome associated with COX enzyme expression. The elucidation of potential new proteins and protein-protein interactions regulated by COX enzyme products in neoplastic cervical cells, may provide us with a tool towards developing novel therapeutics against the action of COX-enzymes and their products in the process of tumorigenesis.

Finally, detailed information is available on the pharmacological and pharmacokinetic properties of tetracycline and its analogues. This may facilitate application of the HeLa COX-1 Tet-Off system as an *in vivo* model in mice. Inducible expression of COX-1 and localised tumorigenesis may allow for the *in vivo* testing of novel pharmacological agents against the effects of enhanced synthesis of eicosanoids brought on by the COX-1 overexpression. This will also assist in determining efficacy and toxicity of any compounds developed as therapeutic regimens.

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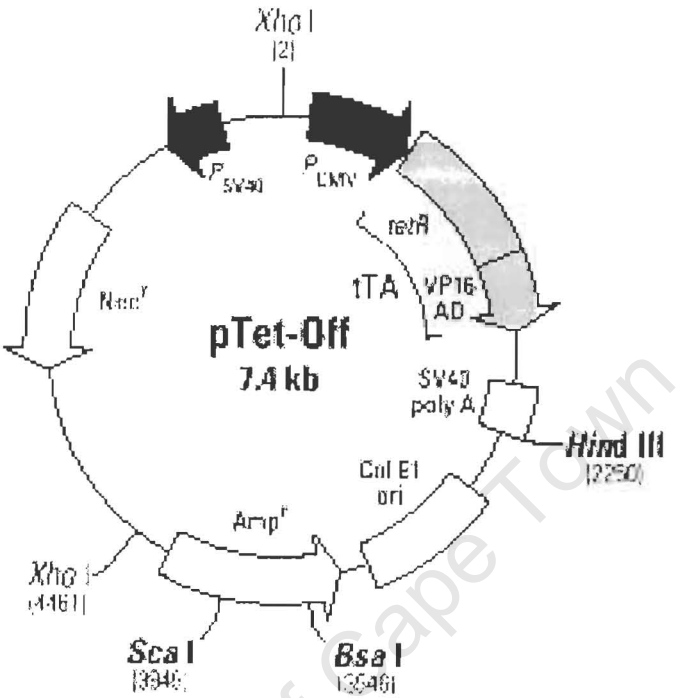
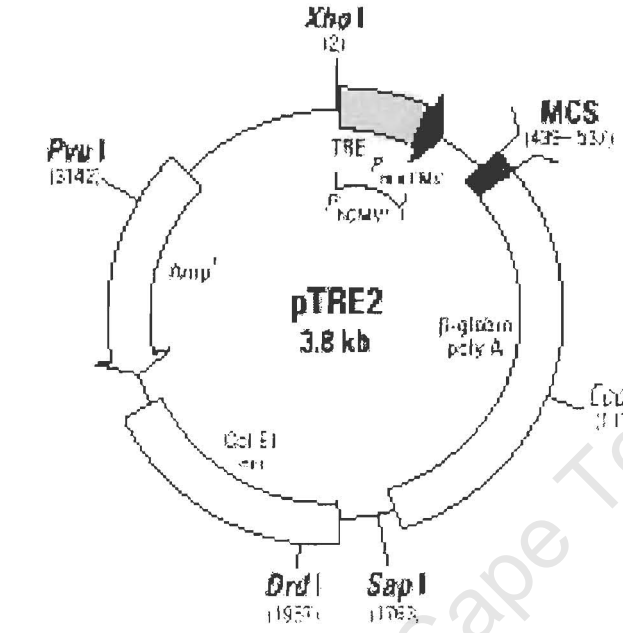
APPENDIX I

Luria Bertani (LB) agar plates	15g agar 10g Bacto-tryptone 5g Bacto-yeast extract 10g NaCl (pH 7.0), to 1 litre of H_2O.
LB broth	10g Bacto-tryptone 5g Bact-yeast extract 10g NaCl (pH 7.0), to 1 litre of H_2O.
Soc medium	20g Bacto-tryptone 5g Bacto-yeast extract 10mM NaCl 2.5mM KCl 10mM $MgCl_2$, to 1 litre of H_2O.
Loading buffer	0.25% w/v bromophenol blue 30% w/v glycerol 0.25% w/v xylene cyanol FF 0.5M EDTA, pH 7.0
1XTBE buffer	89mM Tris-borate 2mM EDTA

APPENDIX II



The pTet-Off vector encoding the tetracycline-controlled transactivator protein (tTA), adapted from the Clontech web site (www.clontech.com).

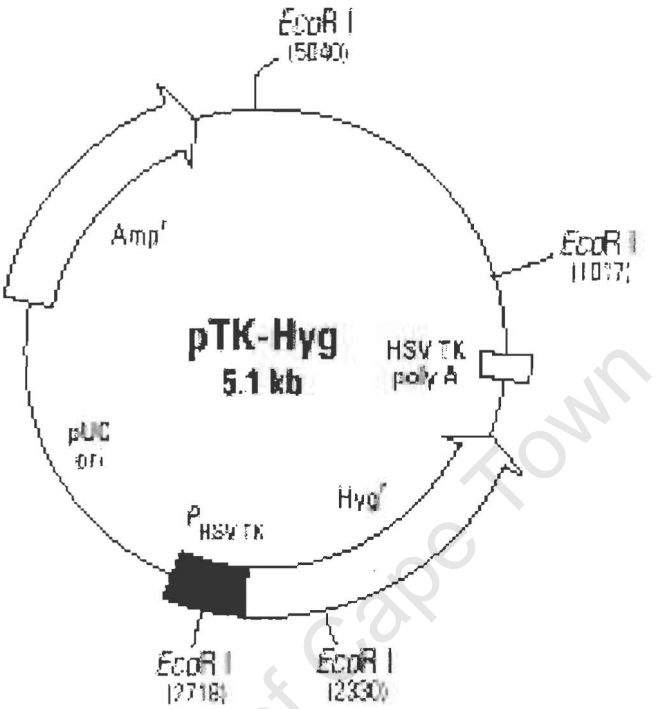


433
CCGCGGCCCCGAATTCGAGCTCGGTACCCGGGGATCCTCTAGTCAGCTG
Sac II EcoR I Kpn I BamH I Pvu II

469
ACGCGTGCTAGCGCGGCCGCATCGATAAGCTTGTCGACGATATCTCTAGA
Mlu I Nhe I Eag I Not I Cla I Hind III Sal I Acc I EcoR V Xba I

The pTRE2 vector containing the gene of interest cloned into the multiple cloning site (MCS). Adapted from the Clontech web site (www.clontech.com).

APPENDIX IV



The pTK-Hyg vector containing the gene for hygromycin resistance to allow for selection of stable transfectants using hygromycin. Adapted from the Clontech web site (www.clontech.com).

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Cyclooxygenase-2 Expression and Prostaglandin E_2 Synthesis Are Up-Regulated in Carcinomas of the Cervix: A Possible Autocrine/Paracrine Regulation of Neoplastic Cell Function via EP2/EP4 Receptors

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ABSTRACT

The prevalence of cervical cancer in South African women is reported as being the highest in the world, occurring, on the average, in 60 of every 100,000 women. Cervical cancer is thus considered an important clinical problem in sub-Saharan Africa. Recent studies have suggested that epithelial tumors may be regulated by cyclooxygenase (COX) enzyme products. The purpose of this study was to determine whether cyclooxygenase-2 (COX-2) expression and PGE_2 synthesis are up-regulated in cervical cancers. Real-time quantitative RT-PCR and Western blot analysis confirmed COX-2 ribonucleic acid and protein expression in all cases of squamous cell carcinoma (n = 8) and adenocarcinoma (n = 2) investigated. In contrast, minimal expression of COX-2 was detected in histologically normal cervix (n = 5). Immunohistochemical analyses localized COX-2 expression and PGE_2 synthesis to neoplastic epithelial cells of all squamous cell (n = 10) and adenocarcinomas (n = 10) studied. Immunoreactive COX-2 and PGE_2 were also colocalized to endothelial cells lining the microvasculature. Minimal COX-2 and PGE_2 immunoreactivity were detected in normal cervix (n = 5). To establish whether PGE_2 has an autocrine/paracrine effect in cervical carcinomas, we investigated the expression of two subtypes of PGE_2 receptors, namely EP2 and EP4, by real-time quantitative RT-PCR. Expression of EP2 and EP4 receptors was significantly higher in carcinoma tissue (n = 8) than in histologically normal cervix (n = 5; $P < 0.01$). Finally, the functionality of the EP2/EP4 receptors was assessed by investigating cAMP generation after *in vitro* culture of cervical cancer biopsies and normal cervix in the presence or absence of 300 nmol/L PGE_2. cAMP production was detected in all carcinoma tissue after treatment with exogenous PGE_2 and was significantly higher in carcinoma tissue (n = 7) than in normal cervix (n = 5; $P < 0.05$). The fold induction of cAMP in response to PGE_2 was 51.1 ± 12.3 in cervical carcinoma tissue compared with 5.8 ± 2.74 in normal cervix. These results confirm that COX-2, EP2, and EP4 expression and PGE_2 synthesis are up-regulated in cervical cancer tissue and suggest that PGE_2 may regulate neoplastic cell function in cervical carcinoma in an autocrine/paracrine manner via the EP2/EP4 receptors. (*J Clin Endocrinol Metab* 86: 2243–2249, 2001)

CANCER OF THE uterine cervix is one of the leading causes of cancer-related death in women world-wide. It is reported as being particularly common in less developed countries, including South and Central America, Southeast Asia, and sub-Saharan Africa (1–3) where 80% of the world's cervical cancers occur (4). The prevalence of cervical cancer in South African women is reported to be the highest in the world, occurring, on the average, in 60 of every 100,000 women (3, 5, 6). Cancer of the cervix is the most common cancer in black (31.2%) and colored (22.9%) South African women, the second most common cancer in Asian women (8.9%), and the fourth most common cancer in white South African women (2.7%) (3, 7). The lifetime risk of developing cervical cancer is 1:34 for black women and 1:93 for white women (7). Three histological categories of epithelial tumors of the cervix are recognized by the WHO (8). These are squamous cell carcinoma, adenocarcinoma, and other less common types of epithelial tumors. The most common histological type of cervical carcinoma is squamous cell carcinoma, which accounts for 60–80% of all cervical cancers. Adenocarcinoma accounts for approximately 20% of invasive cervical carcinoma.

Cyclooxygenase (COX) enzymes, also called PG endoperoxide synthase, catalyze the rate-limiting step in the conversion of arachidonic acid to PGH_2 and other eicosanoids, including PGE (9). There are at least two isoforms of the COX enzyme, COX-1 and COX-2 (10, 11). COX-1 is constitutively expressed in many tissues and cell types and generates PGs for normal physiological function (11). By contrast, the expression of COX-2 is rapidly induced after the stimulation of quiescent cells by growth factors, oncogenes, carcinogens, and tumor-promoting phorbol esters (10–12). PGE_2 elicits its autocrine/paracrine effects on target cells through interaction with seven transmembrane G protein-coupled receptors, which belong to the rhodopsin family of serpentine receptors (13). Four main subtypes of PGE_2 receptors have been identified (EP_1, EP_2, EP_3, and EP_4); these use alternate and, in some cases, opposing intracellular pathways (14). To date,

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the roles of the different PGE_2 receptors, their divergent intracellular signaling pathways, as well as their target genes involved in mediating the effects of PGE_2 on normal or neoplastically transformed cervical epithelium remain to be elucidated.

Recently, a relationship between COX-2, its synthesized product PGE_2, and neoplastic transformation of epithelial cells has been established (15, 16). Transcription of COX-2 is up-regulated in numerous cancers, including colon, pancreas, esophagus, lung, prostate, and bladder (17–22). It has been proposed that COX-2 overexpression and PGE_2 synthesis mediate neoplastic transformation of epithelial cells by increasing their proliferation rate, resistance to apoptosis, and invasiveness. These effects are mediated by suppressing the transcription of target genes that may be involved in cellular growth/transformation (*e.g.* p53) and adhesion (*e.g.* E-cadherin) (16, 23). Moreover, COX-2 and PGE_2 promote cancer development and invasiveness by mediating the transcription of angiogenic factors that promote both the migration of endothelial cells and their arrangements into tubular structures (24, 25).

The present study was designed to investigate whether COX-2 expression and PGE_2 synthesis are up-regulated in human squamous cell carcinomas and adenocarcinomas of the cervix. In addition, a possible autocrine/paracrine role for PGE_2 in cervical carcinogenesis was assessed by investigating 1) the expression of EP2/EP4 receptors in cervical carcinoma tissue and 2) the effect of exogenous treatment of carcinoma tissue with PGE_2 on cAMP turnover.

Materials and Methods

Tissue collection and processing

Cervical specimens were obtained at the time of surgery/biopsy from patients who were attending the Gynecologic Oncology Clinic at Groote Schuur Hospital (Cape Town, South Africa) and who had previously been diagnosed with invasive carcinoma of the cervix. Punch biopsies were taken from the lesion by an experienced gynecologist with a special interest in oncology. A portion of the biopsy was excised and fixed in formalin, followed by paraffin wax embedding for histopathological typing. The remaining portion was snap-frozen in either dry ice or liquid nitrogen and stored at −70 C for RT-PCR and Western blot analysis or was transported at 4 C for *in vitro* culture and PGE_2 stimulation. Histologically normal cervical samples (N1–N25) were obtained from patients undergoing Wertheim's hysterectomy for nonmalignant conditions. Pathological typing was defined according to the International Federation of Obstetricians and Gynecologists (26) staging upon physical examination. The extent of invasiveness and racial distribution of carcinoma biopsies (C1–C50) are presented in Table 1. The ages of the patients ranged from 29–81 yr, with a median age of 50.5 yr. The study was approved by the University of Cape Town research ethics committee, and informed consent was obtained from all patients before tissue collection. The data in this study were analyzed by ANOVA using StatView 5.0 (Abacus Concepts, Berkeley, CA).

Real-time quantitative PCR

Real-time quantitative RT-PCR was performed to assess COX-2, EP2, and EP4 expression. Ribonucleic acid (RNA) samples were extracted from cervical tissue (squamous cell carcinomas, C1–C8 and C31–C37; adenocarcinomas, C9, C10, and C38; normal cervix, N1–N5 and N11–N15) using Tri-Reagent (Sigma, Dorset, UK) according to the manufacturer's protocol. RNA samples were reverse transcribed using $MgCl_2$ (5.5 mmol/L), deoxy (d)-NTPs (0.5 mmol/L each), random hexamers (1.25 μmol/L), oligo(deoxythymidine) (1.25 μmol/L), ribonuclease inhibitor (0.4 U/μL), and multiscribe reverse transcriptase (1.25 U/μL; all from PE Applied Biosystems, Warrington, UK). The mix was aliquoted into individual tubes (16 μL/tube) and template RNA was added (4 μL/tube of 100 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 mM $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 400 μM dUTP), ribosomal 18S forward and reverse primers and probe (all at 50 nM), forward and reverse primers for COX-2, EP2 or EP4 receptor (300 nM), COX-2, EP2 or EP4 receptor probe (200 nM), AmpErase UNG (0.01 U/μL) and AmpliTaq Gold DNA Polymerase (0.025 U/μL; all from PE Biosystems). A volume of 48 μL of reaction mix was aliquoted into separate tubes for each complementary DNA sample and 2 μL/replicate of complementary DNA was added. After mixing 23 μL of sample were 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 reaction was run on an ABI Prism 7700 using standard conditions. COX-2 and EP receptor primers and probe for quantitative PCR were designed using the PRIMER express program (PE Applied Biosystems). The sequences of the COX-2 primers and probe were as follows: forward, 5′-CCT TCC TCC TGT GCC TGA TG-3′; reverse, 5′-ACA ATC TCA TTT GAA TCA GGA AGC T-3′; and probe (FAM labeled), 5′-TGC CCG ACT CCC TTG GGT GTC A-3′. The sequences of the EP2 receptor primers and probe were as follows: forward, 5′-GAC CGC TTA CCT GCA GCT GTA C-3′; reverse, 5′-TGA AGT TGC AGG CGA GCA-3′; and probe (FAM labeled), 5′-CCA CCC TGC TGC TGC TTC TCA TTG TCT-3′. The sequences of the EP4 receptor primers and probe were as follows: forward: 5′-ACG CCG CCT ACT CCT ACA TG-3′; reverse, 5′-AGA GGA CGG TGG CGA GAA T-3′; and probe (FAM labeled), 5′-ACG CGG GCT TCA GCT CCT TCC T-3′. The ribosomal 18S primers and probe sequences were as follows: forward, 5′-CGG CTA CCA CAT CCA AGG AA-3′; reverse, 5′-GCT GGA ATT ACC GCG GCT-3′; and probe (VIC labeled), 5′-TGC TGG CAC CAG ACT TGC CCT C-3′. Expression of COX-2, EP2, and EP4 was normalized to RNA loading for each sample using the 18S ribosomal RNA as an internal standard. Relative gene expression in carcinoma tissue compared with normal cervix was calculated by dividing the expression in carcinoma tissue by the expression in normal cervix. The data are presented as the mean ± SEM.

Western blotting

COX-2 protein expression was assessed by Western blotting. Proteins were extracted from cervical tissue (squamous cell carcinomas, C1–C8; adenocarcinomas, C9 and C10; normal cervix, N1–N5) using Tri-Reagent (Sigma, St. Louis, MO) following the manufacturer's instructions. A total of 100 μg protein were resuspended in 38 μL sample buffer [125 mmol/L

TABLE 1. Extent of invasiveness and racial distribution of cervical carcinoma biopsy samples of South African women

Sample no.	Histological typing	FIGO stage	Race
C37, C46	Squamous carcinoma	1B; poorly differentiated	Black
C1–C3, C8, C11–C20, C39, C47	Squamous carcinoma	2B; well differentiated	Black
C4, C5, C44, C49	Squamous carcinoma	3B; well differentiated	Black
C9	Adenocarcinoma	1B; moderately differentiated	Black
C10, C21–C30, C38, C50	Adenocarcinoma	2B; well differentiated	Black
C6, C7, C31, C32, C34, C40–C42, C45	Squamous carcinoma	1B; poorly differentiated	Colored
C33, C43	Squamous carcinoma	1B; moderately differentiated	Colored
C35, C36, C48	Squamous carcinoma	2B; well differentiated	Colored

Tris-HCl (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 10% SDS-polyacrylamide gel. Proteins were transferred onto polyvinylidene difluoride membrane (Millipore Corp., Watford, UK) and subjected to immunoblot analysis. Membranes were blocked for 1 h at 25 C in 5% skimmed milk powder diluted in washing buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.05% (vol/vol) Tween-20]. Thereafter, membranes were incubated with goat anti-COX-2 primary IgG antibody (sc-1745, Autogenbioclear, Wiltshire, UK) at a dilution of 1:500 at 4 C for 18 h. Control samples were incubated with goat anti-COX-2 antibody preadsorbed to blocking peptide (sc-1745p, Autogenbioclear) according to the manufacturer's protocol. Membranes were subsequently incubated for 1 h, respectively, with rabbit antigoat secondary IgG antibody conjugated to biotin (DAKO Corp., High Wycombe, UK; 1:500) and streptavidin-biotin-horseradish peroxidase complex (Amersham Pharmacia Biotech, Aylesbury, UK). Proteins were revealed by chemiluminescence (ECL Plus kit, Amersham Pharmacia Biotech) following the manufacturer's instructions. The molecular mass of the COX-2 protein was approximately 72 kDa, as determined from the relative mobility on SDS-PAGE compared with the molecular mass standard.

Immunohistochemistry

The site of COX-2 expression and PGE_2 synthesis was localized in cervical tissues by immunohistochemistry using archival cervical blocks (squamous cell carcinomas, C11–C20; adenocarcinomas, C21–C30; normal cervix, N5–N10) obtained from the Department of Anatomical Pathology, University of Cape Town (Cape Town, South Africa). Five-micron paraffin wax-embedded tissue sections were cut and mounted onto coated slides (TESPA, Sigma). Sections were dewaxed in xylene, rehydrated in graded ethanol, and washed in water followed by TBS (50 mM Tris-HCl and 150 mM NaCl, pH 7.4) and blocked for endogenous endoperoxidase (1% H_2O_2 in methanol). Antigen retrieval was performed by pressure cooking for 2 min in 0.01 mol/L sodium citrate, pH 6 (for COX-2 and PGE_2). No antigen retrieval was performed for CD34 immunohistochemistry. Sections were blocked using 5% normal rabbit serum (for COX-2), 5% swine serum (for PGE_2), or 5% normal goat serum (for CD34) diluted in TBS. Subsequently, the tissue sections were incubated with polyclonal goat anti-COX-2 antibody (sc-1745, Autogenbioclear) at a dilution of 1:400, rabbit anti-PGE_2 antibody (supplied by Prof. R. W. Kelly, Medical Research Council Human Reproductive Sciences Unit, Edinburgh, UK) at a dilution of 1:100, or monoclonal mouse antihuman CD34 primary antibody (mca-547, Serotec, Oxford, UK) at a dilution of 1:25 at 4 C for 18 h. The rabbit antiserum that was raised against PGE_2-complexed keyhole limpet hemocyanin has been previously characterized (27). Control tissue was incubated with 5% antisera (for CD34) or goat anti-COX-2 antibody preadsorbed to blocking peptide (sc-1745p, Autogenbioclear) according to the manufacturer's protocol. Control tissue for PGE_2 was incubated with rabbit anti-PGE_2 antibody preadsorbed to excess exogenous PGE_2. Briefly, the PGE_2 antibody was incubated together with a 10-fold excess of exogenous PGE_2 (Sigma) at 37 C for 2 h. Thereafter, the antibody-ligand mixture was diluted, and immunohistochemistry was performed as described above. After thorough washing with TBS, the tissue sections probed with the goat antihuman COX-2 and rabbit anti-PGE_2 primary antibodies were incubated with biotinylated rabbit antigoat secondary IgG antibody (for COX-2; DAKO Corp.) or swine antirabbit secondary IgG antibody (for PGE_2; DAKO Corp.) at a dilution of 1:500 at 25 C for 40 min. Thereafter, the tissue sections were incubated with streptavidin-biotin peroxidase complex (DAKO Corp.) at 25 C for 20 min. Tissue sections probed with the mouse antihuman CD34 antibody were developed using a mouse EnVision Kit (DAKO Corp.) according to the manufacturer's instructions. Color reaction was developed by incubation with 3,3'-diaminobenzidine (DAKO Corp.). The tissue sections were counterstained in aqueous hematoxylin, followed by sequential dehydration using graded ethanol and xylene, before mounting and coverslipping.

PGE_2 stimulation and cAMP measurement

Determination of basal cAMP levels in cervical tissues. Initially, basal cAMP levels were measured in cervical tissue (squamous cell carcinomas, C39–C44; normal cervix, N16–N20; Fig. 5A). Carcinoma and normal cervical tissues were obtained on the day of surgery/biopsy, sectioned finely, and divided equally into three aliquots. The tissue was transported at 4 C and then incubated in 35-mm tissue culture dishes containing 2 mL DMEM (Sigma), 10% FCS, 0.3 mg/mL L-glutamine, 100 IU penicillin, and 100 μg streptomycin for 1.5 h. One aliquot of tissue was snap-frozen to determine the basal cAMP concentration in the tissue at the time of collection. The other two aliquots were incubated overnight at 37 C in humidified 5% CO_2 in the presence or absence of 3 μg/mL indomethacin (a dual COX enzyme inhibitor). Subsequently, tissue sections were harvested by centrifugation at 2000 $\times$ *g*. The supernatant was discarded, and the tissue was homogenized in 0.1 mol/L HCl. The cAMP concentration was quantified by ELISA using a cAMP kit (Biomol, Affiniti, Exeter, UK) according to the manufacturer's protocol and normalized to the protein concentration of the homogenate. Protein concentrations were determined using protein assay kits (Bio-Rad Laboratories, Inc., Hemel Hempstead, UK).

cAMP production in cervical tissues in response to exogenous PGE_2. Cervical tissues (squamous carcinoma, C45–C49; adenocarcinoma, C50; normal cervix, N21–N25) were sectioned finely, divided equally into three aliquots, and incubated overnight in DMEM (Sigma), containing 10% FCS, 0.3 mg/mL L-glutamine, 100 IU penicillin, 100 μg streptomycin, and 3 μg/mL indomethacin. After overnight incubation, samples were incubated in the same medium containing isobutylmethylxanthine (Sigma) to a final concentration of 1 mmol/L for 30 min at 37 C and then stimulated with 0 nmol/L PGE_2, 300 nmol/L PGE_2, or 50 μmol/L forskolin (forskolin treatment in sample C45 was excluded due to the small size of the biopsy) for 5 min. Tissue sections were harvested by centrifugation at 2000 $\times$ *g*. The supernatant was discarded, and the tissue homogenized in 0.1 mol/L HCl. cAMP and protein concentrations were determined as described above.

Results

Expression of COX-2 in cervical carcinomas was investigated using real-time quantitative RT-PCR (Fig. 1A) and Western blot analysis (Fig. 1B). Expression of COX-2 was

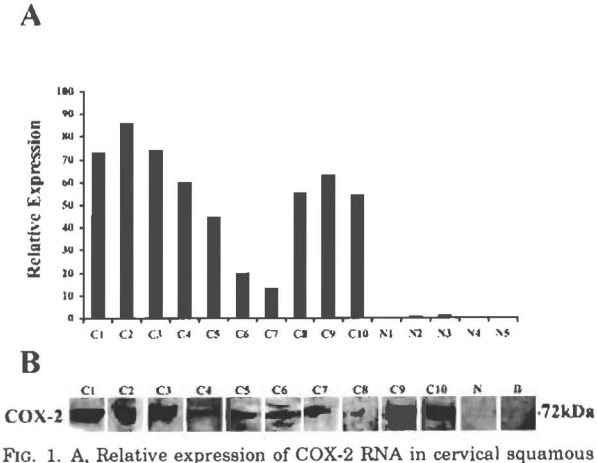

FIG. 1. A, Relative expression of COX-2 RNA in cervical squamous cell carcinoma (C1–C8), adenocarcinoma (C9 and C10), and normal cervix (N1–N5), as determined by real-time quantitative RT-PCR. B, Western blot analysis of 100 μg total protein isolated from human cervical carcinoma tissue. The proteins were loaded onto a 10% SDS-gel, electrophoresed, and subsequently transferred to a polyvinylidene difluoride membrane. The immunoblot was probed with antibody raised against the C-terminus of human COX-2. A specific band of approximately 72 kDa was detected in all squamous (C1–C8) and adenocarcinoma (C9 and C10). No signal was detected in normal cervical tissue (a representative sample is shown). Moreover, preadsorbing the antibody with the blocking peptide (B) abolished the COX-2 signal in all carcinoma samples (a representative sample is shown).

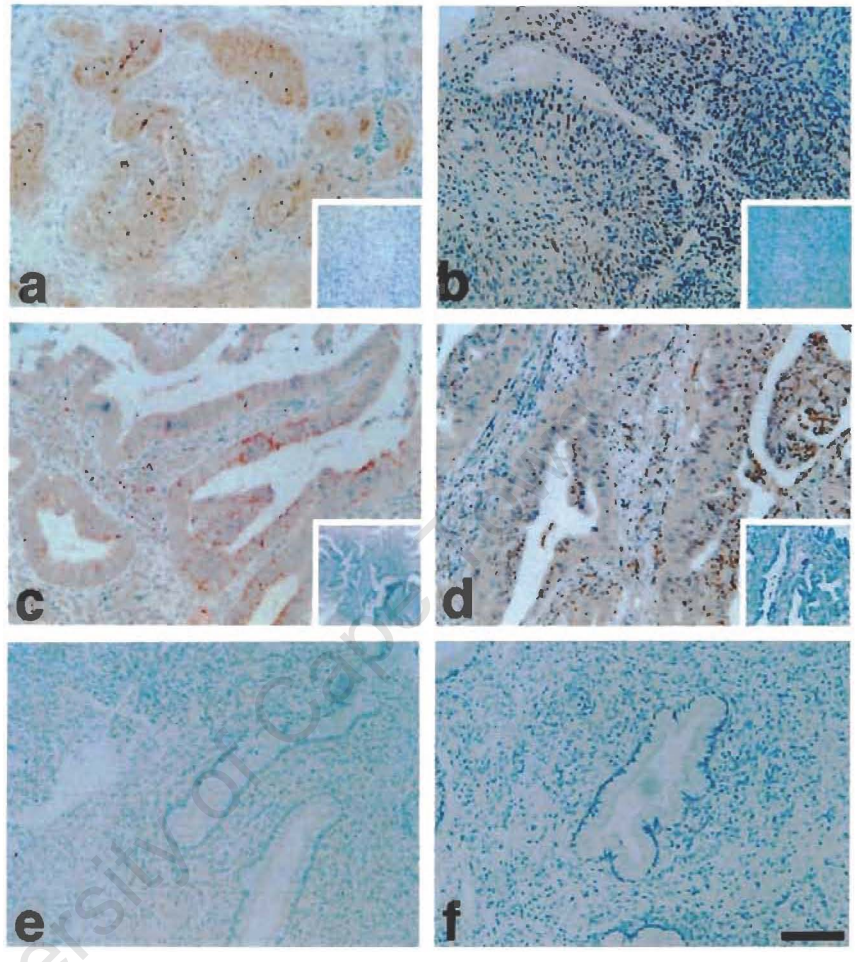

FIG. 2. COX-2 expression and PGE_2 synthesis are detected in epithelial cells of squamous cell carcinoma (A and B, respectively) and columnar and glandular epithelium of adenocarcinomas (C and D, respectively). Minimal COX-2 and PGE_2 signal was detected in normal cervical tissue (E and F, respectively). *Insets* are serial sections that were stained with preadsorbed COX-2 or PGE_2 serum, respectively (negative controls). *Scale bar*, 100 μm.

significantly up-regulated in all cases of squamous cell carcinoma and adenocarcinoma investigated. COX-2 expression as assessed by quantitative RT-PCR was 150.8 ± 43.18-fold greater in cervical carcinoma tissues than in normal cervical tissue ($P < 0.05$). Western blot analysis on these cervical carcinomas revealed immunoreactive bands of approximately 72 kDa. Minimal levels of COX-2 transcript was detected in normal cervical tissue by quantitative RT-PCR, and no COX-2 protein was detected in any of the normal cervical samples. Preadsorbing the primary antibody with the blocking peptide abolished the COX-2 signal in the carcinoma samples, thus confirming the specificity of detection of the 72-kDa COX-2 protein in the carcinoma samples.

The site of COX-2 expression and PGE_2 synthesis in the carcinoma tissue was investigated by immunohistochemistry. Immunoreactive COX-2 and PGE_2 were up-regulated in all carcinoma samples. COX-2 and PGE_2 were localized to the neoplastically transformed squamous epithelium in squamous cell carcinoma (Fig. 2, A and B, respectively), and to neoplastically transformed columnar epithelium lining the endocervical canal and the glandular epithelium of the endocervical glands in adenocarcinomas (Fig. 2, C and D, respectively). In addition, COX-2 and PGE_2 immunostaining was observed in endothelial cells lining the vasculature in all squamous cell carcinoma and adenocarcinoma sections investigated (Fig. 3, A and B). To confirm that COX-2 expression and PGE_2 synthesis were localized to the endothelial cells of blood vessels, immunohistochemistry was performed on tissue sections using antibodies raised against the CD34 endothelial cell marker. The pattern of expression with CD34 was identical to that observed with COX-2 and PGE_2, thus confirming that COX-2 expression and PGE_2 synthesis are localized to the endothelial cell layer of blood vessels in human cervical carcinoma (Fig. 3C). Negligible staining was observed in the stromal compartment of all carcinoma tissue investigated. Moreover, little or no staining for COX-2 and PGE_2 was observed in the normal cervical tissues (Fig. 2, E and F, respectively). Preadsorbing the antibody with the blocking peptide (COX-2-negative control) or incubating sections with PGE_2 preincubated with exogenous PGE_2 ligand (PGE_2-negative control) abolished the COX-2 and PGE_2 signals in all carcinoma samples (*insets* in figures show negative

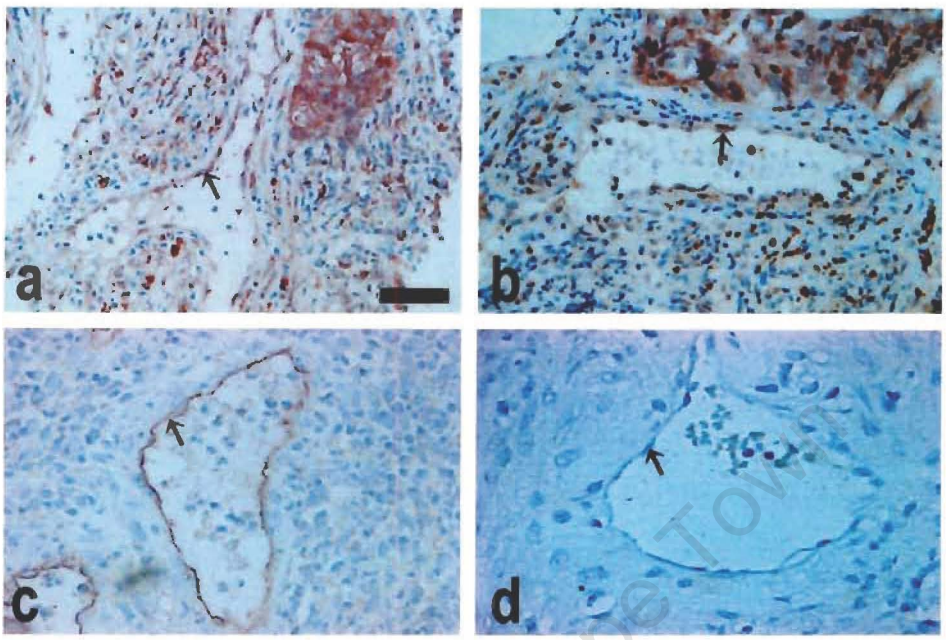

FIG. 3. COX-2 (A) expression and PGE_2 (B) synthesis are detected in endothelial cells (*arrowed*) of all carcinoma tissues. Vascular endothelial cells in cervical cancer tissues were localized using antibodies raised against the human CD34 endothelial cell marker (C). D, A representative section incubated with nonimmune goat serum (CD34-negative control). *Scale bar*, 50 μm.

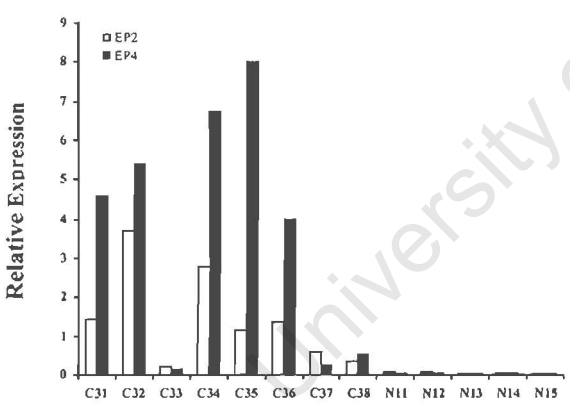

FIG. 4. Relative expression of EP2 (□) and EP4 (■) receptors in cervical squamous cell carcinoma (C31–C37), adenocarcinoma (C38) and normal cervix (N11–N15) as determined by real-time quantitative RT-PCR.

controls for COX-2 and PGE_2 performed on serial sections). No CD34 staining was observed in sections incubated with nonimmune serum in place of primary antibody (Fig. 3D).

The expression of two subtypes of PGE_2 receptors, namely EP2 and EP4, was investigated by real-time quantitative RT-PCR in cervical carcinoma and normal cervix (Fig. 4). Expression of both receptors was significantly up-regulated in all carcinoma tissues compared with that in normal cervix ($P < 0.01$). The relative expressions of EP2 and EP4 receptor in carcinoma tissue were 14.5 ± 3.2- and 106 ± 25.8-fold (respectively) greater than that in normal cervix. To assess the activity of the EP2/EP4 receptors in the cervical tissue, basal levels of cAMP were determined at the time of tissue collection and after overnight incubation in the absence or presence of 3 μg/mL indomethacin (Fig. 5A). The cAMP concentration immediately after tissue excision was significantly higher in carcinoma compared with normal cervix (77.9 ± 30.9 *vs.* 32.5 ± 8.7 pmol cAMP/mg protein; $P < 0.05$). cAMP concentrations in carcinoma tissue after overnight incubation in the absence of indomethacin was similar to that detected in the tissue at the time of excision (64.2 ± 5.1 pmol cAMP/mg protein), but was significantly reduced when the tissue was cultured in the presence of indomethacin (2.59 ± 0.64 pmol cAMP/mg protein). In normal cervical tissue, levels of cAMP were significantly reduced after overnight incubation in the absence or presence of indomethacin (11.96 ± 1.35 and 4.0 ± 0.7 pmol cAMP/mg protein, respectively; $P < 0.05$). Subsequently, we determined the effect of exogenous PGE_2 and forskolin treatment on cAMP production in carcinoma and normal cervical tissues (Fig. 5B). Stimulation of cervical carcinoma tissue with 300 nmol/L PGE_2 or 50 μmol/L forskolin (positive control) yielded a greater cAMP response than in normal cervical tissue treated in the same manner. Overall, the inductions of cAMP generation after PGE_2 and forskolin stimulation were 51.1 ± 12.3- and 55.3 ± 15.84-fold, respectively, in cancer tissue and 5.8 ± 1.68- and 9.18 ± 1.59-fold, respectively, in normal cervix ($P < 0.01$).

Discussion

This study confirms up-regulation of COX-2 expression and PGE_2 production in squamous cell carcinoma and adenocarcinoma of the human cervix, as demonstrated by real-

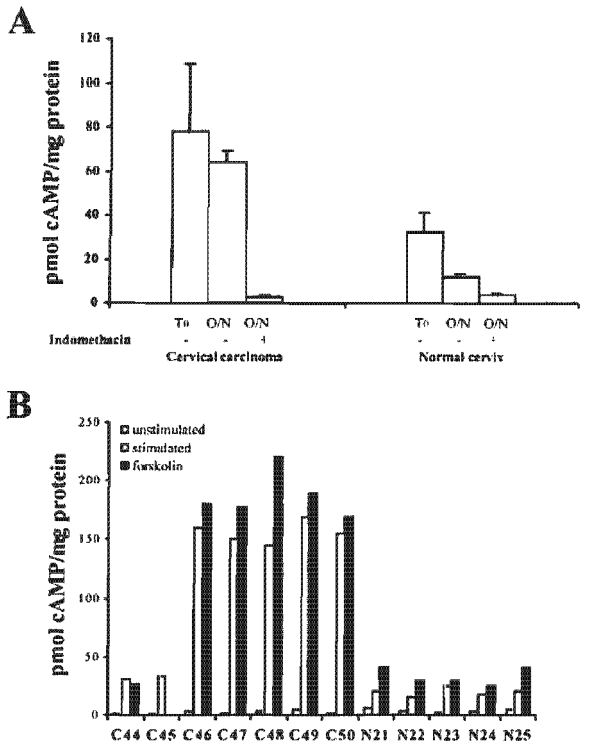

FIG. 5. A, Basal cAMP levels (picomoles of cAMP per mg protein) in cervical tissues (mean ± SEM of squamous cell carcinomas, C39–C43, and normal cervix, N15–N20). Basal cAMP levels were determined shortly after biopsy (T0) and after overnight (O/N) culture in the absence (−) or presence (+) of indomethacin. B, cAMP response (picomoles of cAMP per mg protein) in squamous cell carcinoma (C44–C49), adenocarcinoma (C50), and normal cervix (N21–N25). Cervical tissues were treated with indomethacin overnight and either stimulated with 300 nmol/L PGE_2 (□) or 50 μmol/L forskolin (■; positive control) or left unstimulated (▨).

time quantitative RT-PCR, Western blot analysis, and immunohistochemistry. These data suggest a similar pattern of expression of COX-2 in cancer of the cervix as demonstrated in other carcinomas (17–22). In addition, in this study increased COX-2 expression is associated with increased synthesis of PGE_2, as both COX-2 and PGE_2 colocalized in neoplastic epithelial cells and endothelial cells of the microvasculature. Previous studies have suggested that PGE_2 is the predominant PG synthesized from arachidonic acid by COX-2 (28). The exact role of up-regulated COX-2 and PGE_2 in cervical cancers remains to be elucidated. In other model systems, enhanced synthesis of PGE_2 resulting from up-regulated COX-2 induces malignant change in epithelial cells through immunosuppression (9), inhibiting apoptosis (23), increasing metastatic potential of epithelial cells (29), and promoting angiogenesis (24, 25). COX-2 and PGE_2 control the process of angiogenesis in tumors either directly or indirectly. In an *in vitro* model, overexpression of COX-2 and PGE_2 in colon epithelial cells enhances the expression of angiogenic factors that act on endothelial cells, resulting in enhanced cell migration and microvascular tube formation (24). More recently, it was suggested that COX-2 and PGE_2 produced by endothelial cells may also directly regulate the process of angiogenesis (25). The arrangement of rat aortic endothelial cells into tubular structures is reduced after treatment with selective COX-2 inhibitors, and this effect is partially reversed by cotreatment with PGE_2 (25). Hence, it is feasible to suggest that in cervical carcinomas the process of angiogenesis is regulated by COX-2 and PGE_2 through an epithelial-endothelial and/or endothelial-endothelial cell interaction. This is supported by our data demonstrating COX-2 expression and PGE_2 synthesis in neoplastic epithelial cells as well as endothelial cells.

PGE_2 acts on target cells through interaction with G protein-coupled receptors. To date, several of these receptors have been cloned (termed EP1–EP4) that use alternate intracellular signaling pathways (14). In this study we investigated a possible autocrine/paracrine role for synthesized PGE_2 in neoplastic cervical carcinoma tissue. For this we assessed the expression and functionality of two subtypes of PGE_2 receptors, namely EP2 and EP4, which mediate their effects on target cells via the protein kinase A pathway by activating adenylate cyclase and increasing intracellular cAMP levels via $G_s\alpha$ (13). *In vitro* studies have suggested that cAMP is the primary secondary messenger in regulating COX activity, as cAMP activity accompanies a concomitant increase in COX activity (9). The data presented in this study confirm up-regulation of expression of EP2 and EP4 receptors compared with normal cervical tissue. This is associated with elevated basal cAMP concentrations in carcinoma tissue compared with normal cervix. Treatment of cervical tissue with the COX enzyme inhibitor indomethacin significantly reduced the cAMP concentration. This suggests that the elevated basal cAMP concentration in the carcinoma tissue is mediated by COX enzyme products. Moreover, treatment of cervical carcinoma tissue with exogenous PGE_2 or forskolin after overnight incubation with the COX enzyme inhibitor indomethacin resulted in a rapid cAMP response that was greater in carcinoma tissue than in normal cervical tissue. Taken together, these data confirm that PGE_2 synthesized in cervical carcinoma tissue mediates an autocrine/paracrine effect via interaction with EP2/EP4 receptors. It is possible that other receptor subtypes may also be associated with PGE_2 function in the cervical carcinoma tissue. Due to limitations in the sizes of the biopsies obtained at surgery, it was not possible to investigate other intracellular signaling pathways that may be associated with PGE_2 function in cervical cancers (14).

COX-2 inhibitors exhibit dramatic antineoplastic activity in a number of tumor model systems investigated to date, including colon cancer cells implanted into nude mice, tumor production in APC mutant mice, and carcinogen-induced tumors in rats (30–32). This is mediated partially by reducing PGE_2 synthesis in the COX-2-overexpressing cells, which, in turn, down-regulates the survival, metastatic, and angiogenic potentials of the cancerous tissue (23, 24, 29). This has prompted the suggestion that the inhibition of PGE_2 secretion by the application of COX-2 inhibitors may have an effect on growth and invasiveness of various carcinomas (24, 25, 29, 30). Such treatments may also be of benefit in regulating the growth of cervical carcinoma. Treatment of cervical

carcinoma with NSAIDs will suppress endogenous expression of COX-2 and synthesis of PGE_2, which may act in an autocrine/paracrine manner via the EP2/EP4 receptors. However, it is important to emphasize that in sexually active women the use of selective COX-2 inhibitors may be of partial therapeutic benefit. In these women, the growth and invasiveness of neoplastic cells may be under the direct influence of PGE_2 present in seminal plasma. The PG concentration in seminal plasma is 10,000 times higher than that at the site of inflammation, and PGE is the predominant type of PG detected (33). Future studies to elucidate the relative contributions of endogenous and seminal plasma PGs on the phenotypic behavior of neoplastically transformed cervical epithelial and endothelial cells may assist in implementing improved therapy for women with cervical carcinomas.

Acknowledgments

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Cyclooxygenase-1 Is Up-Regulated in Cervical Carcinomas: Autocrine/Paracrine Regulation of Cyclooxygenase-2, Prostaglandin E Receptors, and Angiogenic Factors by Cyclooxygenase-1

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ABSTRACT

This study was designed to investigate the expression and molecular signaling of cyclooxygenase-1 (COX-1) in cervical carcinomas. Real-time quantitative reverse transcription-polymerase chain reaction and Western blot analysis confirmed enhanced expression of COX-1 RNA, and protein in squamous cell carcinomas and adenocarcinoma of the cervix. COX-1 expression in all carcinoma tissues was associated with enhanced expression of COX-2 RNA and protein. The site of COX-1 expression was localized by immunohistochemistry to the neoplastic epithelial cells in all squamous cell carcinomas and adenocarcinomas studied. Minimal COX-1 immunoreactivity was detected in normal cervix. To explore events associated with COX-1 up-regulation, we developed a doxycycline-regulated expression system in HeLa (cervical carcinoma) cells. Overexpression of COX-1 in HeLa cells resulted in induced expression of cyclooxygenase-2 (COX-2) and prostaglandin E synthase (PGES) concomitant with increased prostaglandin E_2 (PGE_2) synthesis. Treatment of HeLa cells overexpressing COX-1 with the dual COX enzyme inhibitor indomethacin or selective COX-2 inhibitor NS-398 significantly reduced PGE_2 synthesis. Indomethacin, but not NS-398, treatment abolished the up-regulation of expression of COX-2 and PGES in HeLa cells, suggesting that the observed up-regulation of COX-2 and PGES was mediated by COX-1-enzyme products. To assess whether enhanced PGE_2 synthesis after COX-1 induction would act in an autocrine/paracrine manner, we investigated the effect of COX-1 on the expression of the different isoforms of PGE_2 receptors (EP1–EP4). We found that the cAMP-linked PGE_2 receptors were significantly up-regulated by COX-1 overexpression coincident with enhanced cAMP responsiveness of these cells to exogenous PGE_2 ligand. Finally, overexpression of COX-1 was associated with enhanced expression of the angiogenic factors basic fibroblast growth factor, vascular endothelial growth factor, angiopoietin-1, and angiopoietin-2. This up-regulation of angiogenic factor expression was abolished by indomethacin and partially reduced by NS-398. These data indicate that COX-1 up-regulation modulates the expression of factors that may act in an autocrine/paracrine manner to enhance and sustain tumorigenesis in neoplastic cervical epithelial cells. It is likely that similar mechanisms may act *in vivo* to modulate tumorigenesis of cervical carcinomas.

INTRODUCTION

Uterine cervical cancer is considered an important clinical problem in developing countries, with a high incidence of invasive disease reported for South African women (1). Three histological categories of epithelial tumors of the cervix are recognized by the World Health Organization (2): squamous cell carcinoma, adenocarcinoma, and other less common types of epithelial tumors. The most common histological type of cervical carcinoma is squamous cell carcinoma, which accounts for 60–80% of all cervical cancers. Adenocarcinoma accounts for <20% of invasive cervical carcinomas. Numerous studies have demonstrated that epithelial tumors may be regulated by COX-2-enzyme products (3–7). Two distinct isoforms of the COX enzyme, COX-1 and COX-2, have been reported (8–10). The relative contributions of COX-1- and/or COX-2-derived products in mediating events associated with cervical neoplasia remain to be elucidated. COX-1 expression is considered to be constitutive and generates prostaglandins for normal physiological functions (4, 11, 12). Transcription of COX-2 RNA and protein is up-regulated in several epithelial carcinomas (3, 12–15), including carcinomas of the cervix (16–18). This has prompted the suggestion that the increased level of prostaglandins and other eicosanoids present in cancer tissue is a consequence of induced COX-2. More recently, however, it has been demonstrated that both COX isoforms are inducible. In some cell types, including pulmonary artery endothelial cells, COX-1 levels are induced during differentiation (19, 20). COX-1 expression can be induced *in vitro* by VEGF (21), arachidonic acid, forskolin, dibutyryl-cAMP, and PGE_2 (22). In addition, elevated COX-1 expression has been reported in mouse lung tumors (23), human breast cancer (24), and human prostate carcinoma (25). These data suggest that both COX enzymes and/or their products may function in promoting and maintaining the neoplastic state. COX catalyzes the double oxygenation and reduction of arachidonic acid after its release from membrane glycerophospholipids by phospholipase A_2 to the intermediate form prostaglandin H_2. This intermediate serves as the substrate for terminal prostanoid synthases, which produce their specific prostaglandins such as PGE_2, being synthesized by PGES (26–28). PGE_2 has been shown to stimulate gene transcription (29), influence mitogenesis of normal human bone cells (30), and promote growth and metastasis of tumors (31). More recently, enhanced synthesis of PGE_2 resulting from up-regulated COX-2 has been shown to induce malignant change in epithelial cells through immunosuppression (32), inhibiting apoptosis (13), increasing metastatic potential of epithelial cells (6), and promoting angiogenesis (33, 34). Two segregated biosynthetic pathways have been described for PGE_2 biosynthesis. These pathways synthesize PGE_2 via PGES functionally and preferentially coupled with either COX-1 or COX-2 (27). The biological actions of PGE_2 have been attributed to its interaction with G-protein-coupled seven-transmembrane-domain receptors, which belong to the rhodopsin superfamily of serpentine receptors (35). Four main subtypes of PGE_2 receptors have been identified (EP_1, EP_2, EP_3, and EP_4), which use alternate and, in some cases, opposing intracellular pathways (36). Most studies have focused on neoplastic events associated with COX enzyme products as a consequence of COX-2 overexpression. In this study, we investigated (*a*) COX-1 expression and localization in cervical squamous cell carcinomas and adenocarcinomas compared with normal cervical tissue, and (*b*) a possible autocrine/paracrine role for COX-1 enzyme products in regulating the expression of COX-2,

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[2] The abbreviations used are: COX, cyclooxygenase; VEGF, vascular endothelial growth factor; PGE_2, prostaglandin E_2; PGES, prostaglandin E synthase; bFGF, basic fibroblast growth factor; Ang, angiopoietin; Hyg, hygromycin; DOX, doxycycline; RT-PCR, reverse transcription-PCR; TK, thymidine kinase; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline.

PGES, PGE_2 receptors, and angiogenic factors in cervical epithelial carcinoma cells using an inducible expression system.

MATERIALS AND METHODS

Materials

The following antibodies used for Western blotting were purchased from Santa Cruz Biotechnology (Autogenbioclear, Wiltshire, United Kingdom): COX-1 goat polyclonal (sc-1752), COX-2 goat polyclonal (sc-1745), bFGF goat polyclonal (sc-1360), VEGF rabbit polyclonal (sc-152), Ang-1 goat polyclonal (sc-6319), Ang-2 goat polyclonal (sc-7016), and β-actin goat polyclonal (sc-1616), as well as the COX-1 and COX-2 blocking peptides (sc-1752p and sc-1745p). The PGES antibody raised against the microsomal glutathione-dependent inducible PGES (27) was purchased from Caymen Chemical Co. (Cheshire, United Kingdom); antigoat-alkaline phosphatase, antirabbit-alkaline phosphatase, cloning cylinders, G418, Hyg, DOX, and indomethacin were purchased from Sigma Chemical Co. (Dorset, United Kingdom). Samples and synthetic standards for the PGE_2 ELISA were purchased from Applied Therapeutics (Paisley, United Kingdom), and NS-398 was purchased from Calbiochem (Nottingham, United Kingdom). HeLa Tet-Off cells and Tet system-approved fetal bovine serum were purchased from Clontech (Hampshire, United Kingdom). DMEM nutrient mixture F-12 was purchased from Life Technologies, Inc. (Paisley, United Kingdom), and penicillin-streptomycin was purchased from PAA (PAA Laboratories Ltd., Middlesex, United Kingdom). ECF chemiluminescence system was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, United Kingdom).

Tissue Collection and Processing

Cervical specimens were obtained at the time of surgery/biopsy from patients who were attending the Gynaecological Oncology Clinic at Groote Schuur Hospital, Cape Town and who had been diagnosed previously with invasive carcinoma of the cervix. Punch biopsies were taken from the lesion by an experienced gynecologist with a special interest in oncology. A portion of the biopsy was excised and fixed in formalin, followed by paraffin wax embedding for histopathological typing. The remaining portion was snap-frozen in either dry ice or liquid nitrogen and stored at −70°C for RT-PCR and Western blot analysis. The extent of invasiveness of carcinoma biopsies (C1–C58) is represented in Table 1. Histologically normal cervical samples (N1–N21) were obtained from patients undergoing Wertheims hysterectomy for nonmalignant conditions. Pathological typing was defined according to the International Federation of Obstetricians and Gynaecologists (37) staging upon physical examination. The ages of the patients ranged from 29 to 80 years with a median age of 50 years. The study was approved by the University of Cape Town Research Ethics Committee, and informed consent was obtained from all patients before tissue collection.

Cell Culture

HeLa Tet-Off cells containing the regulatory plasmid (pTet-Off) were routinely maintained in DMEM nutrient mixture F-12 with Glutamax-1 and pyridoxine, supplemented with 10% fetal bovine serum, 100 μg/ml G418, and 1% antibiotics (stock, 500 IU/ml penicillin and 500 μg/ml streptomycin) at 37°C and 5% CO_2 (v/v).

Table 1 *Extent of invasiveness of cervical carcinoma biopsy samples of South African women*

Sample no.	Histological typing	FIGO stage[a]
C10, C14; C28–C32	Squamous carcinoma	1B; well differentiated
C5–C9; C24–C27; C37–C47	Squamous carcinoma	2B; well differentiated
C1–C4; C19–C23	Squamous carcinoma	3B; well differentiated
C36	Adenocarcinoma	1B; moderately differentiated
C15–C18; C33–C35; C48–C58	Adenocarcinoma	2B; well differentiated

[a] FIGO, Fédération Internationales des Gynaecologistes et Obstetristes.

Cell Transfections

The Tet-Off expression system we used was developed by Gossen *et al.* (38) to deliver doxycycline-regulated expression based on the high specificity of the *Escherichia coli* tet repressor-operator-doxycycline interaction. In the Tet-Off expression system each clonal cell line is used as its own control (cells cultured in the presence of DOX), and the overexpression of the integrated target gene is modulated solely by removing DOX from the culture medium. This eliminates the need for a control clonal cell line transfected with vector alone (as used with constitutive stable expression systems), thereby overcoming the inherent variation that arises from different sites of integration of DNA between different clones. HeLa Tet-Off cells containing the pTet-Off vector stably transfected and constitutively expressing the tetracycline-controlled transactivator tTA (composed of a fusion of the TetR and VP16 activation domain) were purchased from Clontech. The pBS(SK-)/PSHI cDNA containing the full-length *COX-1* gene (kindly supplied by Dr. Stephen Prescott, University of Utah, Salt Lake City, UT) was used as the template plasmid. The response plasmid pTRE2 (containing the minimal cytomegalovirus promoter containing Tet-operator sequences cloned upstream of the cDNA to be expressed) and the plasmid for antibiotic selection (pTK-Hyg) for use with the Tet-Off system were purchased from Clontech. The *COX-1* gene was excised from the template plasmid and ligated at the *Bam*HI site of the pTRE2 vector. The orientation of the insert was verified by dideoxy DNA sequencing using the sequence- specific primers 5′-CGCCTGGAGACGCCATCC-3′ and 5′-CCACACCTCCCCCTGAAC-3′ (Clontech). Cells were plated in 12-well dishes in complete medium containing 100 μg/ml G418 per well and were allowed to attach and grow overnight. The pTRE2 vector containing the *COX-1* gene (2 μg) was cotransfected with pTK-Hyg (0.1 μg, which contains the *Hyg* gene under control of the minimal TK promoter) into the HeLa Tet-Off cell line at about 80% confluency using pfx-5 (Invitrogen, De Schelp, Netherlands) diluted in Optimem (Life Technologies, Inc.). Cells were incubated for 4 h at 37°C in 5% humidified CO_2. Thereafter, the medium was replaced with fresh complete medium containing no G418. Cells were allowed to grow for 72 h. Transfected cells were then seeded together with wild-type cells. Clones were selected against 200 μg/ml Hyg in the presence of 1 μg/ml DOX. At least 50 Hyg-resistant clones were picked using cloning cylinders. Clones were allowed to grow under continuous selection with Hyg in the presence of DOX and then screened for the ability to express COX-1 in the presence and absence of DOX by immunoblot analysis. Three clones with the greatest inducible overexpression of COX-1 (clones 1.2, 2.2, and 3.1) were selected for additional experiments. All clones were characterized and exhibited identical phenotypic and biochemical alterations. The results of our studies using the COX-1 clone 1.2 are presented here. Similar reproducible results were obtained using clones 2.2 and 3.1. Unless otherwise stated, all clones were maintained uninduced in 1 μg/ml DOX, 200 μg/ml Hyg, and 100 μg/ml G418. COX inhibition studies were conducted by growing cells in medium containing 3 μg/ml indomethacin or 10 μM NS-398.

Real-time Quantitative RT-PCR

Real-time quantitative RT-PCR was performed to determine COX-1 and COX-2 expression in cervical carcinoma biopsies and normal cervical tissue as well as to assess the effect of COX-1 overexpression on expression of the different isoforms of PGE_2 receptors (EP1, EP2, EP3, and EP4) in HeLa Tet-Off cells. RNA samples were extracted from cervical tissue (squamous cell carcinomas, C1–C14; adenocarcinomas, C15–C18; and normal cervix, N1–N8) using Tri-Reagent (Sigma Chemical Co.) as per the manufacturer's instruction. To determine the effect of COX-1 overexpression on expression of EP receptors, cells (2×10^5) were seeded in six-well plates, and allowed to attach and grow overnight in the presence of DOX. The following day, the cells were synchronized by incubating with serum-free medium for 24 h. Thereafter, the medium was replaced with fresh complete medium, and COX-1 overexpression was induced by growing cells in medium containing no DOX. Control cells were maintained in DOX. Cells were harvested after 24, 48, and 72 h with 1 ml/well Tri-Reagent (Sigma Chemical Co.) as per the manufacturer's protocol. RNA samples were reverse transcribed using $MgCl_2$ (5.5 mM), dNTPs (0.5 mM each), random hexamers (1.25 μM), oligodeoxythymidylic acid (1.25 μM), RNase inhibitor (0.4 unit/μl), and multiscribe reverse transcriptase (1.25 units/μl), all from PE Biosystems (Warrington, United Kingdom). 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 5 min at 95°C. A reaction mix was made containing Taqman buffer (5.5 mM $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 400 μM dUTP); ribosomal 18S forward and reverse primers and probe (all at 50 nM); forward and reverse primers for COX-1, COX-2, EP1, EP2, EP3, or EP4 receptor (300 nM); COX-1, COX-2, EP1, EP2, EP3, or EP4 receptor probe (200 nM); AmpErase UNG (0.01 unit/μl); and AmpliTaq Gold DNA Polymerase (0.025 unit/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 were added. After mixing, 23 μl of sample were 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 reaction was run on an ABI Prism 7700 using standard conditions. COX-1, COX-2, and EP receptor primers and probe for quantitative PCR were designed using the PRIMER express program (PE Biosystems). The sequences of the COX-1 primers and probe were as follows. Forward: 5′-TGT TCG GTG TCC AGT TCC AAT A-3′; reverse: 5′-ACC TTG AAG GAG TCA GGC ATG AG-3′; probe (FAM labeled): 5′-CGC AAC CGC ATT GCC ATG GAG T-3′. The sequences of the COX-2 primers and probe were as follows. Forward: 5′-CCT TCC TCC TGT GCC TGA TG-3′; reverse: 5′-ACA ATC TCA TTT GAA TCA GGA AGC T-3′; probe (FAM labeled): 5′-TGC CCG ACT CCC TTG GGT GTC A-3′. The sequences of the EP1 receptor primers and probe were as follows. Forward: 5′-AGA TGG TGG GCC AGC TTG T-3′; reverse: 5′-GCC ACC AAC ACC AGC ATT G-3′; probe (FAM labeled): 5′-CAG CAG ATG CAC GAC ACC ACC ATG-3′. The sequences of the EP2 receptor primers and probe were as follows. Forward: 5′-GAC CGC TTA CCT GCA GCT GTA C-3′; reverse: 5′-TGA AGT TGC AGG CGA GCA-3′; Probe (FAM labeled): 5′-CCA CCC TGC TGC TGC TTC TCA TTG TCT-3′. The sequences of the EP3 receptor primers and probe were as follows. Forward: 5′-GAC GGC CAT TCA GCT TAT GG- 3′; reverse: 5′-TTG AAG ATC ATT TTC AAC ATC ATT ATC A-3′; probe (FAM labeled): 5′-CTG TCG GTC TGC TGG TCT CCG CTC-3′. The sequences of the EP4 receptor primers and probe were as follows. Forward: 5′-ACG CCG CCT ACT CCT ACA TG-3′; reverse: 5′-AGA GGA CGG TGG CGA GAA T-3′; probe (FAM labeled): 5′-ACG CGG GCT TCA GCT CCT TCC T-3′. The ribosomal 18S primers and probe sequences were as follows. Forward: 5′-CGG CTA CCA CAT CCA AGG AA-3′; reverse: 5′-GCT GGA ATT ACC GCG GCT-3′; probe (VIC labeled): 5′-TGC TGG CAC CAG ACT TGC CCT C-3′. Expression of COX-1 and EP receptors was normalized to RNA loading for each sample using the 18S rRNA as an internal standard. Relative COX-1 and COX-2 expression in carcinoma tissue was calculated by dividing the expression in carcinoma tissue by the expression in normal cervix. Relative expression of EP receptors was calculated, from three independent experiments, by dividing the expression in induced cells by the expression in uninduced cells. The data are presented as mean ± SE.

Protein Extraction

Tissue. COX-1 and COX-2 protein expression in cervical carcinomas and normal cervix was assessed by Western blotting. Proteins were extracted from cervical tissue (squamous cell carcinomas, C19–C32; adenocarcinomas, C33–C36; and normal cervix, N9–N16) by homogenization in protein lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS containing 2 mM phenylmethylsulfonyl fluoride). Thereafter, insoluble material was pelleted by centrifugation at 14,000 × *g* for 20 min at 4°C. 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, United Kingdom). A total of 50 μg of protein was resuspended in 20 μl of sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 5% 2-mercaptoethanol, 20% glycerol, and 0.05% bromphenol blue), boiled for 5 min at 95°C, and run on a 10% SDS-polyacrylamide gel before Western blotting.

Cells. Cells were seeded in 5-cm dishes and allowed to attach overnight. The following day, the cells were synchronized by incubating with serum-free medium for 24 h. Thereafter, the medium was replaced with fresh complete medium, and the cells were grown in the presence or absence of DOX for 24, 48, and 72 h, respectively. In parallel, cells were cotreated with indomethacin or NS-398. Cells were harvested by lysing in protein lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.1% SDS). The protein content in the supernatant fraction was determined as described above. The clarified cell lysates (20 μg) were denatured and electrophoresed on 4–20% Tris-glycine gels (NOVEX, Invitrogen).

Western Blotting

Immunoblot analysis was performed on supernatant fractions of cervical tissues and HeLa COX-1 Tet-Off cells. The proteins were transferred onto a PVDF membrane (Millipore, Watford, United Kingdom) and subjected to immunoblot analysis. Membranes were blocked for 1 h at 25°C in 5% skimmed milk powder diluted in TBS-Tween [50 mM Tris-HCl, 150 mM NaCl, and 0.05% (v/v) Tween 20]. Thereafter, membranes were incubated overnight with either COX-1 (1:500)-, COX-2 (1:500)-, β-actin (1:500)-, PGES (1:250), bFGF (1:500)-, VEGF (1:500)-, Ang-1 (1:250)-, or Ang-2 (1:250)-specific antibodies. After transfer, membranes were subsequently incubated for 1 h with rabbit antigoat secondary antibody (for COX-1/2, β-actin, Ang-1/2, and bFGF) at a dilution of 1:30,000 or goat antirabbit secondary antibody (PGES or VEGF) at a dilution of 1:30,000. Thereafter, membranes were washed in TBS-Tween and developed by the ECF chemiluminescence system following the manufacturer's instructions. Proteins were revealed and quantified by PhosphorImager analysis using the STORM 860 system (Molecular Dynamics, Amersham Biosciences, Buckinghamshire, United Kingdom). Fold induction in induced cells was determined relative to uninduced cells, after normalizing to β-actin, by dividing the expression in induced cells by the expression in uninduced cells. The molecular weights of the respective proteins were determined from the relative mobility on SDS-PAGE compared with molecular weight standards. COX-1 and COX-2 negative controls for determination of antibody specificity were performed by incubating membranes with goat anti-COX-1/2 antibody preadsorbed to blocking peptide as per the manufacturer's protocol. Data are presented as mean ± SE from four independent experiments.

Immunohistochemistry

The site of COX-1 expression was localized in cervical tissues by immunohistochemistry using archival cervical blocks (squamous cell carcinomas, C37–C47; adenocarcinomas, C48–C58; and normal cervix, N19–N23) obtained from the Department of Anatomical Pathology, University of Cape Town, South Africa. Five-micrometer paraffin wax-embedded tissue sections were cut and mounted onto coated slides (TESPA, Sigma Chemical Co.). Sections were dewaxed in xylene, rehydrated in graded ethanol, and washed in water followed by TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), and blocked for endogenous endoperoxidase (1% H_2O_2 in methanol). Antigen retrieval was performed by pressure cooking for 2 min in 0.01 M sodium citrate pH 6. Sections were blocked using 5% normal rabbit serum diluted in TBS. Subsequently the tissue sections were incubated with polyclonal goat anti-COX-1 antibody (sc-1752; Autogenbioclear) at a dilution of 1:200 at 4°C for 18 h. Control tissue was incubated with goat anti-COX-1 antibody preadsorbed to blocking peptide (sc-1752p; Autogenbioclear) as per the manufacturer's protocol. After thorough washing with TBS, the tissue sections probed with the goat antihuman COX-1 primary antibody were incubated with biotinylated rabbit antigoat secondary IgG antibody (DAKO, Buckinghamshire, United Kingdom) at a dilution of 1:500 at 25°C for 40 min. Thereafter, the tissue sections were incubated with streptavidin-peroxidase complex (DAKO) at 25°C for 20 min. Color reaction was developed by incubation with 3,3′-diaminobenzidine (DAKO). The tissue sections were counterstained in aqueous hematoxylin, followed by sequential dehydration using graded ethanol and xylene, before mounting and coverslipping.

PGE_2 Assay

HeLa COX-1 Tet-Off cells were seeded in 5-cm dishes at a cell density of 5×10^5 cells/dish and were allowed to grow and attach overnight. The following day, the cells were synchronized by incubating with serum-free medium for 24 h. COX-1 expression was induced for 24, 48, and 72 h, respectively, by DOX withdrawal from the culture medium, in the presence or absence of indomethacin or NS-398. Arachidonic acid to a final concentration of 5 μg/ml was added to the culture medium after induction for 6 h. Thereafter, 1 ml of medium was removed and added to 1 ml of methyloximating solution. Control uninduced cells were treated similarly but maintained with DOX

supplemented daily. PGE_2 secretion into the culture medium was assayed by ELISA (39). The ELISA was performed using 96-well plates (amine-binding plates; Costar, High Wycombe, United Kingdom) coated with donkey antirabbit antibody. Plates were then coated with rabbit IgG (1 mg/ml diluted in PBS with 1% carbonate buffer, pH 9.6) at 200 μl/well for 16 h at 4°C. The solution was aspirated, and blocking solution (50 mM glycine, 10 mg/ml BSA) was added at 25 μl/well for 2 h at 23°C. The plates were then washed, and donkey antirabbit serum (Scottish Antibody Production Unit, Carluke, United Kingdom) was added to a final volume of 150 μl/well, before washing, air drying, and storage with desiccant at 4°C. The link was prepared by ether extraction and reverse-phase chromatography using 20 mg of synthetic PGE_2, 320 μl of dry dimethylformamide, 3 μl butylchloroformate, and 0.05 mM biocytin. Samples and synthetic standards were diluted in ELISA buffer (150 mM NaCl, 100 mM Tris-HCl, 0.05% Tween 20, 50 mM phenol red, 1 mM 2-methylisothiazolone, 1 mM bromonitrodioxane, 2 mM EDTA, 2 mg/ml BSA to a final pH of 7.2), and 100 μl of each were added in duplicate to the plate. The link was diluted $1:1.5 \times 10^6$ in ELISA buffer, and 50 μl were added to each well. Antisera, diluted 1:50,000 in ELISA buffer, were added to a final volume of 50 μl to all wells except those used for measuring nonspecific binding. Plates were incubated at 4°C for 16 h and washed, and 100 μl/well of 0.2 unit/ml streptavidin-peroxidase were added. Plates were then incubated for 20 min at 23°C on an orbital shaker and washed, and substrate (0.3 g/liter urea-hydrogen peroxide, 0.1 g/liter tetramethylbenzene in 100 mM sodium acetate, pH 6.0) was added to a final volume of 200 μl/well for 10 min before quenching with 50 μl/well 1 M sulfuric acid. Color reaction was measured at 450 nm by spectrophotometry. The rabbit antiserum that was raised against PGE_2-complexed keyhole limpet hemocyanin has been characterized previously (40). Data are presented as mean ± SE from three independent experiments.

PGE_2 Stimulation and cAMP Measurement

Functionality of the up-regulated PGE_2 receptors was assessed by measuring cAMP accumulation after COX-1 induction in the presence or absence of indomethacin. Cells (2×10^5) were plated in six-well dishes containing 4 ml/well of complete medium containing DOX. Cells were allowed to attach overnight. The following day, the cells were synchronized by incubating with fresh medium containing no fetal bovine serum for 24 h. COX-1 Tet-Off cells were induced by DOX withdrawal from the culture medium for 48 h at 37°C in humidified 5% CO_2 in the presence or absence of indomethacin. In parallel, control uninduced cells were supplemented daily with DOX. Thereafter the culture medium was removed and replaced with serum-free medium containing 1-methyl-3-isobutylxanthine (Sigma Chemical Co.) to a final concentration of 1 mM for 40 min at 37°C. Cells were then stimulated with 0 or 300 nM PGE_2 for 5, 10, 20, or 30 min, respectively. After stimulation, the medium was removed and the cells were lysed in 0.1 M HCl. cAMP concentration was quantified by ELISA using a cAMP kit (Biomol; Affiniti, Exeter, United Kingdom) as per the manufacturer's protocol and normalized to the protein concentration of the lysate. Protein concentrations were determined using protein assay kits (Bio-Rad). The data are presented as mean ± SE from three independent experiments.

Statistical Analysis

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

RESULTS

Expression of COX-1 and COX-2 in Cervical Carcinomas and Normal Cervix. Expression of COX-1 and COX-2 in cervical carcinomas was investigated using real-time quantitative RT-PCR (Fig. 1*A*) and Western blot analysis (Fig. 1*B*). Expression of COX-1 and COX-2 RNA was significantly up-regulated in 78 and 100% of cases, respectively, of squamous cell carcinoma and 100% of cases of adenocarcinoma investigated. By contrast, minimal COX-1 and COX-2 transcript was detected in normal cervical tissue by quantitative RT-PCR. COX-1 and COX-2 expression, as assessed by quantitative RT-PCR, was 19.9 ± 5.9- and 118 ± 32-fold greater in cervical carcinoma tissues than that observed in normal cervical tissue ($P < 0.01$). Western blot analysis confirmed enhanced expression of COX-1 and COX-2 in cervical squamous cell carcinoma (85 and 100% of cases, respectively; Fig. 1*B*, *panel I*) and adenocarcinoma (100% of cases; Fig. 1*B*, *panel II*). Basal expression of COX-1 protein was detected in 87% of cases of normal cervix. No COX-2 expression was detected in normal cervical tissue by Western blot analysis (Fig. 1*B*, *panel III*). Specificity of detection of the 72-kDa COX-1 and COX-2 protein was performed by competition studies using a specific immunogen (blocking) peptide (data not shown).

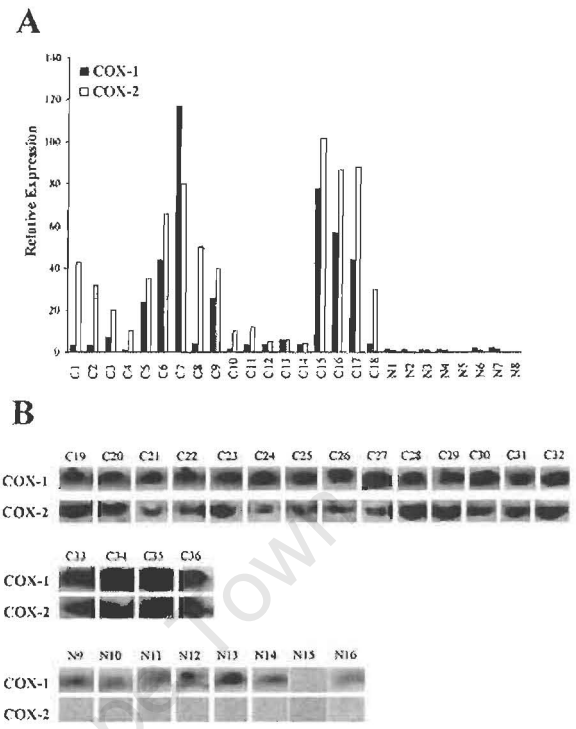

Fig. 1. *A*, relative expression of COX-1 and COX-2 RNA in cervical squamous cell carcinoma (C1–C14), adenocarcinoma (C15–C18), and normal cervix (N1–N8) as determined by real-time quantitative RT-PCR. *B*, Western blot analysis of 50 μg of total protein isolated from human cervical carcinoma tissue. The proteins were loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred to a PVDF membrane. The immunoblot was probed with antibody raised against the COOH terminus of human COX-1 or COX-2. A specific band of approximately 72 kDa was detected in all squamous cell carcinomas (*panel I*; *C19–C32*) and adenocarcinomas (*panel II*; *C33–C36*). Basal COX-1 expression was detected in seven of eight normal cervices. No COX-2 expression was detected in normal cervical tissue (*panel III*, *N9–N16*).

Localization of the Site of COX-1 Expression in Cervical Carcinomas and Normal Cervix. The site of COX-1 expression in the carcinoma tissue was investigated by immunohistochemistry. COX-1 expression was up-regulated in all carcinoma samples. COX-1 was localized to the neoplastically transformed squamous epithelium in squamous cell carcinoma (Fig. 2*A*), and to the neoplastically transformed columnar epithelium lining the endocervical canal and the glandular epithelium of the endocervical glands in adenocarcinomas (Fig. 2*C*). Little or no immunoreactivity for COX-1 was observed in the normal cervical tissues (Fig. 2*E*). Preadsorbing the antibody with the blocking peptide (COX-1 negative control) abolished the COX-1 immunoreactivity in all carcinoma samples. Representative sections incubated with the blocking peptide are shown in Fig. 2, *B*, *D*, and *F* for squamous cell carcinoma, adenocarcinoma, and normal cervical tissues, respectively.

Inducible COX-1 Expression in HeLa Cells. To investigate the effect of COX-1 overexpression in HeLa neoplastic cervical epithelial

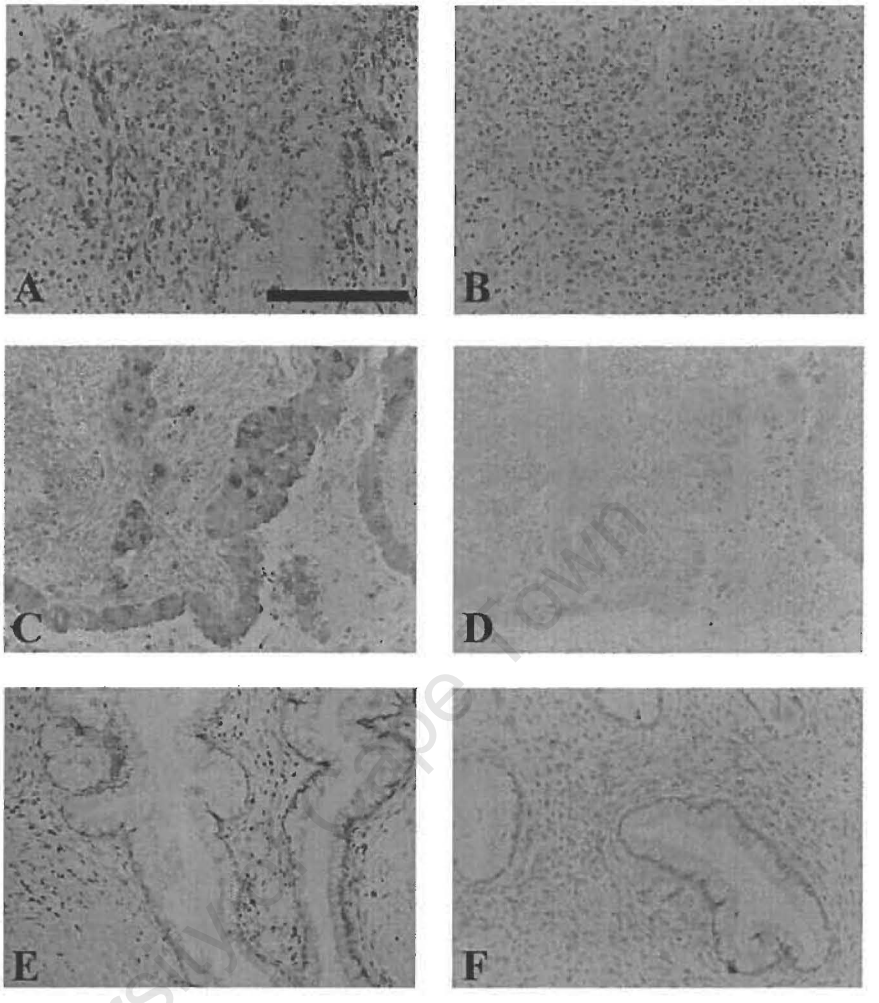

Fig. 2. Localization of COX-1 expression in epithelial cells of squamous cell carcinomas and columnar and glandular epithelium of adenocarcinomas (*A* and *C*, respectively). Minimal COX-1 signal was detected in normal cervical tissue (*E*). Sections that were stained with preadsorbed COX-1 sera are shown in *B*, *D*, and *F* for squamous cell carcinoma, adenocarcinoma, and normal cervix, respectively (negative controls). *Scale bar*, 100 μm.

cells, we established a DOX-regulated expression system. As shown in Fig. 3*A*, a 72-kDa immunoreactive COX-1 band was observed to increase in intensity 48 h after DOX withdrawal from the culture medium. Maximal sustained induction was achieved after 72 h. The fold induction for COX-1 overexpression above basal for 24, 48, and 72 h was determined to be 1.5 ± 0.34-, 3.7 ± 0.45-, and 4.7 ± 0.56-fold, respectively. COX-1 expression was normalized against β-actin on the same blot. Cells maintained in DOX for 72 h showed no elevation of COX-1 expression above basal. Preadsorbing the COX-1 antibody with the blocking peptide abolished the COX-1 immunoreactivity, indicating specificity of the COX-1 antibody. These data indicate that high levels of inducible overexpression of COX-1 were achieved in HeLa cells. To determine whether COX-1 expression was altered by cell confluency or the addition of DOX to the culture medium, wild-type HeLa Tet-Off cells were grown for 72 h in the presence or absence of DOX. No increase in COX-1 expression above basal was observed, suggesting that neither DOX nor cell density affected the expression of COX-1 in wild-type HeLa Tet-Off cells (Fig. 3*A*).

The functionality of the transfected COX-1 cDNA was assessed by measuring PGE_2 secretion into the culture medium after COX-1 induction for 24, 48, and 72 h, respectively. A time-dependent increase in PGE_2 secretion into the culture medium accompanied the induction of COX-1 expression. PGE_2 production was significantly elevated after 48 h (272.2 ± 18.8 nM; $P < 0.05$) and 72 h (537 ± 22.5 nM; $P < 0.01$) when compared with PGE_2 levels in uninduced cells (118 ± 6.75 nM; Fig. 3*B*). The addition of indomethacin reduced the PGE_2 levels to 62 ± 7 nM and 76 ± 0.7 nM after 48 and 72 h, respectively ($P < 0.01$). Cotreatment of cells with NS-398 (selective COX-2 inhibitor) partially reduced PGE_2 levels to 132 ± 26.2 and 268 ± 17 nM after 48 and 72 h, respectively ($P < 0.05$).

COX-1 Overexpression Induces COX-2 and PGES. COX enzyme products including PGE_2 are known to induce COX-2 expression (22). To investigate the effect of COX-1 enzyme products on expression of COX-2 and the microsomal glutathione-dependent inducible PGES, COX-1 Tet-Off HeLa cells were grown in the presence or absence of the dual COX enzyme inhibitor indomethacin or the highly selective COX-2 inhibitor NS-398 for 24, 48, and 72 h. After DOX withdrawal from the culture medium, a time-dependent increase in COX-1 overexpression was observed with maximal sustained overexpression after 72 h (Fig. 4*A*). Concomitant with this increase in COX-1 expression was a 3.2 ± 8.9-fold increase in COX-2 expression after 72 h and a 2.5 ± 0.45- and 1.3 ± 0.78-fold increase in PGES after 24 and 48 h, respectively (Fig. 4*A*). After 72 h, PGES levels had returned to basal. Cotreatment of the HeLa cells, induced for 24, 48, and 72 h, respectively, with indomethacin or NS-398 showed no

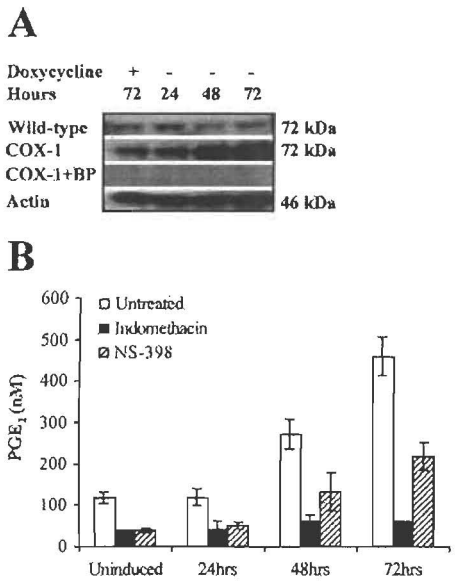

Fig. 3. *A*, Western blot analysis of 20 μg of total protein isolated from wild-type HeLa Tet-Off and HeLa COX-1 Tet-Off cells grown for 24, 48, and 72 h, respectively, in the absence of DOX. In parallel, control uninduced HeLa COX-1 Tet-Off and wild-type HeLa Tet-Off cells were maintained for 72 h under the same conditions supplemented daily with DOX to a final concentration of 1 μg/ml. The proteins were loaded onto a 4–20% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred to a PVDF membrane. The immunoblot was probed with antibody raised against the COOH terminus of human COX-1. A specific band of approximately 72 kDa was detected. No immunoreactivity was detected by preadsorbing the antibody with the blocking peptide (*BP*). COX-1 was normalized for protein loading against β-actin on the same blot. *B*, The functionality of the transfected COX-1 cDNA was assessed by ELISA, by measuring PGE_2 secretion into the culture medium after COX-1 induction in the presence or absence of the COX enzyme inhibitor indomethacin, and treatment of HeLa cells with 5 μg/ml arachidonic acid.

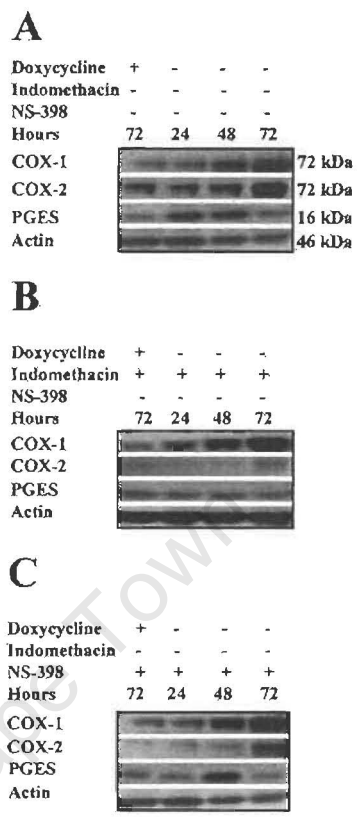

Fig. 4. Western blot analysis of 20 μg of total clarified cell lysate isolated from HeLa COX-1 Tet-Off cells grown for 72 h in the presence of DOX (uninduced) or 24, 48, and 72 h, respectively, in the absence of DOX to induce COX-1 expression. *A*, expression of COX-2 and PGES was induced coincident with COX-1 overexpression in HeLa cells. *B*, cotreatment of HeLa cells with indomethacin abolished the COX-1-mediated up-regulation of COX-2 and PGES. *C*, partial inhibition of the COX-1-mediated up-regulation of COX-2 and PGES expression was observed after cotreatment with the selective COX-2 inhibitor NS-398. Proteins were normalized for loading against β-actin on the same blot.

alteration in COX-1 overexpression (Fig. 4, *B* and *C*). However indomethacin treatment inhibited COX-2 as well as PGES induction (Fig. 4*B*). No significant change in COX-2 expression was observed after treatment of HeLa cells with NS-398 (Fig. 4*C*). Induction of PGES by COX-1 overexpression was delayed by 24 h after treatment of HeLa cells with NS-398 (Fig. 4*C*).

COX-1 Overexpression in HeLa Cells Induces PGE_2 Receptor Expression. The effect of COX-1 overexpression on the four subtypes of PGE_2 receptors, namely EP1–EP4, was investigated by real-time quantitative RT-PCR, after DOX withdrawal from the culture medium and subsequent induction of COX-1. Induced overexpression of COX-1 for 24, 48, and 72 h had no significant effect on EP1 receptor expression when compared with cells grown in the presence of indomethacin. COX-1 overexpression for 48 and 72 h significantly induced expression of EP2 receptor transcript when compared with indomethacin-treated cells (Fig. 5; $P < 0.01$). Levels of EP3 receptor transcript were significantly induced after 48 h of COX-1 overexpression ($P < 0.05$) compared with cells grown in the presence of indomethacin. EP4 receptor transcript was significantly up-regulated after COX-1 overexpression for 24, 48, and 72 h compared with cells cotreated with the COX enzyme inhibitor ($P < 0.05$).

cAMP Production in COX-1-overexpressing Cells in Response to PGE_2. The effect of COX-1-induced up-regulation of the cAMP-linked PGE_2 receptors on cAMP production was determined after overexpression of COX-1 and stimulation with exogenous PGE_2. No significant difference in basal cAMP production was detected in uninduced and induced cells (Fig. 6). Treatment of uninduced cells with 300 nM PGE_2 resulted in a 2.43 $\pm$ 1.07-fold increase in cAMP

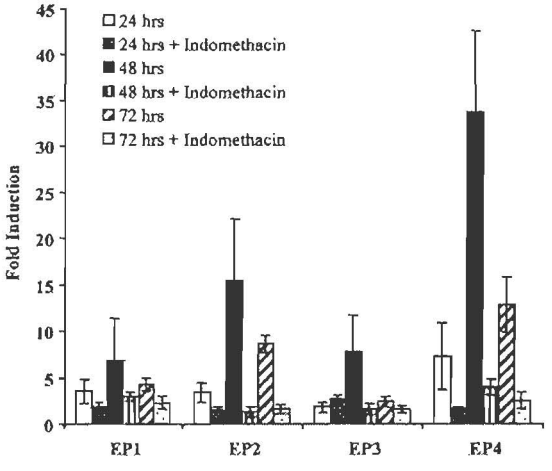

Fig. 5. Fold induction of expression of PGE_2 receptors (EP1–EP4) in HeLa COX-1 Tet-Off cells as determined by real-time quantitative RT-PCR. COX-1 expression was induced for 24, 48, and 72 h in the presence or absence of the COX enzyme inhibitor indomethacin. Fold induction was determined by dividing the relative expression in induced cells by the relative expression in uninduced cells.

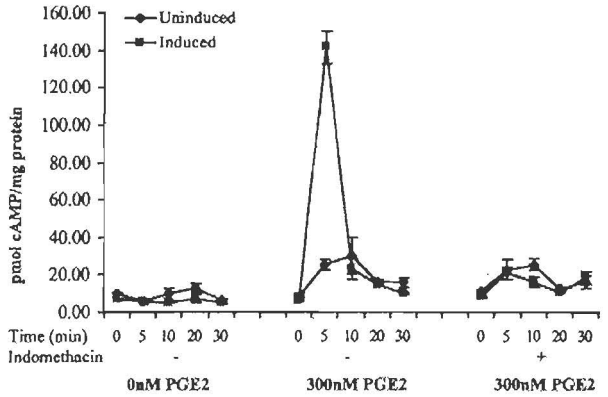

Fig. 6. cAMP levels in HeLa COX-1 Tet-Off after treatment with 0 or 300 nM PGE_2. Cells were either maintained with 1 μg/ml DOX (uninduced) or induced by incubation in culture medium without DOX for 48 h in the presence or absence of the COX enzyme inhibitor indomethacin.

production ($P < 0.05$). Cells in which COX-1 was induced for 48 h before stimulation with exogenous PGE_2 showed a rapid, transient 12.67 ± 3.7-fold cAMP response ($P < 0.01$). This rapid, elevated cAMP production in COX-1-overexpressing cells in response to PGE_2 was abolished when cells were grown in medium containing the COX enzyme inhibitor indomethacin. The activity of EP1 receptor was investigated by measuring inositol phosphate accumulation (41). No inositol phosphate accumulation above basal level was observed in COX-1 Tet-Off HeLa cells after induced expression of COX-1 and PGE_2 stimulation (data not shown).

Induction of Angiogenic Factors in Response to COX-1 Overexpression. The effect of COX-1 on expression of the angiogenic factors bFGF, VEGF, Ang-1, and Ang-2 was assessed by Western blot analysis. Overexpression of COX-1 for 72 h resulted in a 2.3 ± 0.45-fold increase in bFGF (Fig. 7*A*), a 4.5 ± 1.2-fold increase in VEGF (Fig. 7*B*), a 2.3 ± 0.78-fold increase in Ang-1 (Fig. 7*C*), and a 2.1 ± 0.98-fold increase in Ang-2 expression (Fig. 7*D*), respectively. Indomethacin treatment inhibited the COX-1-associated up-regulation of bFGF, VEGF, Ang-1, and Ang-2 (Fig. 7). Treatment of cells with NS-398 partially reduced the up-regulation of bFGF, VEGF, Ang-1, and Ang-2 expression (Fig. 7), suggesting that products from both COX enzymes were modulating expression of these factors.

DISCUSSION

Recent studies have demonstrated up-regulated and inducible expression of COX-1 in different biological models. COX-1 expression is up-regulated in human breast cancer (24), human prostate cancer (25), and murine models of lung tumorigenesis (23). In addition, COX-1 expression can be induced *in vitro* by tobacco carcinogen (42), VEGF (21), arachidonic acid, forskolin, dibutyryl-cAMP, and PGE_2 (22). In an *in vitro* model, COX-1 overexpression in endothelial cells implanted in mice was associated with enhanced tumorigenicity (5). This study confirms up-regulation of COX-1 expression in squamous cell carcinoma and adenocarcinoma of the human cervix as demonstrated by real-time quantitative RT-PCR, Western blot analysis, and immunohistochemistry. The up-regulation of expression of COX-1 was associated with enhanced expression of COX-2. Moreover, the site of COX-1 expression localized to the neoplastic epithelial cells of all squamous cell carcinomas and adenocarcinomas investigated, demonstrating a pattern of expression for COX-1 in cancer of the cervix that is similar to that demonstrated for COX-2 (16, 18) and PGE_2 (18). These data suggest that both COX-enzymes and/or their products may contribute toward the development of cervical cell neoplasias.

To investigate the effect of overexpression of COX-1, we have established a DOX-regulated expression system in HeLa cells. Initial studies performed on wild-type HeLa Tet-Off cells showed no elevation of COX-1 expression above basal levels when wild-type cells were grown for 72 h in the presence or absence of DOX. These data demonstrate that neither cell growth nor DOX affected the basal expression of COX-1. Overexpression of COX-1 in HeLa cells up-regulates expression of COX-2 and PGES concomitant with increased PGE_2 production. These data suggest that COX-2 and inducible PGES are co-regulated. In an *in vitro* model system, administration of interleukin 1β to A549 cells rapidly induced the expression of COX-2 and PGES (43). Similarly, inducible PGES activity has been described in lipopolysaccharide-stimulated rat peritoneal macrophages, coincident with COX-2 expression and PGE_2 biosynthesis (44, 45). Indomethacin, but not NS-398, treatment abolished the up-regulation of expression of COX-2 and PGES and synthesis of PGE_2. Up-regulation

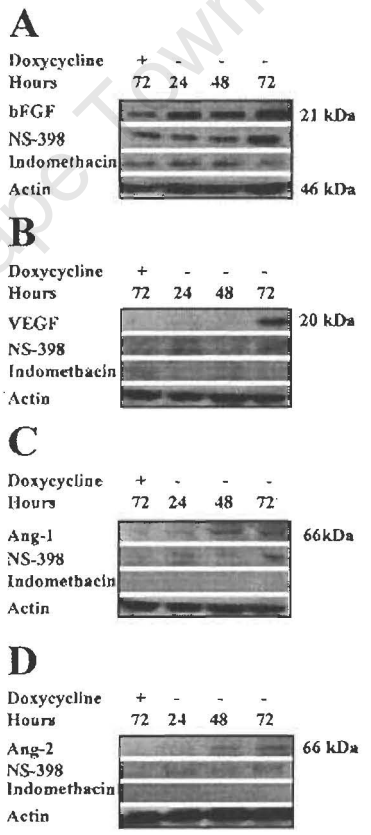

Fig. 7. Western blot analysis of 20 μg of total clarified cell lysate isolated from HeLa COX-1 Tet-Off cells grown for 72 h in the presence of DOX or for 24, 48, and 72 h in the absence of DOX to induce COX-1 expression. *A*, immunoblot of bFGF expression after DOX withdrawal from the culture medium. bFGF expression was induced coincident with COX-1 overexpression. Up-regulated bFGF expression was abolished by indomethacin and partially inhibited by NS-398. *B*, immunoblot of VEGF expression after DOX withdrawal from the culture medium. VEGF was induced after 72 h of COX-1 overexpression. Up-regulated VEGF expression was abolished by indomethacin and partially inhibited by NS-398. *C*, immunoblot of Ang-1 expression after DOX withdrawal from the culture medium. Ang-1 was induced coincident with COX-1 overexpression after 48 h. Up-regulated Ang-1 expression was abolished by indomethacin and partially inhibited by NS-398. *D*, immunoblot of Ang-2 expression after DOX withdrawal from the culture medium. Ang-1 was induced after 48 h of COX-1 overexpression. Up-regulated Ang-2 expression was abolished by indomethacin and partially inhibited by NS-398. Proteins were normalized for loading against β-actin.

of COX-2 and PGES in HeLa cells may thus be mediated by prostanoids produced following overexpression of COX-1. NS-398 treatment significantly reduced PGE_2 synthesis at 72 h but not 48 h. This is not surprising, because COX-2 expression in HeLa cells was only maximally induced at 72 h. This suggests that PGE_2 production detected at 72 h after COX-1 overexpression is enhanced by the activity of both COX enzymes. In other model systems, COX-2 expression is up-regulated by PGE_2 via the cAMP-dependent PGE_2 receptors (22). *In vitro* studies have shown that cAMP activity accompanies a concomitant increase in COX-2 synthesis, suggesting that cAMP is the primary secondary messenger in regulating COX-2, presumably via the upstream cAMP response element located on the *COX-2* gene (46). The biological actions of PGE_2 have been attributed to its interaction with G-protein-coupled receptors, of which four subtypes (EP1–EP4) have been identified (35). COX-1 overexpression in HeLa cells resulted in significant up-regulation of the cAMP-dependent PGE_2 receptors after 48 h of COX-1 overexpression. This up-regulation was inhibited by growing cells in medium containing indomethacin, suggesting that the up-regulation was mediated by COX enzyme products. Previous studies have demonstrated enhanced PGE_2 synthesis in cervical carcinomas together with up-regulated expression of EP2 and EP4 receptors and enhanced cAMP-responsiveness of cervical tumor tissue to PGE_2 (18). Because COX-1 overexpression in HeLa cells induces COX-2 and EP receptor expression, it is feasible that PGE_2 may facilitate the process of cervical tumorigenesis in an autocrine/paracrine manner after enhanced EP receptor expression and ligand-receptor interaction. A direct role for EP receptors in tumorigenesis has been reported recently in colon cancer cells. In this model, enhanced proliferative and tumorigenic effects were mediated by PGE_2 after interaction with the EP4 receptor (47). It is likely that similar mechanisms may exist in cervical carcinomas to enhance growth and proliferation via EP receptors in a cAMP-dependent manner. Because both COX enzymes catalyze the same reaction, enzyme products such as PGE_2 from both COX enzymes may regulate EP receptor expression. The choice of COX enzyme for biosynthesis of prostaglandins may depend on the relative expression of each COX isoform in the cell because, in many cells, COX-2 levels are typically only 20–30% of COX-1 levels (46).

Functionality of the induced EP receptors in our model system was assessed by measuring cAMP in response to stimulation with exogenous PGE_2. cAMP activity was measured in HeLa cells after overexpression of COX-1 for 48 h and stimulation with exogenous PGE_2. A significant fold increase in cAMP production was observed after 5 min of PGE_2 stimulation in COX-1-induced compared with uninduced cells. This augmented cAMP response was abolished by growing cells in medium containing indomethacin. These data suggest that PGE_2 produced by COX-1 overexpression may be acting in an autocrine/paracrine manner via the cAMP-linked PGE_2 receptors to mediate its effect on target genes, such as COX-2, via the cAMP-dependent protein kinase pathway by activating adenylate cyclase and increasing cAMP. Because COX-1 overexpression had no significant effect on EP1 expression, and stimulation of HeLa cells with PGE_2 resulted in no increase in inositol phosphate accumulation above basal levels, this suggested that although PGE_2 may be functioning via EP1 receptors coupled to inositol phosphate production and release of intracellular calcium in these cells, its contribution to events associated with COX-1 up-regulation was minimal.

Cancer cells produce a wide variety of factors that contribute to angiogenesis, including bFGF, VEGF, bFGF-binding protein, and platelet-derived growth factor (34). Our data demonstrate that COX-1 overexpression in HeLa cells results in the up-regulation of expression of proangiogenic factors. Induced overexpression of COX-1 resulted in an increase in bFGF, VEGF, Ang-1, and Ang-2 expression. Cotreatment of these cells with indomethacin abolished the up-regulation of these angiogenic factors. This suggests that the up-regulation of these factors is mediated by prostanoids produced by COX-1 overexpression. Moreover, because the effects of COX-1 overexpression can be reversed by COX inhibition with indomethacin, this confirms that these effects are not an artifact of forced overproduction of the enzyme. Partial reduction in expression of these factors by treatment with NS-398 suggests that both enzymes (COX-1 and COX-2) converge to regulate expression of target genes, possibly through common prostanoid synthetic pathways. In another model system, COX-2 overexpression and increase in PGE_2 synthesis in colon carcinoma cells results in the up-regulation of bFGF and VEGF and this is associated with arrangement of endothelial cells into tubular structures (34). The up-regulation of angiogenic factors by COX enzymes is important in regulating angiogenesis and maintenance of the neoplastic tissue. As the demand for nutrients and oxygen increases for tissue development, an increased vascularization is necessary to supply nutrients to the tumor (48). In this study, we also observe the regulation of the angiogenic factors Ang-1 and Ang-2 by COX enzymes. Ang-1 is a Tie-2 receptor agonist, which is required for recruitment of perivascular cells leading to the formation and stabilization of capillaries, vessel maturation, and endothelial cell survival (49, 50). Ang-1 and other angiogenic factors such as VEGF may act synergistically to increase vascular sprouting and branching (51, 52). In addition, Ang-1/Tie-2 interaction enhances the mitogenic effect of VEGF on endothelial cell growth (53). By contrast, Ang-2 is a natural Tie-2 receptor antagonist, destabilizing cell contacts and thus allowing access to angiogenic factors such as VEGF (54). In our model system, enhanced synthesis of prostanoids as a consequence of up-regulated COX-1 may thus act in an autocrine/paracrine manner to up-regulate the expression of COX-2 and target receptors as well as the intracellular signaling to a host of angiogenic factors, which could act on endothelial cells and lead to the recruitment of new blood vessels to enhance tumor mass.

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