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**CYCLOOXYGENASE-2 IN CERVICAL NEOPLASIA
AND THE RELATIONSHIP WITH SPECIFIC
HUMAN PAPILLOMAVIRUS TYPES**

BY

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**Dissertation submitted in fulfilment of Part III of
the requirements for the degree of Masters of**

Medicine (Anatomical Pathology), University of Cape Town.

August 2000.

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To my family, Helene and Matthew
For their support, encouragement and patience.

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DECLARATION

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ACKNOWLEDGEMENTS

I wish to express my sincere thanks and appreciation to the following persons.

Professor Pauline de la M Hall, my supervisor, for her ideas and guidance, and for her help in editing this work.

Professor J. P. Cruse for the opportunity to undertake this study within his department.

Ms Nafiesa Allie for working very hard and expertly to produce all the immunohistochemical stains.

Mrs Patti Kay for her meticulous work in doing all the PCR investigations for the HPV typing.

Dr. Henry N. Jabbour from the Medical Research Council Human Reproductive Sciences Unit, Centre for Reproductive Biology, Edinburgh, for his donation of the COX-2 antibody, without which this study would not have been possible.

ABBREVIATIONS

HPV	Human papillomavirus
CIN	Cervical intraepithelial lesion
HIV	Human immunodeficiency virus
SIL	Squamous intraepithelial lesion
DNA	Deoxyribo nucleic acid
ELISA	Enzyme linked immunosorbant assay
COPV	Canine oral papillomavirus
COX	Cyclooxygenase
PG	Prostaglandins
AA	Arachidonic acid
NSAIDs	Non-steroidal anti-inflammatory drugs
ER	Endoplasmic reticulum
TNF- α	Tumour necrosis factor- α
ADP	Adenosine diphosphate
GIT	Gastrointestinal tract
CNS	Central nervous system
IL-1	Interleuken-1
RA	Rheumatoid arthritis
OA	Osteoarthritis
FAP	Familial adenomatosis polyposis
APC	Adenomatous polyposis coli
HNPCC	Hereditary non-polyposis coli carcinoma
EGFR	Epidermal growth factor receptor
PCR	Polymerase chain reaction
APES	3- Aminopropyltriethoxysilane
PBS	Phosphate buffered saline
RFLP	Restriction Fragment Length Polymorphism

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ABSTRACT

Introduction

The rate-limiting enzyme in arachidonate metabolism is mediated by enzymes known as cyclooxygenases (COX's). These catalyse the biosynthesis of prostaglandin H₂, the precursor of molecules such as prostaglandins, prostacyclin and thromboxanes. The COX enzyme family consists of COX-1, constitutively expressed in many tissues, and COX-2, which is induced by stimuli such as mitogens and cytokines and is involved in many inflammatory reactions. Recent studies have shown increased COX-2 expression in various malignancies, including colorectal carcinoma, gastric carcinoma, oesophageal carcinoma, lung carcinoma and squamous carcinomas of the head and neck.

Aim

1. To investigate the expression of Cyclooxygenase-2 (COX-2) in the normal cervix.
2. To investigate the expression of COX-2 in the diseased cervix. Specifically to evaluate expression in preneoplastic disease (cervical intraepithelial neoplasia) and invasive carcinoma.
3. To evaluate any association between HPV types and COX-2 expression within a spectrum of cervical disease.

Methods

59 cases were selected, all previously having undergone PCR for HPV typing. These included:

Eighteen "normal", four CIN 1, nine CIN 2, sixteen CIN3 and twelve squamous carcinomas.

Immunohistochemical analysis for COX-2 expression was performed using a polyclonal anti-COX-2 antibody (sc-1745; autogen bioclear). Both the intensity and extent of COX-2 expression were scored.

Results and Conclusions

1. No COX-2 expression was found in the “normal” ectocervix.
2. Minimal COX-2 expression was found in the “normal” endocervix and in mononuclear inflammatory cells within the cervical stroma.
3. All cases of dysplasia and carcinoma showed increased expression of COX-2.
4. The increased expression was incremental from CIN-1 through to invasive carcinoma, which had the highest expression.
5. COX-2 expression in dysplasia and carcinoma was highest when associated with infection by HPV 16 + 33 together compared to HPV 16 or HPV 33 alone.
6. There may be a role for COX-2 inhibitors in the prevention and treatment of cervical dysplasia and carcinoma.

CHAPTER ONE**CERVICAL NEOPLASIA**

1.1 DYSPLASIA AND CARCINOMA OF THE UTERINE CERVIX

Carcinoma of the uterine cervix is one of the leading causes of cancer-related death in women world-wide and the leading cause of cancer-related death in women in South Africa [1]. The crude annual incidence rate of cervical cancer in 1990 – 1991 was 26/100 000 overall and 28/100 000 in black women [2]. Given the limited resources for an effective cervical screening program in South Africa, and the impact of Human Immunodeficiency Virus (HIV), the incidence of cervical dysplasia and carcinoma can be expected to increase steadily.

1.1.1 Nomenclature

The histological classification of tumours of the uterine cervix has recently been revised by the International Society of Gynaecological Pathologists under the auspices of the World Health Organization (WHO). In the WHO histologic classification, preinvasive disease is referred to as cervical intraepithelial neoplasia (CIN) and is graded from 1-3, which corresponds to mild, moderate and severe dysplasia [3]. In addition a new cervical cytologic nomenclature to replace the previous Papanicolaou designation has been developed, in accordance with the recommendations of a workshop convened by the National Cancer Institute in Bethesda, Maryland [4]. The generic term used in the Bethesda classification is squamous intraepithelial lesion (SIL). SIL encompasses the entire morphological spectrum of precursors to invasive squamous cell carcinoma, previously called “dysplasia”, “carcinoma-in-situ”, “borderline lesion”, and “cervical intraepithelial neoplasia”. In addition, the Bethesda

System avoids any potential misunderstandings as to the definitions of dysplasia, carcinoma-in-situ or neoplasia. SIL is subdivided into low-grade SIL, which corresponds to CIN 1 and HPV related lesions not qualifying as CIN, and high-grade SIL, which corresponds to CIN 2 and CIN 3. The Bethesda system recognizes that it is inherently difficult to distinguish flat condylomata from CIN 1 and, given the fact that most of these lesions will not progress, they have therefore been grouped together as low-grade SIL [5].

The most prevalent invasive carcinoma involving the uterine cervix is squamous cell carcinoma. The WHO classification has incorporated a number of squamous carcinoma subtypes including: keratinizing, nonkeratinizing, verrucous, warty (condylomatous), papillary (transitional), and lymphoepithelioma-like. Adenocarcinoma and its subtypes form a separate category and then there is a group of miscellaneous epithelial tumours grouped together which include: adenosquamous carcinoma, glassy cell carcinoma, adenoid cystic carcinoma, adenoid basal carcinoma, carcinoid tumour, small cell carcinoma and undifferentiated carcinoma [3].

1.2 HUMAN PAPILLOMA VIRUS

Human papillomavirus (HPV) has been linked to various types of cervical disease ranging from the relatively innocuous condyloma acuminatum to the sometimes-fatal invasive squamous cell carcinoma [6,7] and most likely explains the well-known association between sexual history (early age of first sexual contact and increased number of sexual partners) and increased risk of cervical cancer [7,8]. A recent study, involving 22 countries and over 1000 specimens, has confirmed that HPV is the main aetiological factor in cervical cancer worldwide [9].

In 1949 Ayre [10] was the first to describe the HPV altered squamous cell subsequently known as a koilocyte. He originally named these cells “precancer cell complex” and speculated that they may be precursors to cancer. Papanicolaou described these cells as “dyskaryotic” in his *Atlas of Exfoliative Cytology* and the term “koilocytosis” was introduced, by Koss and Durfee [11] in 1956 after the Greek word koilos (hollow). This was because of the cytological features characterized by the prominent, sharply defined cytoplasmic cavities of these cells. It was two decades later that two independent groups of investigators, Meisels et al [12] and Syrjanen et al [13], made the connection between koilocytosis and HPV. HPV was initially thought to be involved in the pathogenesis of only condylomata acuminata although similar koilocytic changes were observed in cervical smears from women with cervical dysplasia and invasive carcinomas. The inability to prove a direct link between HPV and human neoplasia was due to the fact that HPV could not be propagated in tissue culture and a suitable animal model could not be found initially. It was only after recent advances in electron microscopy (Figure 1.1), molecular biology and immunohistochemistry that the role of HPV in cervical neoplasia could be appreciated [14,15].

1.2.1 HPV Types

More than 80 different distinct types of HPV have been identified to date, and partial sequences have been obtained from more than 30 putative novel genotypes [14-16]. All HPV's infect the squamous epithelia of skin or mucous membranes, but different HPV genotypes show a specific tropism for distinct cell types. It is according to this specific tropism and DNA sequence homology that HPV can be divided into three categories: genital mucosal or anogenital genotypes, nongenital or cutaneous genotypes, and HPV genotypes

associated with epidermodysplasia verruciformis, a rare genetic cutaneous disorder characterized by widespread chronic nongenital HPV lesions [15,16] (Table 1.1).

Furthermore the anogenital HPV's can be further divided into three groups based on their ability to induce viral associated tumours and their oncogenic potential. The high risk HPV's are associated with intraepithelial neoplasia that may progress to invasive anogenital neoplasia (HPV genotypes 16 and 18 most frequently detected), and the low risk HPV's are seen frequently in benign epithelial tumours such as condylomata acuminata and laryngeal papillomas (HPV genotypes 6 and 11 most frequently detected) [15,17]. Studies have lead to the suggestion that the high-risk group (HPV genotypes 16 and 18) be further subdivided to allow HPV 18 to be categorised separately. The rationale for this is that HPV 16 is found equally in CIN 2, CIN 3 and invasive carcinoma, whereas HPV 18 is detected much more frequently in invasive carcinoma than CIN [17].

Table 1.1 Classification of HPV Genotypes

<p><u>Genital - mucosal (anogenital) HPV Genotypes:</u></p> <p>High-risk HPV geotypes: HPV 16, 18, 31, 45</p> <p>Low-risk HPV genotypes: HPV 6, 11, 42-44, 53-55</p> <p>Moderate-risk HPV genotypes: HPV 33, 35, 39, 41, 52, 56, 58, 59, 68</p> <p><u>Nongenital (cutaneous) HPV genotypes:</u> HPV 1-4, 7, 10, 26-29, 37, 38, 41, 48, 49, 65</p> <p><u>HPV genotypes associated with epidermodysplasia verruciformis:</u> HPV 5, 8, 9, 12, 14, 15, 17, 19-25, 36, 47, 50</p>

A number of studies have reported the high incidence of multiple HPV infection with the incidence ranging from 11.6% [18] to 39.4% [19]. Mixed infections involving three or four HPV types are less frequent but have been noted. The prevalence of the mixed infections seems to vary with the severity of the lesion, with one study showing 21% of condylomata and 50% of CIN 3's having dual infections [18]. Other studies have found that a significantly higher proportion of double infections were detected in women with normal or inflamed cervixes than those with CIN or invasive carcinoma [19,20]. The most common combinations, in cases of dual infection, are HPV types 11 and 16, 16 and 33, and 16 and 58 together. A number of hypotheses have been suggested to explain the apparent low risk of disease progression in dual infection. One such hypothesis suggests the possibility that infection of individual cells, by a HPV type with low transforming potential, precludes infection by a second virus type with greater transforming potential [19]. Another hypothesis suggests that for a cervical cell infected with multiple HPV types to progress to malignancy, E2 inactivation events will have to occur in each of the existing types to avoid the E2 – encoded protein of one type suppressing E6/E7 proteins of the other HPV type [18,21].

70% of all cervical squamous carcinomas are associated with HPV types 16 or 18, and the remaining 30% are made up by subtypes 31, 33, 35, 51, 52 and 58 [22,23]. In addition, HPV 16 is more commonly associated with keratinizing squamous carcinoma compared with nonkeratinizing squamous carcinoma [24], and HPV 18 is more commonly associated with poorly differentiated carcinomas and lymph node metastases [25].

1.2.2 HPV Structure

The papillomaviruses were originally grouped together with the polyomaviruses in a single taxonomic group, the Papovaviridae. They were later split into separate groups on the basis of

genomic organization, virion size, and on different molecular patterns of replication [26,27]. HPV are small, nonenveloped DNA viruses, which are approximately 55nm in diameter and contain double-stranded circular DNA, which is covalently closed. This DNA is 7800 – 7900 base pairs in length and packaged in a protein coat [15,28]. The DNA sequences coding all of the putative proteins are termed *open reading frames* and are all located on one DNA strand (Fig. 7). The non-coding regions, termed *upstream regulatory regions*, are located on the same DNA strand and promote mRNA synthesis, regulate gene expression and DNA replication [15,27].

The *open reading frames* are referred to as early (E) and late (L). The early region codes for several proteins (E1 – E8) which are involved in viral replication and transforming properties. The late region is expressed in the final phase of infection and encodes structural proteins involved in the assembly of viral particles [15,27,28].

The E2 region is thought to regulate gene expression, independently of E6, via activation of the tumour suppressor gene, p53 [26]. Proteins E6 and E7 stimulate cell proliferation by activating cyclins E and A, and interfere with the functions of tumour suppressor proteins p53 and the retinoblastoma gene (Rb) respectively. The binding of E6/E7 to p53/Rb respectively is linked to the oncogenicity of the HPV type. For example HPV 16 and 18 show stronger binding compared to HPV types 6 and 11 [29].

The two “late” reading frames extend over almost half of the HPV genome and encode the two viral capsid proteins (L1 and L2). L1, a major capsid protein, is highly conserved amongst HPV genotypes whereas the L2 capsid protein, a minor protein, is highly variable among different HPV genotypes [15,30]. In addition, the L2 protein appears to be important in virus–host cell interactions [27].

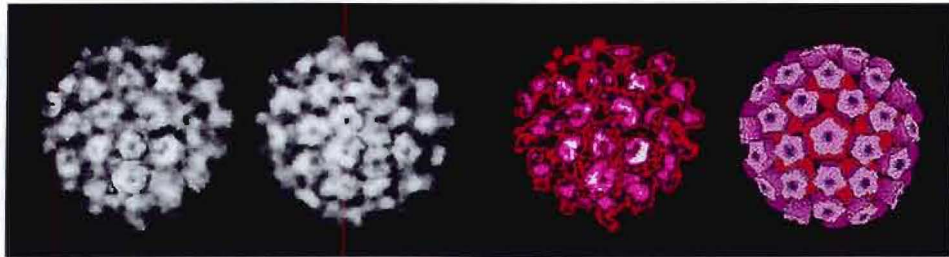


Figure 1.1 – Electronmicroscopic image of HPV with adjacent computer colourised image . Note the geometrical configuration of the outer capsid (L1), which is composed of 72 morphological units. The capsomers located at each of the 12 vertices, are pentavalent (i.e. each is surrounded by five adjacent capsomers), and the other 60 are hexavalent (each adjacent to six capsomers). (see 1.2, page 14).

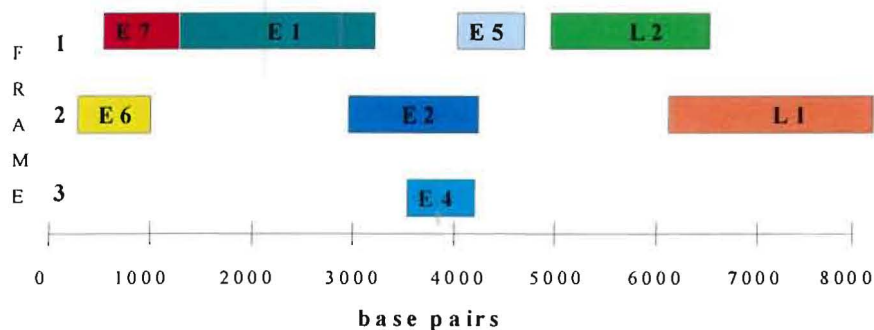


Figure 1.2 – Linear illustration of the HPV 16 genome. This is a circular arrangement of a single strand of DNA containing open reading frames “early”(E), and “late” (L) which are arranged in a specific sequence. Adapted from Vousden KH et al [29]. (see 1.2.2, page 16,17)

HPV typing is based on differences demonstrated by DNA hybridization, in particular differences in the E6/E7 and L1 regions. A new HPV type is, by definition, less than 90% homologous with previously identified types with respect to these regions, as determined following complete viral cloning. A subtype shares 90 – 98 % homology in these regions and a variant more than 98 % homology [26,27].

1.2.3 HPV Life Cycle

HPV infection of the cervix is a sexually transmitted disease and the virus life cycle is tightly linked to squamous differentiation. HPV must gain access to the basal epithelial cells, particularly those of the metaplastic squamous epithelium, for infection to occur. The alpha-6 integrin protein has recently been suggested as a cellular receptor for HPV [31]. The virus may remain dormant, that is in the latent phase, for a variable period from a few weeks to over 18 months, and in most individuals remains subclinical and does not produce clinically apparent lesions [27,31]. The viral DNA remains as a separate extrachromosomal piece of circular DNA, which is termed an “episome”. These episomes may replicate within the infected cells without producing cytological atypia [30]. The transition from latent to productive infection is initiated by the differentiation of cells as they undergo maturation towards the surface. HPV types 16 and 18 have been shown to have a significantly increased risk of disease progression [32]. In addition, persistent viral infection with an associated high viral load, has also been associated with persistent cervical dysplasia [33]. At a critical point of epithelial differentiation, transcription of the late structural reading frames L1 and L2 predominate resulting in viral capsid production and the formation of complete viral particles. Subsequently, the viral DNA becomes integrated into the host cellular genome, which is the hallmark of malignant progression [27,28,34]. The CIN 3 (high-grade dysplasia) and

malignant lesions characteristically show evidence of loss of L gene expression but increased E gene expression. The koilocytic appearance of infected cells is related to viral E4 protein expression, which disrupts the intracellular cytokeratin matrix facilitating the release of viral particles [3]. Disruption of the E1 and E2 genes during viral integration allows unregulated expression of the E6 and E7 viral proteins, which inactivate p53 and Rb, leading to loss of cell cycle control and cellular transformation [30,34]. Viral DNA integration is rarely seen in CIN 1 (low grade cervical dysplasia), but is seen in the majority of CIN 3 (high-grade dysplasia) and invasive squamous cell carcinomas [35]. It is widely accepted that HPV infection alone is not responsible for cervical neoplasia and several cofactors for disease progression, including smoking [36], oral contraceptive use [37] and immunosuppression [38], have been implicated. It is more than likely that other cofactors, yet to be identified, do exist.

1.2.4 HPV and the Immune Response

Immunosuppressed patients are more likely to develop multiple and intractable condylomata accuminata (genital warts) and are at an increased risk of developing anogenital dysplasia and neoplasia [27]. HIV-infected individuals have higher incidences of HPV infection (as defined by the presence of HPV DNA), CIN, and anogenital condylomata [39]. Although no significant increase in the incidence of cervical cancer has been noted in this population yet, life expectancy for HIV-infected patients has increased due to therapeutic advances, and cervical cancer in this group may yet become a significant health care problem. In addition anal cancer is found predominantly in gay men with or without concomitant HIV infection [40,41]. Renal transplant patients and other immunosuppressed individuals are also at higher

risk for developing large, disabling condylomata acuminata and premalignant anogenital lesions due to HPV [42,43].

It has been established that cell mediated immune responses are crucial to the pathogenesis of HPV infection [44]. Regression of genital warts is characterized by a delayed type hypersensitivity reaction with prominent CD4 T lymphocytes and macrophages present [45]. In addition, various cytokines, which are present in high concentrations, such as IL-12, may have a direct antiviral effect [46]. A recent study showed that cytotoxic T lymphocyte (CTL) responses to E6/E7 are more commonly detectable in women with HPV 16 infection not associated with CIN, than in HPV 16 positive women with CIN. This suggests a possible protective role for the CTL response [47]. The role of humoral immunity in HPV is unclear. Enzyme linked immunosorbant assay (ELISA) tests using HPV 16 L1/L2 genetically engineered virus-like particles, have shown that recent or current exposure generates a good initial antibody response which then declines with time [48]. Seroconversion occurs at an average of eight months after cervical HPV DNA detection and the antibody persists for at least forty months after cervical samples become HPV negative. Not all HPV infections result in seroconversion with between 50% and 94% of HPV 16 infected women developing antibodies. Those failing to seroconvert may have less persistent infection [49]. In addition, HPV 16 seropositivity is associated with a 3 to 6 times increased risk of developing CIN [50].

1.2.5 HPV Vaccine

HPV causes significant disease but treatment options are limited at present. The development of a suitable vaccine to prevent HPV-related disease is therefore essential. This is particularly relevant to developing countries, like South Africa, where there is a high prevalence of HPV-related cervical dysplasia and carcinoma, and where treatment resources are limited. The two

main objectives for a HPV vaccination programme are – firstly, to prevent initial infection, and, secondly, to eliminate an already established infection or the infection associated with carcinomas.

One difficulty in establishing a vaccine is which HPV types to include [51]. For example, HPV 6 and –11 constitute approximately 90% of genital condylomata acuminata, while many other types constitute the rest [52]. HPV 16 and –18 are found in 70% to 80% of cervical cancers; many other types are associated with the remainder [9].

In addition there are many antigenic targets for immunologic-based therapeutic agents. The replication proteins, E1 and E2, are required to establish genital infection whereas E4 and E5 RNA transcripts, although still of uncertain function, are the most abundant making them attractive targets for investigation. E6 and E7 oncoproteins are expressed during the late stages of disease, which also makes them possible therapeutic targets [51,53]. L1 and L2 proteins are not always expressed late in disease limiting their usefulness as target proteins [53].

Although currently no suitable animal model is available for testing potential HPV vaccines, animal papillomaviruses can be used to assess critical requirements for human vaccine development [54]. Some animal studies have lent support for the use of E6 and E7 proteins as immunological targets for new therapeutic agents [54,55]. Studies using the newly discovered canine oral papillomavirus (COPV), which causes disease on a mucosal surface and hence may be a better model for human disease, have shown that only native empty capsids of COPV are protective; denatured capsids afforded no protection [56]. Studies using a rhesus monkey papillomavirus, similar to the HPV types 16 and 18, have been encouraging but limited due to the expense of working with monkeys. Nevertheless, the rhesus monkey model could serve to test potential human vaccines [57].

Phase 1 and 2 trials of HPV vaccines or immunologic-based therapeutics are planned in humans [58]. Genital wart vaccines are being tested as the disease has a high prevalence and a reasonable time period to show efficacy. One method has targeted HPV-6 and -11 infections with either a prophylactic approach (using empty capsids) or a therapeutic approach using E7 proteins as immunogens [59].

Cumulative research has shown the major capsid protein, L1, to be the most likely antigen of choice. However, which HPV types are to be included and when to vaccinate are issues which still have to be resolved. It is essential that human trials are initiated as soon as possible for both the treatment and prophylaxis of diseases caused by this ubiquitous group of viruses.

CHAPTER TWO THE CYCLOOXYGENASE ENZYMES

Cyclooxygenase (COX) or prostaglandin H₂ synthase is the enzyme that catalyses the first two steps in the biosynthesis of the prostaglandins (PGs) from the substrate arachidonic acid (AA). These are the oxidation of AA to the hydroperoxy endoperoxide PGG₂ and its subsequent reduction to the hydroxy endoperoxide PGH₂. PGH₂ is transformed by a range of enzymes and nonenzymatic mechanisms into the primary prostanoids: PGE₂, PGF_{2α}, PGD₂, PGI₂ and TXA₂ [60,61] (Figure 2.1).

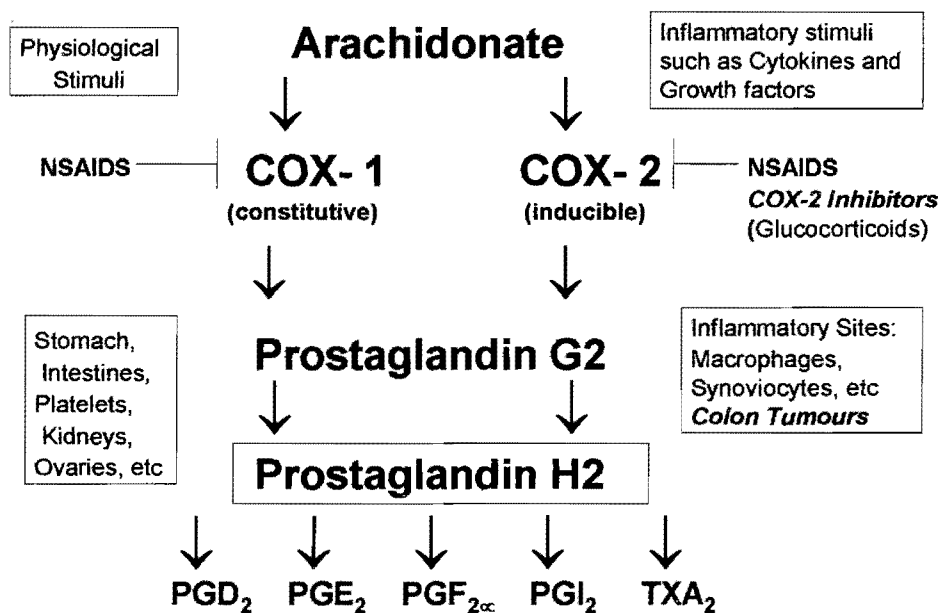


Figure 2.1 Synthesis of prostaglandins from arachidonate by COX-1 and COX-2. Modified from Taketo MM [61].

Since its discovery about a century ago, aspirin, and other nonsteroidal anti-inflammatory agents have revolutionised the treatment of pain and inflammation associated with arthritis [62]. However, it was not until 1971, that the principle mechanism of action of these agents was discovered when Vane et al. [61] and Smith et al [63] reported that aspirin and other NSAIDs inhibited the COX enzyme. It was also more than 20 years ago that prostaglandins were reported in high concentrations in human and animal anal tumours [64]. Since these initial reports there has been intense investigation into the COX enzymes in an attempt to characterise them and hence to produce more specific treatment agents for inflammatory diseases i.e. arthritis. Further studies, to be discussed later, have also revealed the association between COX enzymes and certain malignancies.

2.1 STRUCTURE

PGs are short-lived substances that act as local hormones and are important in normal physiology and certain pathological conditions [65]. Amongst other effects, PGs maintain normal gastric mucosa, influence kidney function and, via thromboxane-A₂ (TXA₂), aid blood clotting by promoting platelet aggregation [61,62,64]. In addition PGs contribute to the signs and symptoms of inflammation (PGE₂ being the major eicosanoid detected in inflammatory conditions) [62]. Production of PG is initiated by phospholipase A₂, which liberates arachidonic acid, the substrate for COX. Until the 1980's, the availability of arachidonate was thought to be the rate-limiting step in PG synthesis. However, the cloning of COX by three separate groups in 1998, and the identification of a second form of COX in 1991, revealed the regulatory role of these enzymes in PG synthesis [61,62,64,66].

The genes for COX-1 and -2 are located on chromosomes 9 and 1 respectively [61,64,67]. Fluorescence in situ hybridisation revealed the exact locations to be 9q32-q33.3 for COX-1

and 1q25.2-1q25.3 for COX-2 [64]. The intron/exon arrangements are identical except that exons 1 and 2 of COX-1 are condensed into a single exon in COX-2. The net result is that the COX-2 gene introns are smaller at 8kb whereas the COX-1 introns are 22kb [67,68]. Both isoforms have a molecular weight of 71K and are almost identical in length with just over 600 amino acids, of which 63% are in identical sequence. The gene products also differ with the mRNA of COX-1 being 2.8kb and that of COX-2 being 4.5kb [61].

The three-dimensional x-ray crystalline structure of COX-2 (Figure 2.2) can be superimposed on that of COX-1. The residues that form the substrate binding channel, the catalytic sites, and the residues immediately adjacent are all identical except for two small variations at positions 434 and 523 (Ile exchanged for Val in COX-2). It is these two differences, which account for the biochemical differences between the two iso-enzymes [61,62,64].

Another striking structural difference between the two, but of unknown significance, is the absence of a sequence of 18 amino acids at the C terminus of COX-2 in comparison to COX-1. This insert does not alter the last four amino acid residues, which in both form the signal for attachment to the membrane of the endoplasmic reticulum (ER) [69]. However, COX-2 is located on the nuclear as well as the ER membrane, while COX-1 is found attached only to the ER membrane [67,70]. Both enzymes are integral membrane proteins, but instead of having a transmembrane domain they are monotopic i.e. they sit within one leaflet of the lipid bilayer via 3 amphipathic α -helices. The COX active site is located in a channel formed in the centre of the membrane associated α -helices, allowing the fatty acid substrate access without leaving the membrane. This channel containing the active site of COX-2 seems to be more flexible, allowing acceptance of a broader range of substrate than COX-1 [67]. These include a range of fatty acids including γ - and α - linoleic acid. In addition specific inhibitors will differentiate between COX-1 and COX-2 with over 1000-fold selectivity [71].

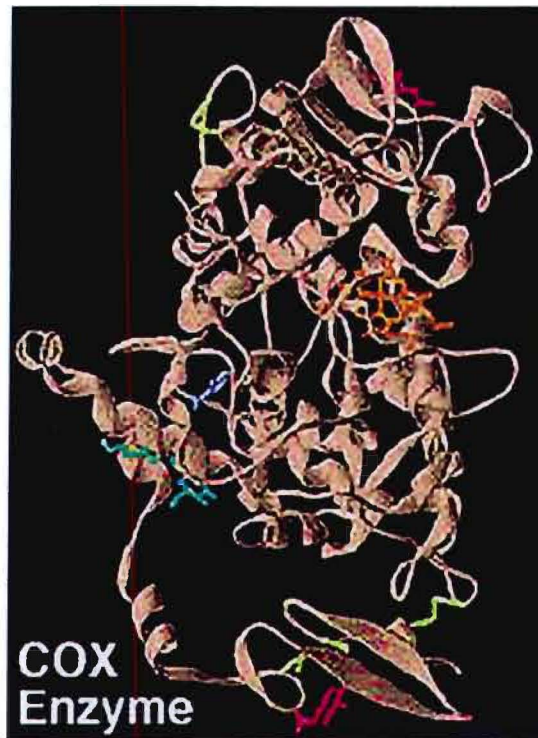


Figure 2.2 Ribbon structure of the basic COX enzyme. Both COX-1 and COX-2 have identical substrate binding channels, catalytic sites and adjacent residues. They differ only at positions 434 and 523 (Ile exchanged for Val in COX-2).

2.2 NORMAL FUNCTION OF COX-1 & -2

Instead of classifying PG biosynthesis into physiological and pathological, it may be better to use the classification applied to COX isoforms: either constitutive (COX-1) or induced (COX-2). COX-1 is constitutively expressed in nearly all cell types at a constant level [61,62,64,67,72,73,74]. The physiological roles of COX-1 have been deduced by studying the deleterious side effects of NSAIDs. While inhibiting PG synthesis at inflammatory sites (COX-2), NSAIDs also inhibit constitutive biosynthesis (COX-1). COX-2 is induced by most of the stimuli associated with inflammation including bacterial lipopolysaccharide, cytokines such as interleukin-1 and -2 (IL-1, IL-2), and tumour necrosis factor- α (TNF- α) [75]. The COX-2 gene contains regions that allow for rapid upregulation in response to these stimuli. In addition the COX-2 mRNA has labile regions which result in rapid degradation and hence allow for rapid turnover and diminished gene expression in the absence of continued stimulation [75,76]. This regulation of COX-2 is seen in macrophages, synoviocytes, endothelial cells and chondrocytes [76].

2.2.1 Gastrointestinal Tract

Cytoprotective PGs in the stomach are synthesized by COX-1 and have vasodilatory effects enhancing mucosal blood flow. Inhibition of COX-1 by NSAIDs causes gastric erosions, ulceration, haemorrhage and perforation [61,76,77]. In addition animal models have shown COX-1 to be involved in epithelial regeneration within the crypts of Lieberkuhn following radiation injury [78]. Hence the PGs produced by COX-1 appear to promote crypt stem cell survival and proliferation. COX-2 is not entirely absent within the gastrointestinal tract. Following intestinal infection, intestinal epithelial cells express COX-2, which leads to production of PGE₂, which in turn creates a chloride and fluid flux that helps flush bacteria

from the gut [79]. In addition, the now well established role of COX-2 in inflammation has implications in certain GI conditions. In tissue on the edges of ulcers, COX-2 is induced, and it has been suggested that blocking COX-2 may delay gastric ulcer healing in animals [80].

2.2.2 Kidney

The role of PGs in the kidney is well established. COX-1 induced production of PGI₂, PGE₂ (synthesized by both the glomerulus and medullary interstitial cells) and PGD₂ diminish vascular resistance by dilating renal vascular beds and enhance organ perfusion [77,81]. This in turn leads to redistribution of blood flow from the renal cortex to the nephron in the juxtamedullary region [82]. NSAID blocking of COX-1 leads to relative renal ischaemia. Animal studies have shown upregulation of COX-2 within the macula densa in salt deprivation states suggesting a role for COX-2 in renal sodium excretion [83]. Thus it appears that the two COX isoenzymes may exert differential renal effects by synthesizing distinct prostanoids that react with specific receptors at different locations. These effects of the COX isoenzymes also appear to be intimately linked to the rennin-angiotension-aldosterone axis [84].

2.2.3 Platelets

Inhibition of COX-1 leads to diminished formation of TXA₂, a pivotal autocrine stimulator of platelet aggregation and vasoconstriction [76,85]. This is also the therapeutic aim of “half an aspirin a day” prophylaxis against thromboembolic disease [86]. Platelet adhesion is primary mediated by von Willebrand factor, the molecular “glue” that binds to specific receptors on the platelet surface localised in the platelet membrane glycoprotein 1b-IX-V complex. This von Willebrand factor binding initiates a cascade of intraplatelet signalling events that

activates the adherent platelets and induces the release of factors that recruit additional platelets to the site of injury. Two important platelet-activating factors elaborated during this platelet release reaction are adenosine diphosphate (ADP) and thromboxane A₂ (TXA₂) [85]. NSAID inhibition of COX-1 results in irreversible inhibition of TXA₂ for the platelet lifetime of 8 – 10 days, as the platelets do not form new isoenzymes. Some of the new COX-2 inhibitors do still retain some platelet TXA₂ inhibitory properties but their antiplatelet potency is several orders lower than standard NSAIDs. This implies a minor role by COX-2 in normal platelet function [87]. In normal subjects, NSAID inhibition of COX-1 has a mild clinical effect. However, in subjects with impaired haemostasis, disruption of TXA₂ becomes a significant problem. In patients with NSAID related gastrointestinal tract (GIT) ulceration, the NSAID related platelet dysfunction could bring about a potentially disastrous synergy resulting in severe haemorrhage [88].

2.2.4 Female Genital Tract

PGs are important in gestation and parturition. Both COX-1 and COX-2 are expressed in the uterine epithelium at different times in early pregnancy and may be important for the implantation of the ovum and in the angiogenesis needed for the establishment of the placenta [61,89]. While COX-1 related PGs appear to be involved in preparing the wall for interaction with the embryo, COX-2 and PG receptors seem to mediate implantation [77]. In addition the PGs originating from COX-2 may play a role in the birth process, since COX-2 mRNA in the amnion and placenta increases substantially immediately before and after the start of labour [77,90]. It has also been postulated that COX-2 plays a role in preterm labour as intrauterine infection results in release of endogenous factors that increase PG production by upregulating COX-2. Hence selective inhibitors of COX-2 should be useful in delaying premature labour

without the side effects of indomethacin [61,91]. However, COX-2 inhibition appears to have little effect in preventing cervical ripening as nitric oxide appears to be the major metabolic mediator of this process, with COX-2 having only a minor contributing role [92]. Studies involving COX-2 null mice show reproductive failure at ovulation, fertilization, implantation and decidualization [93].

2.2.5 Central Nervous System

Both COX-1 and COX-2 appear to play integral functions in the central nervous system (CNS) [61,77,94]. Human brain contains equal amounts of mRNA for COX-1 and COX-2. COX-1 is distributed in neurones throughout the brain, but is most prevalent in the forebrain, where PGs may be involved in complex integrative functions, such as modulation of the autonomic nervous system and sensory processing. COX-2 expression is limited to certain parts of the CNS, notably the cortex, hippocampus, hypothalamus and the spinal cord [95]. COX-2 is upregulated by normal or by abnormal (convulsive) nerve activity and is found in neuronal and nonneuronal cells. These findings suggest a role for PGs in CNS transmission and raise the possibility that selective COX-2 inhibitors may modulate CNS function [96]. The major PGs in the CNS are PGE₂ and PGD₂. Lipopolysaccharides cause the release of cytokines such as IL-1, which in turn induce COX-2 in brain endothelial cells. The resulting PGE₂ then acts on the temperature-sensing neurones in the preoptic area and induce fever. This is also the mechanism by which NSAIDs reduce fever [61,77,94,97]. Another central effect of PGs is pain or, more accurately, hyperaesthesia. COX-2 is known to be induced in peripheral terminals of sensory neurones with resultant PG synthesis in the setting of inflammatory pain [97]. Studies have also shown that NSAIDs appear to decrease by half the severity and incidence of Alzheimer's disease as it is recognised that Alzheimer's disease may

have an inflammatory component. An integral component of this disease is the formation of discrete lesions termed neuritic plaques, which contain depositions of β -amyloid. This substance is thought to be elaborated as part of an inflammatory cascade involving microglial cells, which express COX-2 and PGs. This is postulated as a mechanism by which NSAIDs decrease the severity of Alzheimers disease [61,77,96,98].

2.3 COX-2 AND INFLAMMATION

As mentioned earlier, inflammatory mediators and cytokines are the main stimulus for the upregulation of COX-2. Initial animal studies done by Sano et al [99], and later by Anderson et al [100], showed increased levels of COX-2 in inflammatory arthritis. Analysis of human synovial tissues in patients with rheumatoid arthritis (RA), osteoarthritis (OA) and nonarthritic patients with traumatic injury showed increased expression of COX-2 within the synovial lining. RA showed the most intense staining for COX-2, which appeared to correlate with the degree of mononuclear inflammation present, whereas in OA the synovial tissue showed less staining. Little immunoreactive COX-2 was seen in nonarthritic synovial tissues [67,100]. Experimental evidence has also suggested that PGE₂ is intimately involved in mediating the inflammatory cascade with levels of COX-2 mRNA and COX-2 protein correlating with elevated PGE₂ levels [75,76]. PGE₂ is involved in the oedema of acute inflammation, in mediating inflammatory pain as well as mediating chronic inflammation associated with arthritis [101].

NSAIDs are the mainstay of treatment for patients with RA and OA as well as for the treatment of acute pain. In addition, more than 70% of people in developed countries, over the age of 65 years, show some radiological evidence of OA and over 17 million people in the USA alone, take prescription NSAIDs [102]. It is of little surprise that the pharmaceutical

companies have gone on to develop a specific COX-2 inhibitor, of which Celecoxib is one such example. Recent studies with Celecoxib in patients with RA and OA have shown excellent results in relieving pain and joint stiffness. In addition the patients suffered none of the unwanted side effects of COX-1 inhibition such as gastric erosions, platelet dysfunction and renal impairment [74,101,102].

2.4 COX-2 AND TUMOURIGENESIS

Evidence that arachidonic acid and its metabolites may be mediators of carcinogenesis comes from experimental, clinical and epidemiological studies in which inhibition of prostaglandin synthesis suppresses carcinogenesis. The initial work was done in colorectal carcinomas but other malignancies have subsequently also been studied [103].

Peculiar to COX-2 is its perinuclear location. One of the prostaglandin receptors, PGJ₂, has proven to be a ligand for the retinoid X receptor family – a diverse group including the retinoid and thyroid nuclear receptors, all acting as transcription factors for genomic DNA [61]. This action as a transcription factor might form the link between COX-2 activity and the progression of preneoplastic epithelial cells to fully malignant phenotypes [61,104]. Another interesting finding is an increase in BCL-2 protein, an anti-apoptotic protein, following COX-2 activity in epithelial cells [61,67,105]. In addition enhanced synthesis of PGs, a consequence of upregulation of COX-2, promotes angiogenesis and inhibits immune surveillance, both of which favour the growth of malignant cells [106].

2.4.1 Gastrointestinal Malignancies

Initial epidemiological studies showed a lower incidence of *colorectal carcinoma* in people on long term NSAID therapy [61,107]. Subsequent studies showed that both animal and

human colon tumours express high levels of COX-2, whereas normal intestinal mucosa has very little COX-2 expression [61,73,77,108]. NSAIDS also appear to reduce both the number and size of polyps in patients with familial adenomatous polyposis (FAP) and hence an association between COX-2 and a mutation of the adenomatous polyposis coli (APC) gene has been postulated [61,72,73,103,108]. Another group with a clear genetic predisposition to colorectal carcinoma are patients with hereditary non-polyposis colorectal cancer (HNPCC) as they have a germline mutation in one set of genes required to repair mismatched bases in DNA. A recent study has shown a significantly lower level of COX-2 expression in colorectal carcinomas arising through defective DNA mismatch repair, suggesting that these carcinomas arise independently of COX-2 [109].

Increased COX-2 levels have also been identified in the epithelial cells of *gastric carcinoma* [78]. The same study revealed that the degree of COX-2 expression correlated with invasion of lymphatic spaces and metastases to regional lymph nodes [78]. *Oesophageal carcinomas*, including both squamous and adenocarcinomas, have shown elevated COX-2 levels on both immunohistochemical and Western blot analysis. In vitro tests on oesophageal squamous carcinoma cell lines showed increased tumour cell apoptosis with selective COX-2 inhibition [61,110].

2.4.2 Head and Neck Malignancies

Squamous cell carcinomas of the head and neck too have shown to express elevated levels of COX-2. Furthermore, activation of the epidermal growth factor receptor (EGFR)/ Ras pathway contributes to the upregulation of COX-2 and animal studies have shown that NSAIDS prevent head and neck squamous carcinomas [106].

2.4.3 Lung Carcinoma

Carcinoma of the lung has also shown an association with elevated COX-2 expression. This is particularly evident in squamous cell carcinoma and adenocarcinoma in which the COX-2 expression correlated with the degree of tumour differentiation. Small cell carcinomas, however, showed only very low levels of COX-2. Of interest was the presence of elevated COX-2 in hyperplastic bronchial epithelium and atypical alveolar epithelium, both considered to be potential preneoplastic lesions [111,112].

No studies have yet been reported investigating COX-2 expression in preneoplastic and neoplastic lesions of the uterine cervix.

AIMS

1. To investigate the expression of cyclooxygenase-2 (COX-2) in the normal cervix.
2. To investigate the expression of COX-2 in the diseased cervix. Specifically to evaluate expression in preneoplastic disease (cervical intraepithelial neoplasia) and invasive carcinoma.
3. To evaluate any association between HPV types and COX-2 expression within a spectrum of cervical disease.

CHAPTER THREE MATERIALS AND METHODS

In this study immunohistochemical methods were used to assess COX-2 expression in a range of normal and diseased cervixes. These included histological normal cervixes (from cervical biopsies and routine hysterectomies for non-cervical related disease), mild dysplasia (CIN1), moderate dysplasia (CIN2), severe dysplasia (CIN3) and invasive squamous cell carcinoma.

3.1 SELECTION OF CASES

Cervical tissue from a total of 59 cases was obtained from archival material and recent routine surgical specimens. The archival material selected was limited to cases, which had undergone prior HPV typing using polymerase chain reaction (PCR) techniques. A haematoxylin and eosin stained slide was necessary for histological diagnosis and paraffin wax-embedded tissue had to be available on all selected tissue in order for the immunohistochemical investigations to be done. In addition, cases obtained prospectively from the routine surgical load were subjected to PCR technique to determine HPV types.

A total of 18 “normal” cases were selected (8 histological normal cervical biopsies from archival tissue, 10 routine hysterectomies for non-cervical related disease – i.e. uterine leiomyomas), and 41 cases incorporating a range of CIN and invasive squamous cell carcinoma.

For the purposes of the study “normal” was defined as cervical epithelium not showing any histological evidence of dysplasia or invasive carcinoma. Underlying stromal inflammation did not disqualify cases from being included in this group.

Cervical dysplasia, as described in the WHO classification, is characterised histologically by replacement of the normal epithelium by abnormal parabasal cells with abnormal epithelial maturation, nuclear atypia and abnormal mitotic figures. **CIN 1** is characterised by dysplastic changes effecting the lower 1/3 of the epithelium; **CIN 2** when the dysplastic changes extend up to involve 2/3s of the epithelium and **CIN 3** when the dysplastic changes involve the upper 1/3 of the epithelium merging into full thickness dysplasia. In all cases, the stromal inflammation, when present, was scored as: 1 = mild, 2 = moderate, 3 = marked.

3.2 IMMUNOHISTOCHEMISTRY

The antibody used in this study was kindly donated by Dr Henry Jabbour (MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, Edinburgh). In all cases studied, 3 μ sections were cut from formalin fixed, paraffin wax-embedded tissue and mounted onto aminopropyltriethoxysilane (APES) coated glass slides. The sections were dried at 56°C for 1 hour. Sections were dewaxed in xylene, hydrated in graded ethanol and washed in water, and then blocked for endogenous peroxidases (1% H₂O₂ in methanol).

Various heat-mediated and enzymatic digestion antigen retrieval procedures were attempted and included:

- (1) Antigen retrieval by means of enzymatic pre-digestion in a 0.1% porcine pancreas solution at 37°C at graded time intervals.
- (2) Heat-mediated antigen retrieval utilising either microwave or bench top pressure cooking techniques, employing a 0.1M sodium citrate buffer (pH6).

In our laboratory, heat-mediated antigen retrieval by means of pressure cooking was found to be optimal and compatible with the departments processing protocols.

Antigen retrieval was performed by pressure cooking for 2 minutes at full pressure in 0.1M sodium citrate (pH6). The sections were then blocked by using 5% normal rabbit serum (for COX-2) diluted in phosphate buffered saline (PBS). Subsequently the sections were incubated with polyclonal goat anti-COX-2 antibody (sc-1745; Autogenbioclear) at a dilution of 1:300 at 4°C for 18 hours. Negative control tissue was incubated with either 5% antisera (PGE) or goat anti-COX-2 antibody preabsorbed to blocking peptide.

After thorough washing with PBS, the tissue sections were incubated with biotinylated rabbit anti-goat secondary IgG antibody (for COX-2; Dako; code: E0466) at a dilution of 1:500 at 25°C for 40 minutes. Thereafter the tissue sections were incubated with streptavidin-biotin peroxidase complex (Dako; code:P0364) at 25°C for 30 minutes. After washing, peroxidase activity was detected using 3,3-diaminobenzidine (Dako; code: K3466) as a chromagen.

The tissue sections were then counterstained in aqueous haematoxylin followed by sequential dehydration using graded ethanol and xylene, before mounting and coverslipping in Entellan.

3.3 COX-2 SCORING

No standardized scoring system for COX-2 reactivity exists. For the purpose of this study the following scoring system was developed. The **COX-2 score** was obtained for each case by assessing subjectively and by adding together both the **intensity** and the **extent** scores of the COX-2 expression. **The intensity** was scored out of 3: 1 = mild expression, 2 = moderate expression, 3 = marked expression. **The extent** of staining was also scored out of 3: 1 = focal staining, 2 = multiple foci staining, 3 = diffuse staining.

3.4 PCR ANALYSIS FOR HPV TYPES

The HPV typing, utilised in this study, was kindly done by Mrs. P.S. Kay in the department of Virology, UCT Faculty of Health Sciences.

The retrospective cases used in this study had all undergone previous HPV typing using **My09/11 Consensus Primers** and subsequent **Type Specific Primers (11,16,33)** [113]. The prospective cases studied all underwent HPV typing utilizing a **Restriction Fragment Length Polymorphisms RFLP** method in conjunction with a **Nested PCR** method [114] – the current method of HPV typing done in our Virology laboratory.

3.4.1 My09/11 Consensus Primers/Type specific primers

DNA Extraction

A piece of biopsy sample about the size of a match head was macerated in a sterile petri dish using a sterile disposable scalpel. Extreme caution was taken to prevent cross contamination of specimens. A new pair of gloves, sterile petri dish and scalpel was used for handling each specimen. All work was performed in a laminar flow hood.

DNA was extracted from the macerated biopsy sample by incubating the sample in proteinase K lysis buffer (50Mm KCl, 10Mm Tris-HCl Ph 8,3, 2,5 Mm MgCl₂, 0,5% NP 400, 5% Triton x100, 120ug/ml Proteinase K) at 56°C for at least 2 hours or until no pieces of tissue were visible. The proteinase K was then inactivated by incubation at 95°C for 10 minutes. The extracted DNA samples were stored at 4°C.

Polymerase Chain Reaction using My09/11 Primers

PCR using degenerate consensus sequence primers (Ting and Manos) was used to detect the presence of HPV DNA. These primers amplify a 450 bp sequence of the L1 open reading

frame (ORF). PCR master mixes were made up using Southern Cross Biotechnology reagents and Supertherm Taq. Each sample contained:

- Reaction buffer
- 200Um each dNTP
- 2Mm MgCl₂
- 50 pmol each primer
- 2.5 units Taq
- Ultrapure water (BDH Ltd.)

5ul of sample was added to 45 ul of PCR master mix. PCR was performed in a Perkin Elmer GeneAmp 6700 thermocycler. After an initial denaturation step of 95°C for 3 minutes the samples were subjected to 30 rounds of amplification as follows: denaturation for 30 seconds at 95°C, annealing for 30 seconds at 55°C and primer extension for 60 seconds at 72°C. A final extension step of 7 minutes at 72°C was performed at the end of the cycles.

All samples were initially amplified using PCR primers for the human CCR5 gene to ensure that no PCR inhibitors were present and that the sample contained amplifiable DNA. Any samples that did not amplify with the CCR5 primers were excluded from the study.

PCR using Type Specific Primers

Samples showing the presence of HPV DNA after amplification with the HPV consensus primers were then typed by PCR using type specific primers. Each sample was subjected to PCR using type specific primers for the following HPV types: 11, 16, and 33.

PCR reaction mixes were made up as per MY09/11 protocol except that the primers were changed for each type specific reaction. Cycling conditions were also the same except that 35 PCR cycles were performed and the annealing temperatures of each type specific primer were as follows:

- 56°C for HPV 16 type specific primers
- 65°C for HPV 11 type specific primers
- 65°C for HPV 33 type specific primers

3.4.2 RFLP/Nested PCR

DNA extraction.

Biopsy samples were macerated in a sterile petri dish with a sterile disposable scalpel. Extreme caution was taken to prevent cross contamination of specimens. A new pair of gloves, sterile petri dish and scalpel was used for handling each specimen. All work was performed in a laminar flow hood. DNA was extracted from the macerated samples using the Qiagen Qiamp DNA Mini Kit. The principle of this procedure is as follows:

- The macerated tissue sample is lysed using Proteinase K.
- The buffered lysate is applied to a column.
- The DNA is adsorbed to the silica-gel membrane.
- The DNA is recovered from the column by elution with water.

Detection of HPV using group specific primers

A nested PCR using degenerate primers, developed by Williamson and Rybicki [114] was used to detect the presence of HPV DNA. These primers were designed to detect a wide range of mucosal HPV types. The outer pair of primers is specific for a conserved 441-base sequence contained within the L1 gene and the inner pair of primers amplifies a 335-base sequence within the sequence amplified by the outer pair. PCR master mixes were made up using Southern Cross Biotechnology reagents and Supertherm Taq. Each reaction sample contained:

- Reaction buffer

- 200uM each dNTP
- 2mM MgCl₂
- 50pmol each primer
- 0.175 units Taq
- Ultrapure water (BDH Ltd.)

For the first round of amplification with the outer primers, aliquots of 45ul master mix was dispensed into 200ul flat topped PCR tubes and 5ul of sample DNA added. For the second round of amplification with the inner primers, aliquots of 49ul master mix were dispensed into flat topped 200ul PCR tubes and 1 ul of amplified DNA from the first round of PCR was added.

PCR cycling was performed in a Perkin Elmer GeneAmp 6700 thermocycler. After an initial denaturation step of 94°C for 5 minutes the samples were subjected to 30 reaction cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds and primer extension at 72°C for 1 minute. At the end of the cycles a final extension step of 7 minutes at 72°C was performed.

Before amplification with the degenerate nested primers for HPV, all samples were amplified using PCR primers for the human CCR5 gene to ensure that the samples contained amplifiable DNA and that no PCR inhibitors were present. Any samples that did not amplify using the CCR5 primers were not included for amplification with the HPV primers.

All PCR amplified products were cleaned up using the Qiagen PCR clean up kit.

Typing of HPVs using restriction fragment length polymorphisms

A restriction fragment length polymorphism (RFLP) method was used in conjunction with the nested PCR to type HPVs. The DNA sequences of known HPV types were downloaded from the NCBI Genebank database. The section of DNA amplified by the inner primers of

the nested PCR was mapped on the known sequences and restriction sites identified using DNAMAN. Appropriate restriction endonucleases were chosen and the RFLP was performed by digesting approximately 1µg of amplified PCR product with selected restriction endonucleases.

Samples were initially digested with Pst1/Bgl1 and BstE11 endonucleases. The combination of these 2 restriction digests results in a unique pattern for HPV types 16, 33, 35 and 58. HPV 18 requires a further confirmatory restriction digest with Pvu11 and HPV31 with Nco1. The 25µl reaction mixes were made up as follows:

- Reaction buffer
- 2 units enzyme
- Ultrapure water (BDH)
- Amplified PCR product

The reaction mixes were incubated at 37°C for 1 hour in an incubator except for the BstE11 digests which were incubated at 60°C.

Electrophoresis

After digestion the samples in both methodologies were electrophoresed in a 2% and 3% agarose gel respectively, to separate the different size fragments and these were stained with ethidium bromide to visualize the DNA.

The typing results derived from the RFLP were confirmed by direct sequencing of the PCR products.

CHAPTER FOUR

RESULTS

The original haematoxylin and eosin stained sections were reviewed in all cases and the original histological diagnoses confirmed. The study included 18 normal controls, 4 cases of CIN 1, 9 cases of CIN 2, 16 cases of CIN 3 and 12 cases of invasive squamous cell carcinoma (Figure 4.1). PCR analysis for HPV subtypes was available on all cases and revealed 15 cases negative for HPV types tested, 9 cases of HPV 16, 7 cases of HPV 33, 1 case of HPV 18 and 8 cases of HPV 16 + 33 dual infection (Figure 4.2). In addition 2 cases showed HPV positivity on the initial primer, but expressed too weak a signal for specific typing.

The mean COX-2 scores for each HPV group, within each histological category, are given in Table 4.1 and Figure 4.3 (see page 53); and the COX-2 scores for each individual case are listed in Table 4.2.

The pattern of COX-2 staining in all cases was cytoplasmic positivity and nuclear membrane positivity. The nucleus itself did not show any staining.

4.1 NORMAL CERVIX

The normal cervixes consisted of 8 biopsy specimens which all showed only ectocervical squamous epithelium but no endocervical epithelium. The other 10 control cases were all obtained from elective hysterectomies for non-cervical disease and hence tissue showing both ecto-and endocervix.

PCR analysis for HPV was positive in 3 cases (cases 13,14,18). Case 14 was shown to harbour HPV 18, but the other 2 cases had too weak a signal to allow for specific typing.

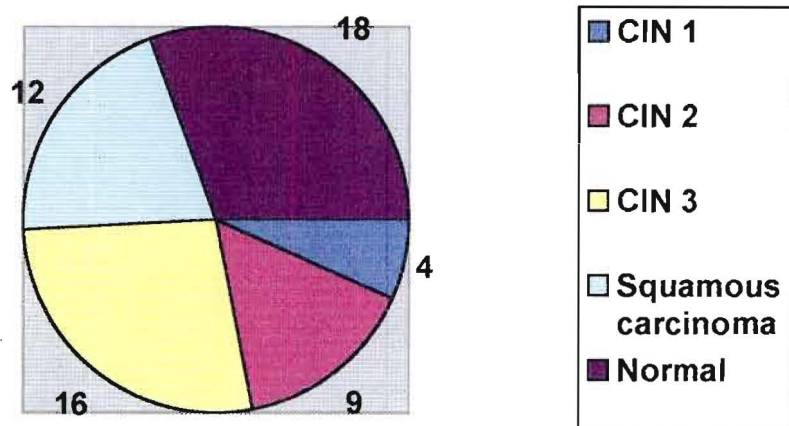


Figure 4.1 – Pie Chart illustrating the number of cases in each histological category. The small study size was due to the fact that only cases which had histological material available and on which HPV typing had been done, were included. The small case number precluded any meaningful statistical analyses.

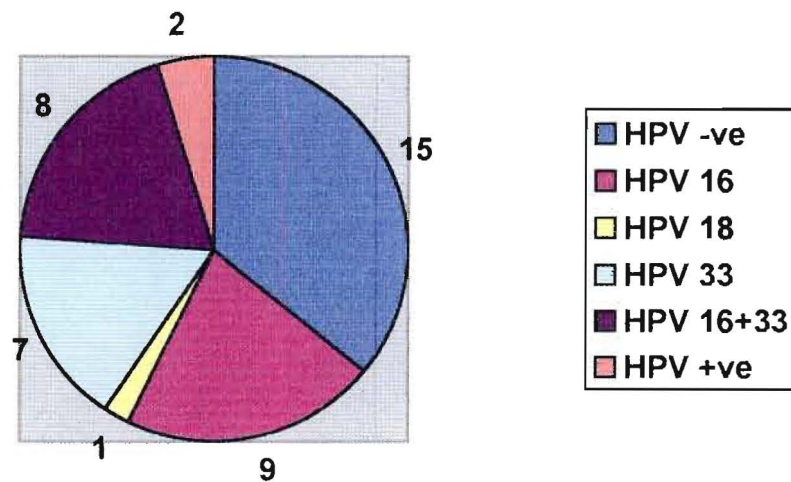


Figure 4.2 – A Pie Chart illustrating the distribution of HPV typing within this study. HPV types 16 and 18 are categorised as “high-risk” whereas type 33 falls within the “intermediate-risk group. Only dual infections with a type 16 and 33 combination were found. A large proportion of cases (n=15) revealed no HPV on typing using the My09/11 primers. These may well be false negative results; the specimens will be re-examined using more sensitive PCR techniques.

11 cases showed a mononuclear inflammatory infiltrate within the underlying stroma. Case 14 was the only 'normal' cervix to score maximum (3/3) for inflammation.

COX-2 immunohistochemical staining showed no staining of the normal ectocervical squamous epithelium (Figure 4.4). All 10 cases containing normal endocervix showed basal COX-2 staining of both the surface and underlying glandular epithelium (Figure 4.5). The three cases positive for HPV showed a more intense COX-2 expression within the endocervical epithelium with case 14 (Figure 4.6) showing the highest COX-2 score.

The inflammatory cells present within the stroma of all cases stained positive for COX-2 but no endothelial cells lining vascular spaces showed any positive staining.

4.2 CIN 1

Only four cases of CIN 1, where the HPV status was known, were available for inclusion in the study.

PCR analysis for HPV was positive in only one case, which showed HPV 16 present. The other 3 cases were negative for the types tested.

COX-2 immunohistochemical staining was positive within the dysplastic epithelium showing a COX-2 score range from 2.0 to 3.0. A mean COX-2 score of 2.3 was present in the HPV negative group, and a score of 3.0 in case 22 - HPV 16 positive (Figure 4.7).

All cases showed a stromal inflammatory infiltrate, which was positive for COX-2. Neither stromal cells nor endothelial cells lining vascular spaces showed any COX-2 positivity.

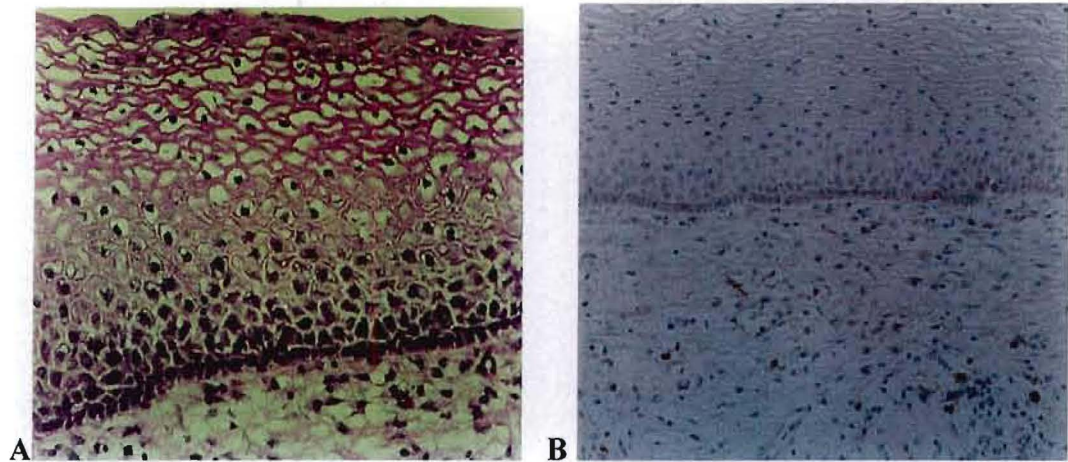


Figure 4.4 “Normal” ectocervix - case 16 (HPV -ve) (A) “Normal” cervical ectocervix showing an intact basal layer, normal maturation and no dysplasia. H&E, objective x 20. (B) COX-2 staining from the same case showing no expression by the ectocervical epithelium. Occasional stromal inflammatory cells show COX-2 expression. COX-2 immunohistochemistry, objective x 20.

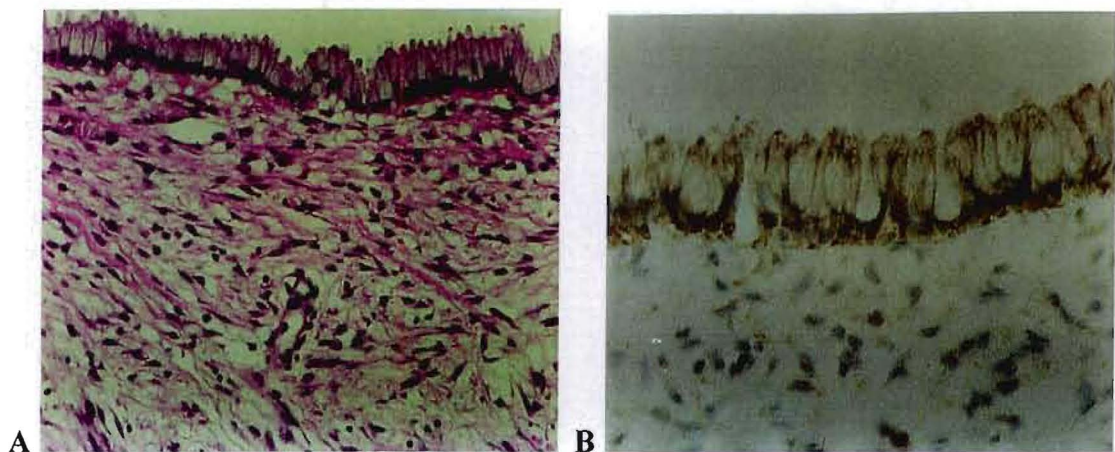


Figure 4.5. “Normal” endocervix – case 16 (HPV -ve). (A) “Normal” endocervical columnar mucin secreting epithelium. H&E, objective x 20. (B) COX-2 staining of the same case showing weak staining in the cytoplasm around the basally located nucleus. Faint expression is also seen around the mucin vacuoles. COX-2 immunohistochemistry, objective x 40.

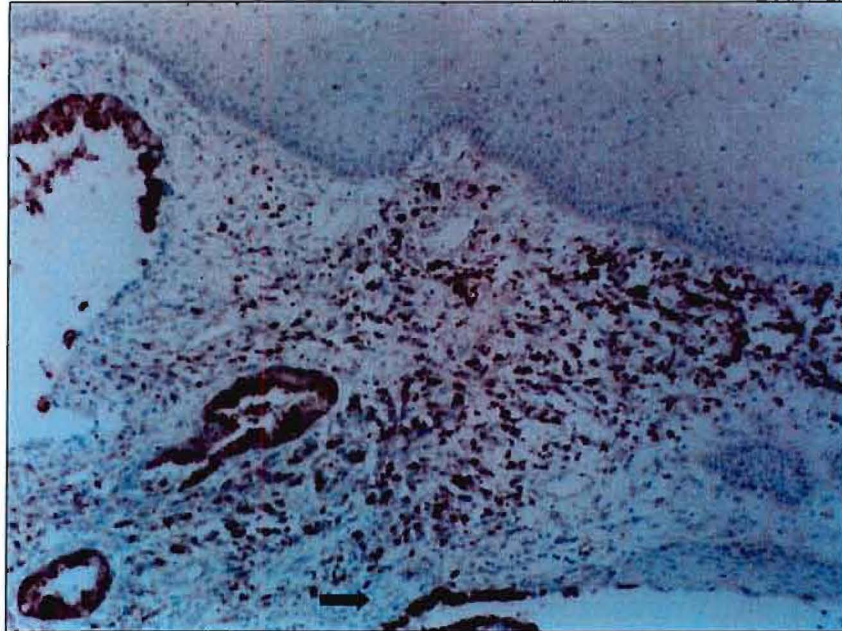


Figure 4.6 “Normal” endocervix – case 14 (HPV 18). Cervical mucosa in the region of the transformation zone. There is “normal” (non-dysplastic) squamous epithelium with underlying endocervical glands and stroma. Evidence of squamous metaplasia involving one gland is present (arrow). The COX-2 expression is markedly increased within the endocervical glands, including the gland showing squamous metaplasia. In addition, there is a prominent stromal inflammatory infiltrate, which also shows marked expression of COX-2. The changes shown here were limited only to this case – the only “normal” case to show HPV positivity. COX-2 immunohistochemistry, objective x 10.

4.3 CIN 2

A total of nine cases, where the HPV status was known, showed histological features of CIN 2.

PCR analysis for HPV showed four cases which were negative for the HPV types tested, three cases were positive for HPV 33, one case positive for HPV 16 and one case showed dual HPV 16 and 33 positivity.

COX-2 immunohistochemical staining was positive within the dysplastic epithelium in all cases. The COX-2 scores ranged from 3.0 to 4.0 with the HPV negative group showing a mean score of 3.0 and the HPV 33 group a mean of 3.3. Both the HPV 16 case and the case of dual 16 + 33 positivity showed COX-2 scores of 3.0 (Figure 4.8).

All cases showed a COX-2 positive inflammatory infiltrate of varying intensity, within the stroma. Once again no stromal cells or vascular endothelial cells showed definitive COX-2 staining.

4.4 CIN 3

Sixteen cases showed histological features of CIN 3.

PCR analysis for HPV showed six cases that were negative for the subtypes tested. There were three cases of HPV 16, three cases of HPV 33 and four cases, which showed dual 16 and 33 positivity.

COX-2 immunohistochemical staining was positive in all cases within the dysplastic epithelium. The COX-2 scores ranged from 2.0 to 6.0. The mean scores were 3.2 in the HPV negative group, 3.3 in the HPV 16 and the HPV 33 groups and 4.7 in the group showing dual HPV 16 and 33 positivity (Figure 4.9).

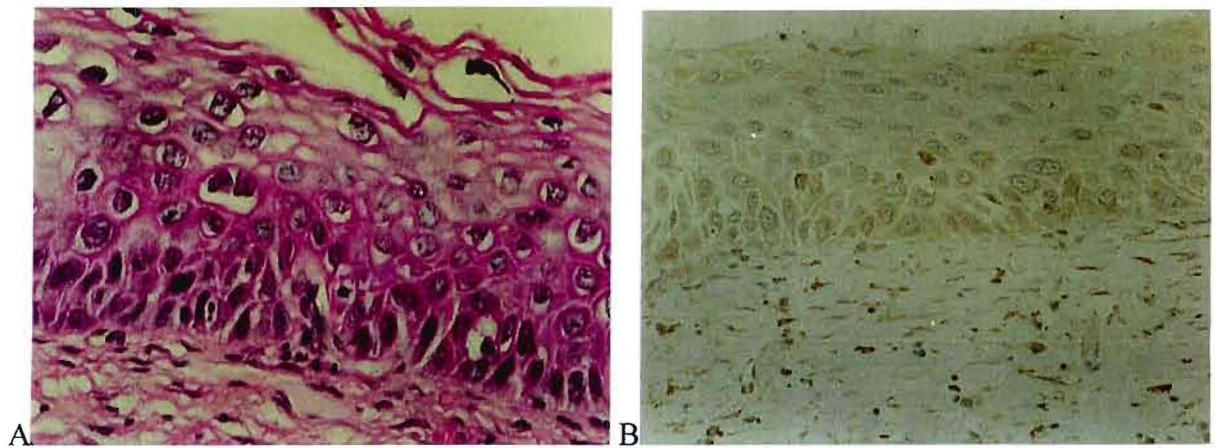


Figure 4.7. CIN 1 – case 22 (HPV 16) **(A)** Cervical epithelium showing typical features of CIN-1. There are dysplastic changes affecting the lower third of the epithelium with a typical binucleated koilocyte (centre). H&E, objective x 20. **(B)** COX-2 staining of the same case showing mild expression of COX-2 predominantly within the dysplastic lower third. Stromal inflammatory cells also stain with COX-2. COX-2 immunohistochemistry, objective x 20.

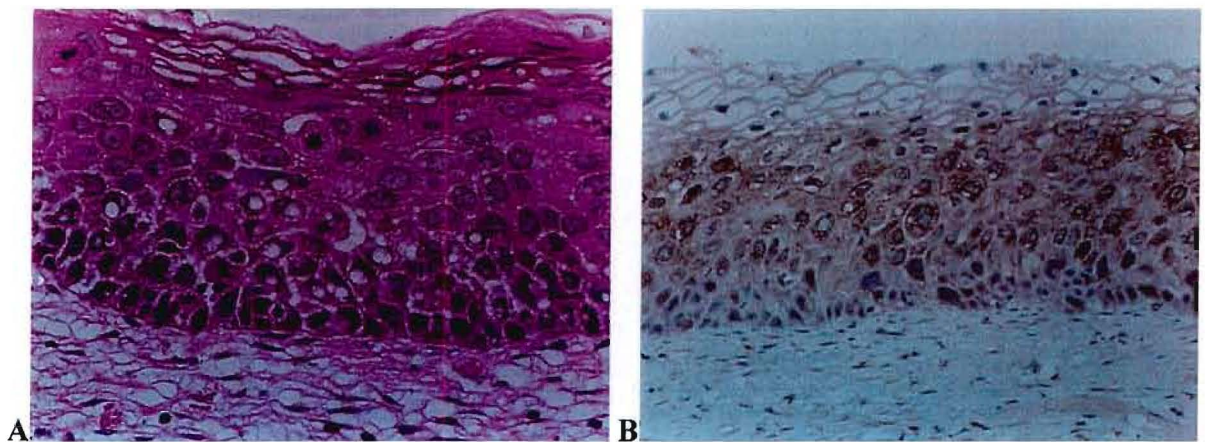


Figure 4.8 CIN 2 – case 29 (HPV 33) **(A)** Cervical epithelium showing features of CIN 2 characterised by dysplastic changes affecting two thirds of the epithelium with the upper third showing normal maturation. Note several atypical mitoses occurring within the dysplastic epithelium. H&E, objective x 20. **(B)** COX-2 staining of the same case showing diffuse staining of the dysplastic epithelium of moderate intensity. COX-2 immunohistochemistry, objective x 20.

Once again a stromal inflammatory infiltrate was noted in all cases except one (case 40). No COX-2 staining was noted in the stromal or endothelial cells.

Cases of CIN 3, where HPV was demonstrated, showed some variability of COX-2 staining intensity and extent (Table 4.2). Cases with HPV 16 or HPV 33 showed a mean COX-2 score of 3.3 (Table 4.1), whereas cases of dual infection with HPV 16 + 33 showed a mean COX-2 score of 4.75 (Table 4.1). These differences are also reflected on the bar graph (Figure 4.3) and illustrated photographically (Figure 4.41).

4.5 SQUAMOUS CARCINOMA

Twelve cases showed features of squamous cell carcinoma.

PCR analysis for HPV revealed four cases negative for the HPV subtypes tested. PCR showed four cases of HPV 16, one of HPV 33 and three cases of HPV 16 and 33 dual positivity.

COX-2 immunohistological staining showed a range of scores from 3.0 to 6.0. The HPV negative group had a mean score of 3.75, the HPV 16 group a mean of 4.25 and the HPV 33 case scored 5.0. The group showing dual HPV 16 + 33 positivity had a mean COX-2 score of 5.6 (Figure 4.10).

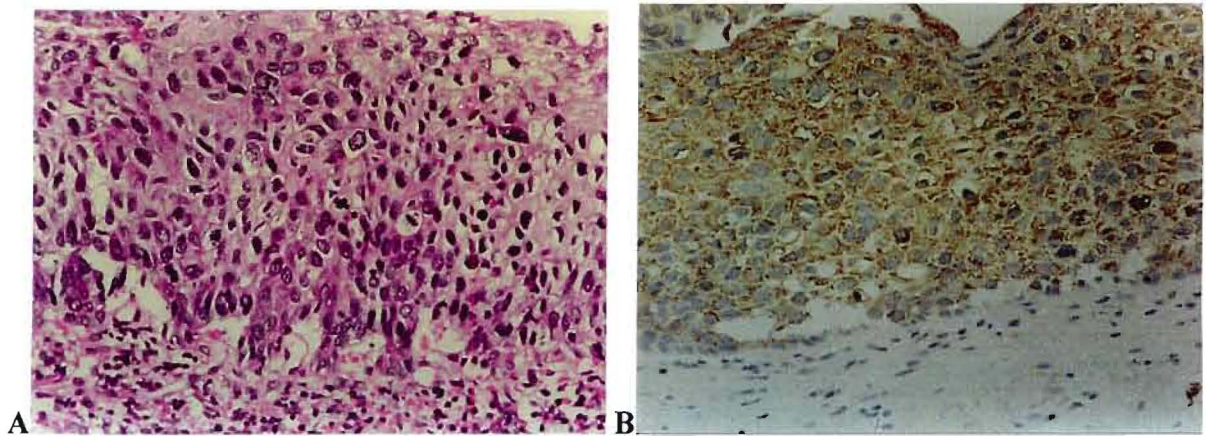


Figure 4.9. CIN 3 – case 44 (HPV 16+33) (A) Cervical epithelium showing full thickness dysplasia. H&E, objective x 20. (B) COX-2 staining of the same case with diffuse expression of COX-2 of a moderate intensity. COX-2 immunohistochemistry, objective x 20.

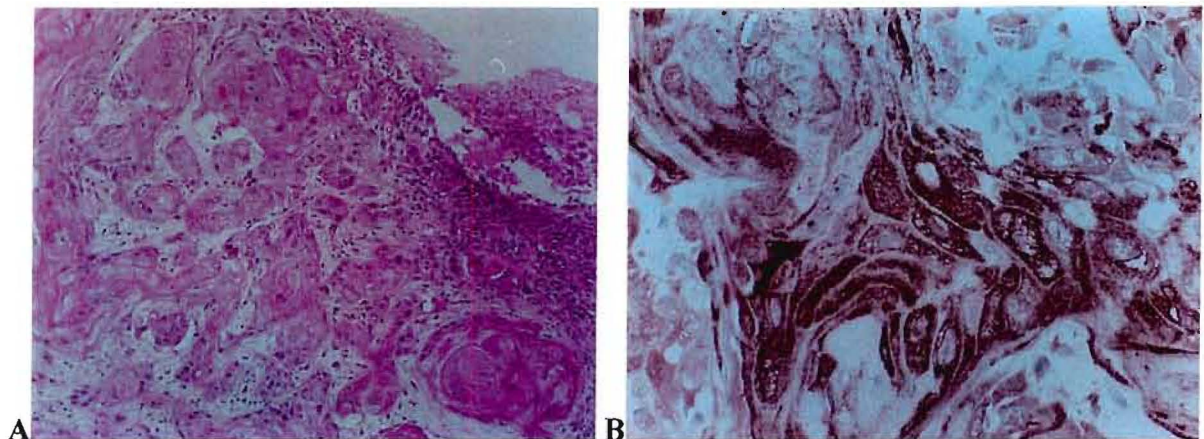


Figure 4.10. Squamous carcinoma – case 58 (HPV 16+33) (A) Typical features of an invasive keratinising squamous cell carcinoma. H&E, objective x 10. (B) COX-2 staining of the same case showing diffuse COX-2 expression of a marked intensity. COX-2 immunohistochemistry, objective x 40.

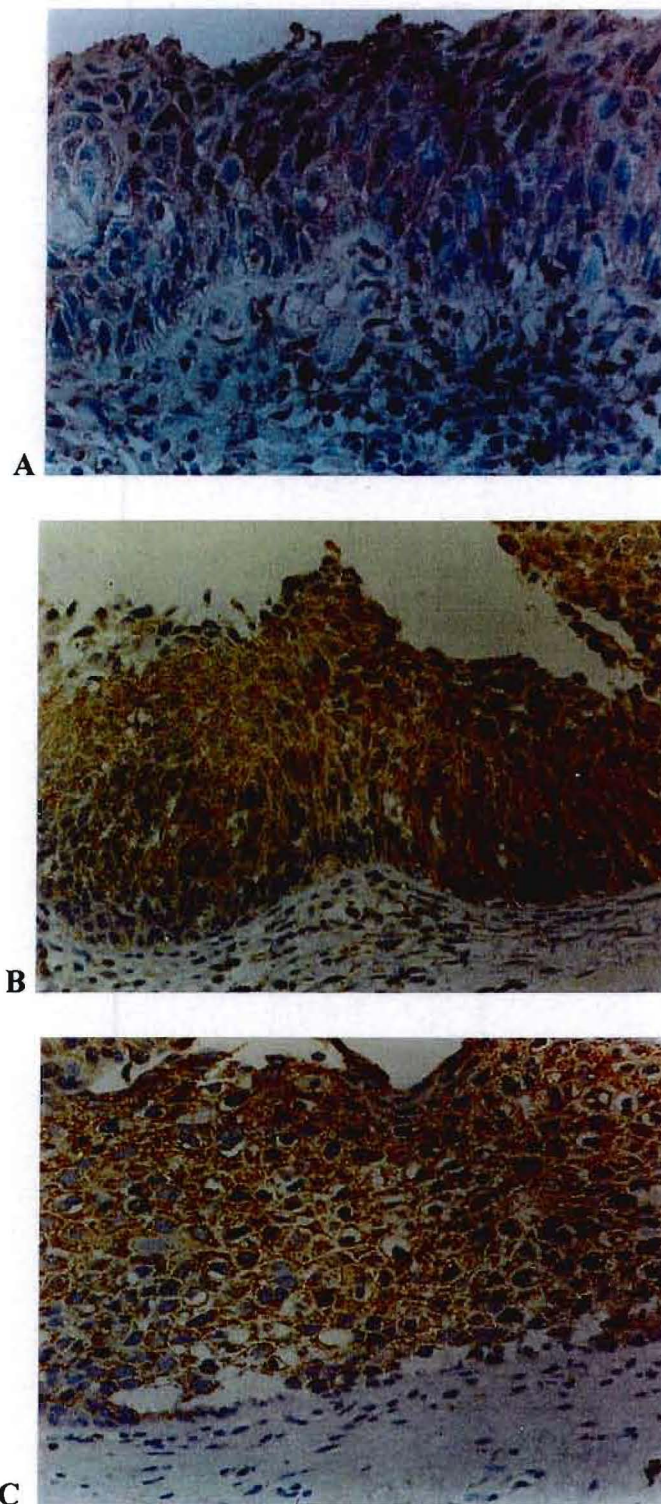


Figure 4.11 CIN 3 Representative sections of COX-2 expression in three different cases of CIN 3, each with a different HPV profile. **A**– case 43 (HPV 16), **B**– case 39 (HPV 33), **C**– case 496 (HPV 16 + 33). There is a marked increase in the COX-2 expression in the dual HPV infection (**C**) compared to the cases showing HPV types 16 and 33 alone.

Table 4.1. Summary of the mean COX-2 scores

	CIN 1	CIN 2	CIN 3	Squamous Ca
HPV 0	2.3 (n = 3)	3.0 (n = 4)	3.2 (n = 6)	3.75 (n = 4)
HPV16	3.0 (n = 1)	3.0 (n = 1)	3.3 (n = 3)	4.25 (n = 4)
HPV 33	-	3.3 (n = 3)	3.3 (n = 3)	5.0 (n = 1)
HPV 16+33	-	4.0 (n = 1)	4.75 (n = 4)	5.6 (n = 3)

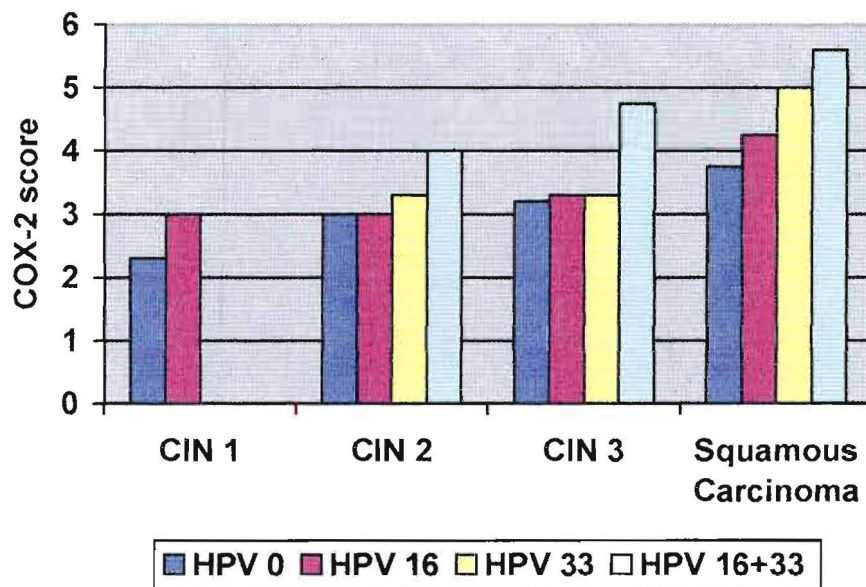


Figure 4.3 Bar graph illustrating the mean COX-2 scores for each HPV type within each histological group (summarised in Table 4.1). There is an incremental increase in COX-2 expression from CIN 1 through to squamous carcinoma. Dual infection with HPV types 16 and 33 showed the highest level of COX-2 expression within each histological group.

Table 4.2 Summary of individual case results

number	specimen	HPV type	pathology	intensity	extent	total	inflam
1	bx	-ve	normal	0	0	0	0
2	bx	-ve	normal	0	0	0	1
4	bx	-ve	normal	0	0	0	1
5	bx	-ve	normal	0	0	0	1
6	bx	-ve	normal	0	0	0	0
7	bx	-ve	normal	0	0	0	1
8	mfu	-ve	normal	1-endo	2-endo	3-endo	0
9	bx	-ve	normal	2-endo	1-endo	3-endo	1
10	mfu	-ve	normal	1-endo	1-endo	2-endo	1
11	mfu	-ve	normal	1-endo	1-endo	2-endo	2
12	mfu	-ve	normal	0	0	0	0
13	mfu	positive	normal	2-endo	2-endo	4-endo	1
14	mfu	18	normal	2-endo	3-endo	5-endo	3
15	mfu	-ve	normal	1-endo	2-endo	3-endo	0
16	mfu	-ve	normal	1-endo	1-endo	2-endo	2
17	mfu	-ve	normal	1-endo	1-endo	2-endo	0
18	mfu	positive	normal	2-endo	2-endo	4-endo	2
19	bx	-ve	cin-1	1	2	3	1
20	bx	-ve	cin-1	1	1	2	1
21	lletz	-ve	cin-1	1	1	2	3
22	lletz	16	cin-1	2	1	3	1
23	lletz	-ve	cin-2	2	1	3	1
24	lletz	-ve	cin-2	2	1	3	1
25	bx	-ve	cin-2	2	1	3	0
26	lletz	-ve	cin-2	1	2	3	1
27	lletz	16	cin-2	2	1	3	1
28	bx	33	cin-2	2	1	3	1
29	lletz	33	cin-2	2	2	4	1
30	lletz	33	cin-2	1	2	3	2
31	lletz	16+33	cin-2	1	3	4	1

number	specimen	hpv type	pathology	intensity	extent	total	inflam
32	lletz	-ve	cin-3	2	1	3	2
33	lletz	-ve	cin-3	2	1	3	1
34	lletz	-ve	cin-3	2	2	4	1
35	lletz	-ve	cin-3	1	1	2	2
36	lletz	-ve	cin-3	1	2	3	2
37	bx	-ve	cin-3	2	2	4	2
38	lletz	33	cin-3	2	1	3	3
39	lletz	33	cin-3	2	2	4	2
40	lletz	33	cin-3	1	2	3	0
41	bx	16	cin-3	1	1	2	1
42	bx	16	cin-3	2	2	4	2
43	bx	16	cin-3	2	2	4	3
44	bx	16+33	cin-3	3	2	5	1
45	lletz	16+33	cin-3	2	2	4	1
46	lletz	16+33	cin-3	3	3	6	3
47	lletz	16+33	cin-3	2	2	4	1
48	bx	-ve	ksc	2	2	4	2
49	bx	-ve	ksc	3	2	5	1
50	bx	-ve	ksc	2	1	3	3
51	bx	-ve	lcnksc	2	1	3	1
52	bx	16	ksc	3	2	5	3
53	bx	16	ksc	2	2	4	1
54	bx	16	ksc	3	2	5	2
55	bx	16	ksc	2	1	3	3
56	bx	33	lcnksc	3	2	5	2
57	bx	16+33	ksc	3	3	6	3
58	bx	16+33	ksc	3	3	6	2
59	bx	16+33	lcnksc	3	2	5	1

KEY: bx – biopsy, lletz – lletz excision, mfu – multi-fibroid uterus, ksc – keratinising squamous cell carcinoma, lcnksc – large cell non-keratinising squamous cell carcinoma

The total COX-2 score is derived by the sum of both the intensity and extent scores, and hence is scored out of a total of 6.

CHAPTER FIVE**DISCUSSION**

This is the first study to examine the expression of COX-2 in the normal and diseased uterine cervix. In addition, this study has correlated the COX-2 expression in cervical dysplasia and carcinoma with specific HPV types. The absence of similar published studies does not allow for direct comparison of results. However, studies of COX-2 in malignancies involving other organ systems, particularly the GIT [103,107,109,110], lung [111,112] and head and neck region [106] have been reported and it is from these studies that analogies will be drawn.

5.1 GENERAL CONSIDERATIONS

The total number of cases of each HPV type, within each histological category, are too few for meaningful statistical analysis. This is due the study being limited to those cases with cervical pathology that had already undergone HPV typing and on which histological material was available. The small prospective component was done in order to obtain a “normal” group. This also accounted for the HPV typing having two methodologies. The retrospective cases had all undergone HPV typing using PCR My09/11 primers with subsequent type specific primers. In the last eighteen months, this method for typing has been replaced by a RFLP method in conjunction with nested PCR to type HPV, a method accepted as being more sensitive and the preferred method of HPV typing in our Virology laboratory at present [114]. A proportion of the cases tested previously were negative for HPV, yet still showed histological evidence of dysplasia or carcinoma. It is possible that these cases harboured HPV types not present in the My09/11 primer, which amplify a 450base pair sequence of the L1 open reading frame, or that the primer was not sensitive enough to detect HPV that was

present in very small quantities i.e. they may be false negative results. These cases are currently being re-examined for HPV using the newer method, as a HPV negative cervical carcinoma, in all probability, does not exist. These results are not yet available for inclusion in this study.

5.2 NORMAL CERVIX

There are no other studies describing the expression of COX-2 in the normal uterine cervix within the current literature. Certainly nothing is known about the role of COX-2 in the normal menstrual cycle – particularly whether there a constant weak level of expression, or whether there is induced cyclical upregulation. All 18 “normal” cases studied showed expression of COX-2 in the histologically normal endocervical epithelium and no expression in the normal ectocervix. 17 of these cases showed weak COX-2 expression within the cytoplasm of the endocervical surface epithelium and glands (Figure 4.4, 4.5). The level of expression was considerably less than that seen in the dysplastic and malignant epithelium. These findings in the normal cervical epithelium are similar to the COX-2 expression in the normal squamous epithelium adjacent to head and neck squamous carcinoma [106] and oesophageal carcinoma [111], where the normal epithelium adjacent to tumour showed either weak expression of COX-2 or no expression at all. 11 of the “normal” cases showed a stromal mononuclear inflammatory infiltrate which exhibited weak expression of COX-2. However one case, case 14, which was found to harbour HPV 18 on PCR, but which showed no histological evidence of dysplasia, showed a strikingly increased expression of COX-2 in the endocervix compared to the other “normal” cases (to be discussed in more detail in section 5.4). In addition the stromal inflammatory infiltrate was more intense than in the other cases, and also showed increased COX-2 expression (Figure 4.6). This is in keeping with the role of

COX-2 in inflammatory states where studies of rheumatoid arthritis [99] and osteoarthritis [100] have shown that the intensity of COX-2 staining of synovial tissue is directly proportional to the degree of adjacent mononuclear inflammation present.

Some expression of COX-2 would be expected in the normal cervix as COX-2 does play a role in prostaglandin regulation in gestation and parturition [61,77,89,90,91] and it is the endocervical epithelium, which undergoes metaplasia and dysplasia in the development of cervical neoplasia. More recent studies however, have shown that the cervical PGE₂ upregulation in the pregnancy state is induced predominantly by nitric oxide – a powerful inducer of COX-2 - rather than by COX-2 itself [92].

In addition COX-2-induced PGE₂ is intimately involved in mediating the inflammatory cascade [75,76]. The stromal inflammatory response present may well be induced by the viral activation of cytokines, which are the major mediators of increased COX-2 activity in inflammatory diseases such as rheumatoid arthritis [75].

5.3 CIN AND CARCINOMA

The number of cases of each HPV type, within the dysplasia and carcinoma categories, are too few to allow meaningful statistical analysis. However, a number of interesting trends are evident, even allowing that some of the results may be “false negatives”. All cases of CIN 1, -2, -3, and carcinoma showed COX-2 expression within the diseased epithelium. In addition there appeared to be an incremental increase in COX-2 expression from CIN 1 through to carcinoma as depicted by the COX-2 scores (Table 4.1, Figure 4.3). The highest COX-2 scores in the categories of CIN 2, CIN 3 and invasive carcinoma were the cases showing dual HPV infection by types 16 and 33, with the carcinoma group showing by far the highest mean COX-2 score (Table 4.1).

Several studies suggest that a dual infection involving a combination of a low-risk and a high-risk type is less likely to progress to a high-grade dysplasia and carcinoma [18,19,20]. However, the results of this study, even though limited by the small number of cases, showed that the cases with dual infection still progressed to CIN 3 and squamous carcinoma (Table 4.1). Dual infection by HPV types 16 and 33, as seen in this study, represents a combination of a moderate-risk type (33) and a high-risk type (16) virus and may have a higher incidence of progression to CIN 3 and carcinoma than the combination of a low-risk and high-risk HPV type. The observation that dual infection resulted in the highest COX-2 expression, and that carcinoma showed more expression than the CIN cases, suggests that the amount of COX-2 upregulation is dependent on the total viral load. It can also be deduced that COX-2 upregulation must occur as a separate, early event, in cervical carcinogenesis and has no direct role in interfering with the Rb protein or the tumour suppressor gene, p53.

5.4 HPV, COX-2 AND CERVICAL CARCINOGENESIS

COX-2 upregulation in malignancies involving the lung, oesophagus, stomach, colon and rectum, and head and neck region are well documented [103,106,109,110,111,112]. Similar to the findings in this study, squamous cell carcinomas in the head and neck region [106], oesophagus [110] and lung [111] have all shown similar upregulation of COX-2. Of interest is the observation that precursor lesions in the lung (atypical alveolar hyperplasia) and dysplastic lesions in the head and neck region, also showed increased COX-2 expression as compared to the normal epithelium.

Case 14, in which the cervical epithelium was histologically normal, was found to harbour HPV 18 on typing. The endocervical epithelium and stromal inflammatory cell expression of

COX-2, as discussed earlier, was far more intense than the staining seen in the HPV-negative “normal” cases. Also of importance is the fact that this case showed no histological evidence of dysplasia, suggesting that the virus was in the episomal form. Even though these features are present in only a single case and no follow-up is available on this patient, it does suggest that COX-2 upregulation is an early event which must occur while the virus is still in the episomal phase and prior to the development of histologically evident cervical dysplasia. This finding is paralleled in studies investigating the role of COX-2 in mouse colonic adenomas [108] which showed that COX-2 upregulation is an early event in the colonic adenoma – carcinoma sequence. Furthermore it has been established that in mouse colonic adenomas, COX-2 expression occurs coincidentally or slightly after the loss of the wild-type APC allele suggesting a direct role of APC loss in COX-2 overexpression [109]. Studies looking at lung carcinomas and precursor lesions have found an association between K-ras oncogene activity and COX-2 upregulation [111].

Clearly there are a number of different events, in different organ systems, which result in COX-2 upregulation. The net effect of upregulation of COX-2 is increased PG synthesis, particularly PGE₂, which has been shown to promote angiogenesis and inhibit immune surveillance [61,67,105]. It has been shown that selective inhibition of COX-2 in oesophageal cancer cells induces apoptotic cell death and reduces proliferative activity, and that these effects correlate with the inhibition of PG synthesis [110].

Another interesting finding in a study looking at epithelial cell lines [105], is an increase in BCL-2 protein, an anti-apoptotic protein, following COX-2 activity. BCL-2 is associated with a number of different malignancies, particularly leukaemias and lymphomas, but it is also expressed in breast cancer, prostate cancer and lung carcinomas [115]. A recent study by Dimitrakakis et al [116] showed that the expression of BCL-2 was found to increase in direct

relation to the grade of CIN. Other studies have also confirmed the presence of BCL-2 expression in cervical squamous cell carcinoma and have shown its' presence to indicate the likelihood of a good response to radiotherapy. [117,118,119]. Interestingly BCL-2 acts synergistically with a number of viruses – human adenovirus, Epstein-barr virus and baculoviruses to inhibit apoptosis [120]. Increased expression of BCL-2 alone is almost certainly not sufficient to induce the tumour state, rather it promotes cell viability and thus allows additional genetic events to occur, the accumulative effect of which is malignant transformation [105]. BCL-2 was not part of the design of this study but will be worth investigating as an additional study in the future, in these and other cases.

It is not yet clear how many factors play a role in cervical carcinogenesis. One possible scenario is that COX-2 expression is induced by HPV infection of the cervical epithelium as an early event via inflammatory associated cytokines. Indeed, this study has shown the stromal inflammatory response to be increased in a case of possible episomal HPV infection. An elevated COX-2 level may well interact with BCL-2 or other oncogenes, such as K-ras, to retard the process of apoptosis. This in turn would aid the well known HPV-associated E6/E7 protein interference of p53 and the Rb gene resulting in the progression to cervical dysplasia and carcinoma. In this way COX-2 would act as a cofactor to cervical carcinogenesis.

Current evidence suggests that our knowledge of the broad spectrum of biological activity of COX-2 is increasing rapidly [61,64,72] and further studies investigating potential alterations of specific physiological or protective mechanisms by inhibition of COX-2, are expected. It remains to be seen whether COX-2 inhibitors, such as NSAIDS or the COX-2 specific agents such as Celocoxib, have any effect on cervical carcinoma cell cultures or on the progression of cervical disease in animal models and subsequently in humans. The work to date in mouse models for colorectal carcinoma [73,108], studies looking at the expression of COX-2 in

human colorectal adenomas and carcinomas [103], and human epidemiological studies investigating the incidence of colorectal carcinoma [107], have shown a definite therapeutic role for COX-2 inhibitors. Certainly more investigative work needs to be done, specifically probing for any association between COX-2 and BCL-2 or other oncogenes such as K-ras, and the role of cervical nitric oxide in inducing COX-2 upregulation. Studies have shown that COX-2 plays an important role in pathological processes other than pain and inflammation and ongoing research is needed to investigate the role of specific COX-2 inhibitors in treating and preventing conditions such as colonic polyposis, colorectal tumours and Alzheimer's disease.

5.5 CONCLUSION

1. COX-2 is expressed in small amounts in the normal cervical endocervix but not in the normal ectocervical squamous epithelium.
2. All cases of cervical dysplasia and carcinoma express COX-2 with the highest levels seen in CIN2, CIN3 and squamous carcinoma. Dual HPV infection correlated with the highest expression of COX-2.
3. Upregulation of COX-2 appears to be an early event which occurs when the HPV virus is still in the episomal form and prior to the development of histologically evident dysplasia.
4. The possible role of COX-2 inhibitors in the prevention and treatment of cervical dysplasia and carcinoma needs to be investigated further.

In conclusion, this study has reported a possible role for COX-2 in cervical carcinogenesis. Further studies are needed to determine whether inhibition of COX-2 has any preventative or therapeutic contributions to make in the field of HPV-associated cervical neoplasia.

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