CRANFIELD UNIVERSITY

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Molecular Basis for Maize as a Risk Factor for Oesophageal Cancer in a South African Population

Cranfield BioMedical Centre

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Molecular Basis for Maize as a Risk Factor for Oesophageal Cancer in a South African Population

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Abstract

Throughout the world squamous cell carcinoma of the oesophagus seems to be an increasing problem. There is a huge variation in prevalence globally; locations such as Japan, Iran, China and Finland can have ten times the prevalence compared to other western countries. One place that is hugely affected is Transkei, a 16,000 square mile area of South Africa. Some of the factors proposed to be implicated with squamous cell carcinoma in this region include tobacco smoking, alcohol consumption, bacterial infections and fungal infection of common food crops. In addition, the 'Sammon theory' links carcinogenesis in Transkei to the high consumption of maize by the population. Through a chain of reactions it is postulated that a component of maize inhibits the breakdown of growth factors, which have already been implicated in cancer.

This study investigates the Transkei population and updates the Sammon theory with current research to predict a theory at a molecular level. This theory is then tested with novel research to show PGE_2, shown here in high concentrations in gastric fluid samples, directly increases the proliferation of oesophageal cell lines. Gastric fluid samples from the Transkei population are then shown to have a mitogenic effect on oesophageal cells, supporting a theory that gastric fluid regurgitation commonly found in this population predisposes them to cancer. Further experimentation on the expression of related proteins shows how high PGE_2 may increase its own production by increasing COX 2 expression, leading to a positive feedback loop causing constant proliferative stimulation of the oesophageal squamous tissue in the presence of the COX 2 substrate, aracadonic acid.

Therefore this thesis suggests that a high maize diet provides the correct conditions for regurgitation of increased concentrations of PGE_2 into the oesophagus leading to squamous hyper-proliferation over long periods of time through self stimulated production, which would normally have ceased over a much shorter time if only localised PGE_2 was produced through natural restitution.

Acknowledgments

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Contents

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Abbreviations

AA – Arachidonic acid

ABC - Avidin-biotin complex

AJCC – American joint commission on cancer

APC – Adenomatous polyposis coli gene

ATCC – American type culture collection

BAX – A pro-apoptotic protein

Bcl-2 – B-cell leukemia oncogene 2

BrdU – 5-bromo-2'-deoxyuridine proliferation assay

CD44 – Cluster of differentiation marker 44

cMyc – Cellular myelocytomatosis oncogene

COX - Cyclooxygenase

CT – Computer tomography

DAB – Diaminobenzidine

DGOR - Duodenogastro-oesophageal reflux

DMEM – Dulbecco's modified eagle medium

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

EAOCT - Environmental Associations with oesophageal cancer in Transkei

ECACC - European collection of cell cultures

EGF - Epidermal growth factor

EGFR - Epidermal growth factor Receptor

ELISA - Enzyme-Linked Immunosorbent Assay

FCS- Fetal calf serum

Het1a – Normal oesophageal squamous epithelium cell line

HGF – Hepatocyte growth factor

HPV - Human papillomavirus

HRP – Horseradish Peroxidase

LOX - Lypoxygenase

Mdm2 - Murine double minute 2 oncogene

MRI- Magnetic resonance imaging

MTT - 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

NDGA – Nordihydroguaiaretic acid

NMBA - Nitrosomethylbenzylamine

NSAID – Non-steroidal anti-inflammatory drug

ND:YAG - Neodymium:yttrium-aluminum-garnet

Oe21 – Oesophageal squamous cell carcinoma cell line.

OSSC – Oesophageal squamous cell carcinoma

p21 - 21 kDa tumour suppressor protein

p53 – 53 kDa tumour suppressor protein

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PGE_2 – Prostaglandin E_2

PGES – Prostaglandin E Synthase

PLA2 – Phospholipase A2

pH – Logarithmic measure of hydrogen ion concentration

Rb - Retinoblastoma tumour suppressor gene

RT-PCR – Reverse transcriptase polymerase chain reaction

SCC – Squamous cell carcinoma

TB - Tuberculosis

TBS – Tris buffered saline

TGF - Transforming growth factor

TNM – Tumour-node metastasis status

VEGF – Vascular endothelial growth factor

1. Introduction

1.1. Introduction

The following chapter will provide the underlying information required to understand the work that has taken place in this thesis. This will start with the basic physiology and histology of the oesophagus and provide information on general oesophageal cancer. Much of this information is provided for a background of oesophageal cancer to complete the readers understanding and not required to understand the study itself. Information relating to the epidemiological aspect of oesophageal cancer factors world wide are briefly explained, along with some molecular pathology and the proteins relating to this study. This study has been based on a theory originally developed by cancer surgeon, Alistair Sammon, therefore his work and research has been explained at the end of the chapter. As one of the aims of this work was to develop his theory and to extend the research by adding a molecular understanding to carcinogenesis in the target population, new concepts and factors are introduced and discussed in subsequent chapters.

According to a full literature survey the research in this thesis has never been carried out before in this target area, presenting interesting novel work. Due to the nature of this research the study is more than the pathological analysis of the target population. A large area of this research is based on the epidemiological effects on the population and how this can drive changes at a molecular level to better understand the local pathology. Although this study is essentially biological, a great amount of work has been necessary to understand the underlying sociological factors of the target population. Therefore, chapter 2 discusses these factors with reference to carcinogenic and molecular effects. Chapter 3 evaluates the environmental investigation of the target population in chapter 2 and links this with previous molecular research and the Sammon Theory outlined below (section 1.9). This postulated molecular pathogenesis provides a platform for the experimental research in this study. Chapter 4 explains the ethics and sample collection carried out in South Africa. Chapter 5 investigates the gastric fluid samples used in the study, studying sample number, Prostaglandin E2

(PGE_2) content, the detrimental effect of transportation on the samples, and checking their significance and validity for the study. Chapter 6 describes the models used to test the proliferative effects the gastric fluid and its components could have on the oesophagus in the target population. This provides the fundamental information as to how a high maize diet may lead to hyperplasia in the oesophagus, predisposing this epithelium to cancer. Chapter 7 investigates suggested molecular changes within the target population caused by a high maize diet. Changes in the amounts of growth promoting proteins acting on these oesophageal cells are therefore studied. An important novel molecular positive feedback pathway is discovered in chapter 7 that is investigated and evaluated in chapter 8. This is then concluded and summarised in chapter 9 and further work is discussed.

1.2. The Oesophagus.

1.2.1. Anatomy of the Oesophagus

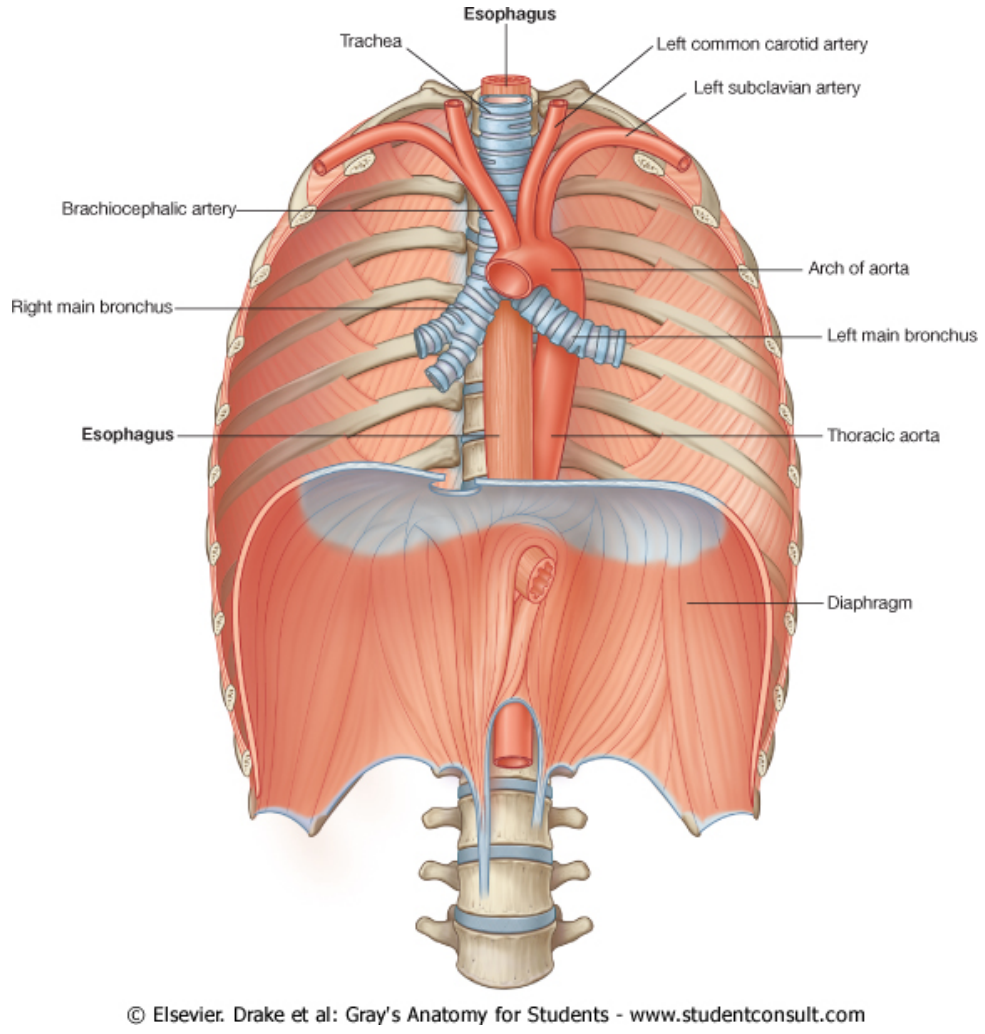


Figure 1.1: The anatomy of the Oesophagus (Drake, Vogel & Mitchell, 2005)

The oesophagus is a tubular muscular organ of around 25-30 cm in length. It runs from the pharynx joining the cardia of the stomach, via the diaphragm. It passes anterior to the trachea, down the front of the spine, and makes up the first part of the digestive system to encounter food after ingestion (fig 1.1) (Rubin & Farber, 1988; Vander *et al.*, 1994). The oesophagus runs straight through the mediastinum of the

thorax and through the muscular right crus of the diaphragm at the oesophageal hiatus, joining the stomach at the cardiac orifice (Drake, Vogel & Mitchell, 2005). There is a large vascular network round the oesophagus consisting of both a cardiovascular system and the lymphatic system (Cotran *et al*., 1999).

The oesophagus can be subdivided in to three separate sections: The cervical oesophagus, the thoracic oesophagus, and finally the lower oesophagus. Throughout these different sections there are narrowings, including two 3cm areas that remain contracted even in the resting phase of the surrounding muscle, which are known as the upper oesophageal and lower oesophageal sphincters. These aid the muscular lumen in carrying out its primary function of channelling food boli and fluids to the stomach via a peristaltic motion avoiding reflux of the gastric contents back into the oesophagus. In order for the upper oesophageal muscle to cause this propulsion there is striated muscle, however the lower oesophagus is mainly comprised of smooth muscle under autonomic neural control (Rubin & Farber, 1988; Gray, 1991), see Figure 1.2. The lower oesophageal sphincter is tonically active but relaxes on swallowing. Control seems to rely on four complementary factors: diaphragmatic contraction, greater intra-abdominal pressure than intragastric pressure being exerted upon the abdominal part of the oesophagus, unidirectional peristalsis and maintenance of correct anatomical arrangements of the structure (Young *et al.* 2006). A contraction of the muscle is present under vagal release of acetylcholine and a relaxation of the muscle is due to nitric oxide and vasoactive intestinal polypeptide release (Ganong, 2003). This tight control prevents reflux of gastric contents back into the oesophagus. The contents of normal fasting gastric fluid contains a number of substances that may have an effect on the oesophagus. The gastric fluid contains cations (Na^+, K^+. Mg^{2+}, H^+), anions (Cl-, HPO_4^{2-}, SO_4^{2-}), pepsins, lipase, mucus and intrinsic factor (Ganong, 2003).

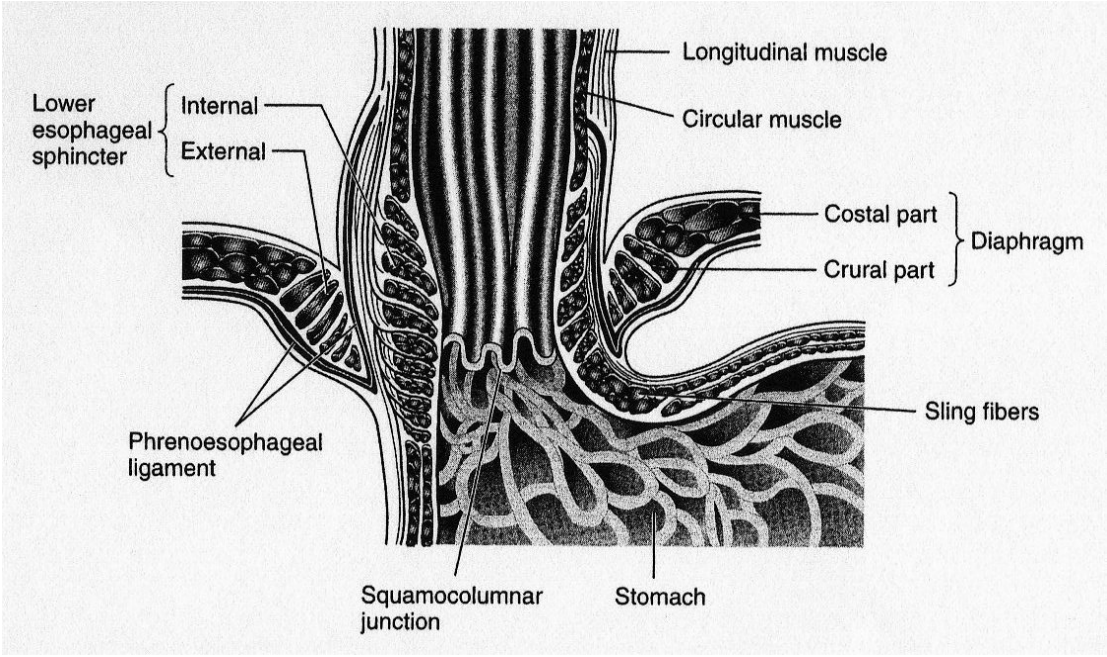


Figure 1.2: Oesophagogastric junction. (Mittal & Balaban, 1997)

1.2.2. The Histology of the Oesophageal Tract

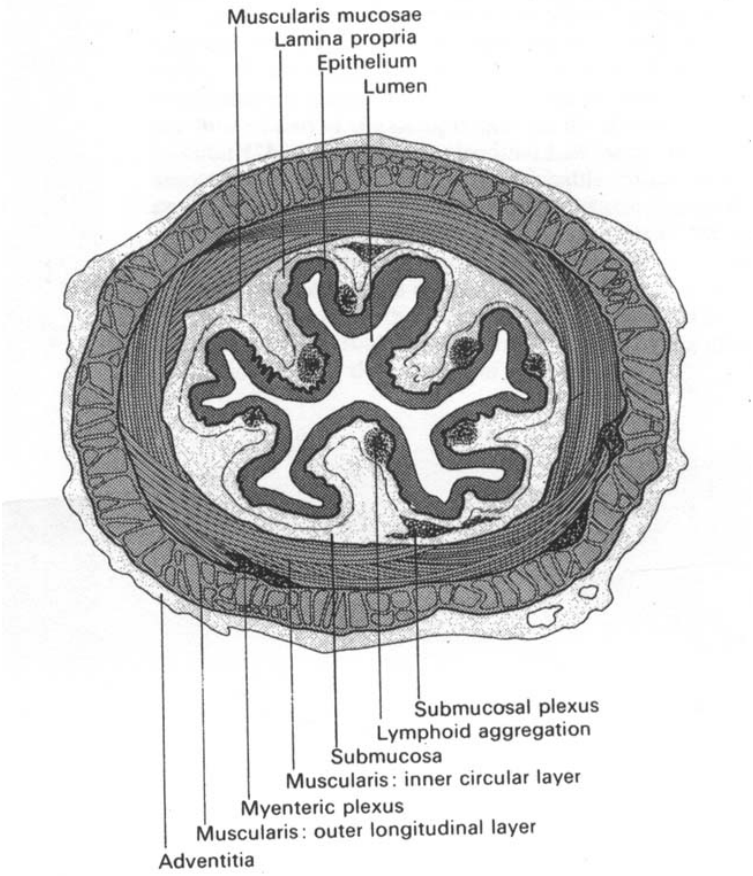


Figure 1.3: The defined cellular layers of the oesophageal tract (Wheater *et al.*, 1979).

There are three distinct layers of the oesophageal tissue, the mucosa, submucosa and muscularis (Fig 1.3). The mucosa is around 500-800 μm thick made up of the non-keratinizing stratified squamous epithelium supported by the connective tissue, the lamina propria. The muscularis mucosae is a 300μm thick smooth muscle layer, which moves and folds the mucosa (Wheater *et al.*, 1979). The squamous epithelial layer has a basal area occupying around 10-15 percent of the epithelium consisting of several layers of cuboidal or oblong basophilic cells with dark nuclei (Day & Dixon, 1995) (Fig 1.4). It is this basal layer that proliferates to replace those cells sloughed

off as food passed through the lumen. Squamous cell carcinoma (SCC) is derived from this layer (King, 2000). Between the squamous cells, Langerhans cells, part of the immune surveillance system, can be found (Day & Dixon, 1995). The lamina propria is the non-epithelial part of the mucosa consisting of lymphocytes, plasma cells, connective tissue, vascular structures and oesophageal cardiac glands, which are mucus secreting (Cotran *et al*., 1999). It is from these glands that adenocarcinoma arises (Rubin & Farber, 1988).

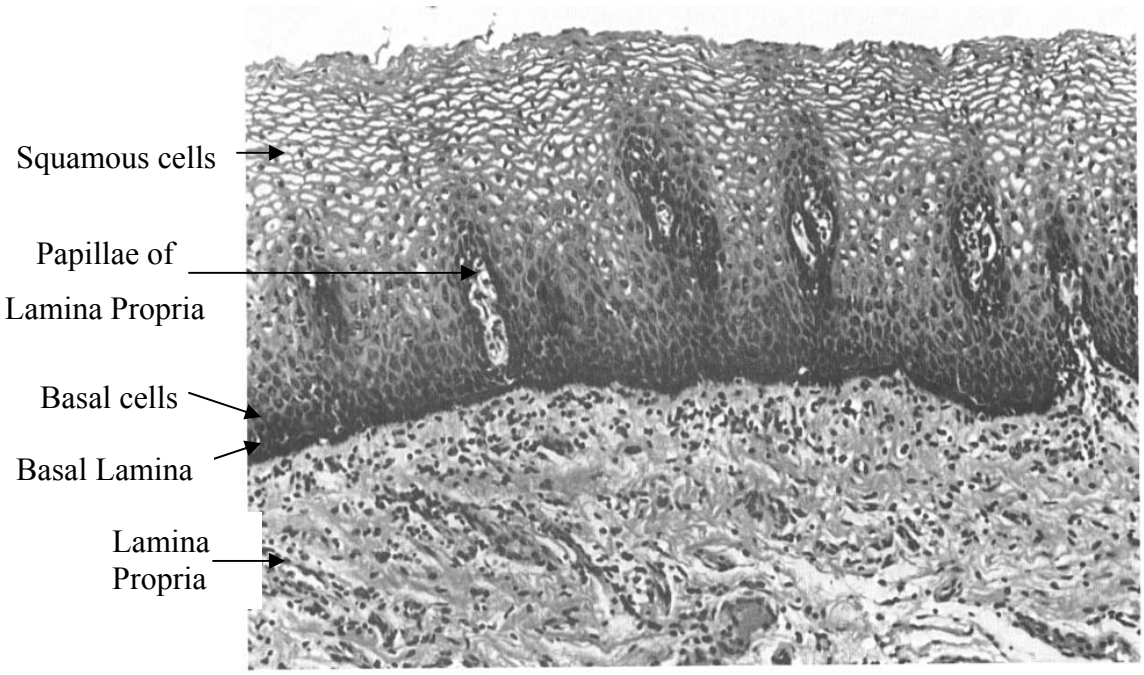


Figure 1.4: Normal oesophageal Squamous epithelium with narrow basal zone (Day & Dixon, 1995).

Just short of the gastro-oesophageal junction, a 3 –5 cm long segment is lined with surface mucin-secreting columnar epithelium, rather than the usual squamous epithelium (Day & Dixon, 1995).

The submucosa consists mainly of loose connective tissue supporting the mucosa containing the blood vessels and a network of lymphatics. As well as parasympathetic nerve fibres and leukocytes, the submucosal glands can be found (Wheater *et al*.,

1979). These vessels are normally found in high amounts around either end of the oesophagus, although they can be found all the way along it (Day & Dixon 1995). The glands are thought to be continuations of minor salivary glands secreting both mucus and sulphomucins (Cotran *et al.,* 1999).

The third layer, the muscularis, is subdivided into two histological layers: an inner circumferential layer and an outer longitudinal layer of smooth muscle. These layers are sitting at right angles to each other therefore offer the basis of the peristaltic movement (Gray, 1991).

In much of the gastrointestinal tract there is a fourth layer, the subserosa/serosa. Instead of this the oesophagus has a fibrous adventitia composed entirely of connective tissue, which blends with surrounding structures along its route. The absence of a subserosa/serosa, exacerbated by a prominent lymphatic network, can facilitate the spread of infections and tumours from the oesophagus into the posterior mediastinum. (Cotran *et al*., 1999).

1.3. Cancer

There is no one factor that causes the development of cancer. There are many different aspects to investigate when assessing why and how a cancer is progressing. Some of the influential factors that can affect processes include cell death, proliferation rate, cell differentiation, surrounding cell response, immune attack, control of blood supply, and composition of extra cellular matrix. All of these have been extensively researched.

1.3.1. Tumourigenesis

The generation of cancer is termed tumourigenesis, and has many different states resulting from accumulation of errors in vital regulatory pathways that control normal cell growth. The process by which this takes place is split into a series of stages termed initiation, promotion and progression (Figure 1.5). The term tumourigenesis is refers to more of a physical genesis and is often staged by changes in tissue growth characteristics. The term carcinogenesis is often characterised as changes in the tissue at a molecular level through changes in chemically induced cancer. In this thesis both tumourigenesis and carcinogenesis are used in the same context, as the overall generation of cancer, both at a tissue and molecular level.

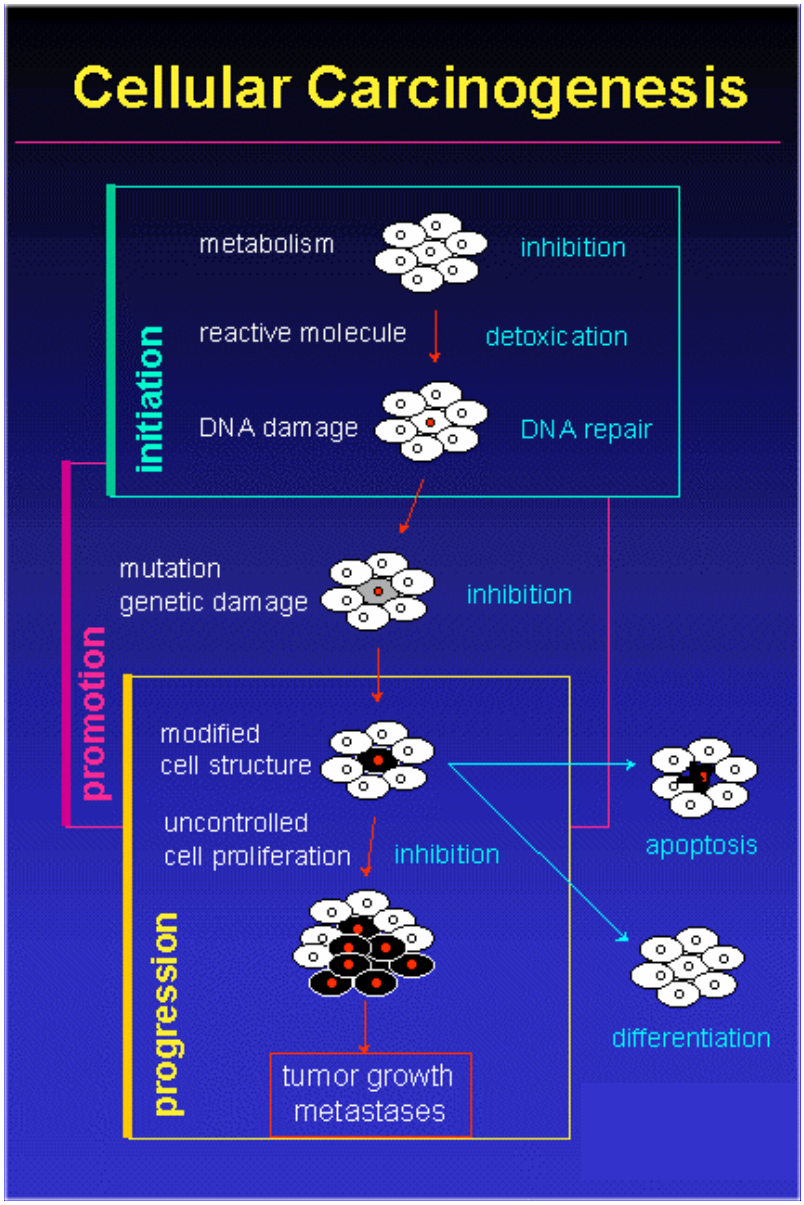


Figure 1.5: The process of carcinogenesis (Gerhäuser, 2002)

The irreversible stage of initiation is presumed to be a consequence of a genetic mutation (such as transitions, transversions and deletions) that increases a cell's propensity to proliferate. This can be via external or internal influences, for example radiation or the presence of free radicals. It is mainly postulated that the points of these mutations can coincide with protooncogenes/cellular oncogenes or tumour suppressor genes. Examples of these are *Bcl-2*, which translates a protein that normally blocks apoptosis, and p53 which encodes a protein which can halt cell division and induce abnormal cells to kill themselves (Larsen, 1994). The cellular

and molecular changes reflecting the stage of initiation in gastrointestinal carcinogenesis have been extensively researched and further explained below (section 1.7).

A promotional agent drives a tumour to the premalignant state and encourages its expansion in cell number; this agent does not have to be geno-toxic, and can be reversible at both genetic and cellular level. With the enhancement or repression of gene expression by receptors, which are specific to the promoting agent, then cellular proliferation or inhibition of apoptosis can promote cell population.

Following promotion, two important processes occur: The formation of blood vessels which is called angiogenesis, allowing tumours to grow larger than 1mm in diameter; and a change in the amount of cellular proliferation, termed hyperplasia. From this point and through the progression, further mutations may take place. This results in the loss of basic control of cellular growth, changing the differentiation state of the cells and the cells surrounding it, leading to dysplasia and *in situ* carcinoma. Common examples of promotional factors that have been researched in squamous oesophageal cancer are tobacco smoke (Yu *et al.,* 1988) and alcohol (Hu *et al.,* 1994).

Progression induces cells into a stage of irreversible promotion via complex genetic alterations such as deletions, translocations and gene amplification, driving phenotypic instability. This state of dedifferentiation demonstrates increased autonomous growth and aggressive behaviour (King, 2000). At this stage the promotion agent no longer needs to be present for the tumour to develop.

Several changes can be seen in progression, including an altered sensitivity for the surrounding cells, changes in the amount of growth factors and receptor changes. This allows the growth characteristics to change and to grow out of its normal tissue. These metastasise round the body via surrounding blood vessels or the lymphatic system (Woodman, 2001).

1.4. Oesophageal Cancer

Oesophageal cancer is the sixth most common cancer worldwide (Morris *et al.*, 1998) with an estimated 335,000 deaths a year (Altorki, 2003). In the UK Oesophageal cancer is the fifth most common cancer with around 7230 deaths in 2004 (Office for National Statistics, 2007) There are various types of oesophageal cancer but most occurrences are restricted to two: Squamous cell carcinoma and Adenocarcinoma. A majority of cases around the world are due to squamous cell carcinoma, the principal cancer investigated in this study, although much of the research has been carried out on adenocarcinoma because of links to western lifestyles like alcohol and tobacco consumption, stress etc.

1.4.1. Squamous Cell Carcinoma

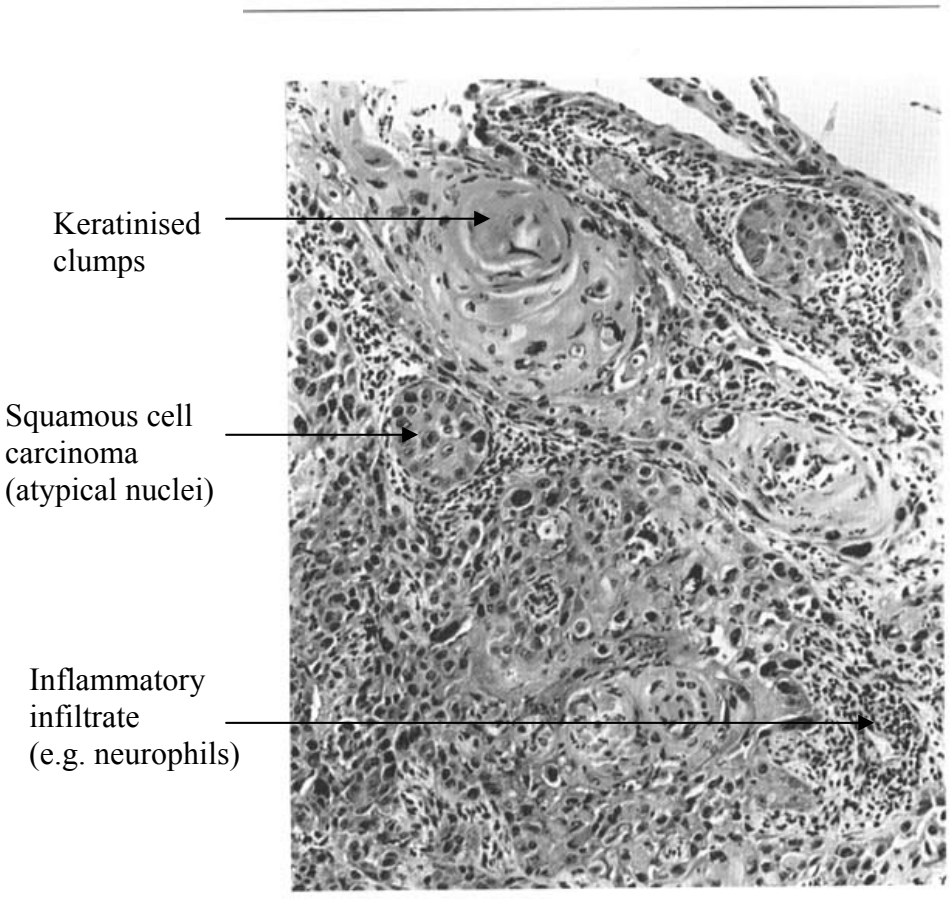


Figure 1.6: Well-dedifferentiated Squamous cell carcinoma with central keratinisation in many of the cell clumps. (Day & Dixon, 1995)

Squamous cell carcinoma (Fig 1.6) arises from the mucosa of the oesophagus in the squamous epithelium. It is histologically characterized by being invasive, with a distinct ragged stromal-epithelium (Anderson & Lad, 1982) or "exophytic, ulcerating or infiltrating lesions or a combination of these, and often result in a stricture which is usually irregular and haemorrhagic" (Day & Dixon, 1995). These are mainly located in the thoracic oesophagus; about 60 percent of cases in the middle third and 30 percent of cases in the distal third (Anderson & Lad, 1982).

1.4.2. Adenocarcinoma

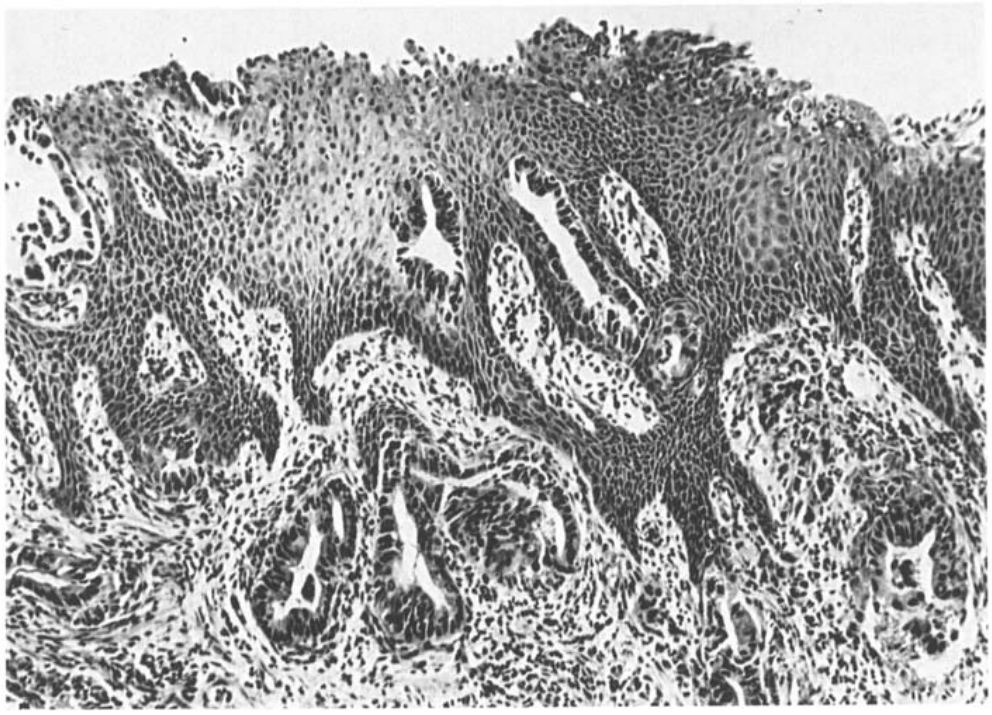


Figure 1.7: Well differentiated adenocarcinoma underling oesophageal epithelium from the lower end of the oesophagus. (Day & Dixon, 1995)

Adenocarcinoma is usually found in the lower third of the oesophagus (Cotran *et al*., 1999) (Figure 1.7) arising from the oesophageal glands. Gastric cancers arising at the gastro-oesophageal junction are often mistakenly assumed to be oesophageal adenocarcinoma (Day & Dixon 1995). A considerable amount of research has gone into the pathogenesis of a condition called Barrett's oesophagus/mucosa. This is where squamous epithelium is replaced by columnar epithelium resembling the cells that line the stomach, and is associated with chronic gastric reflux. This is thought to be a major premalignant predisposing condition that leads to adenocarcinoma. The incidences of this are increasing, mainly in white men. This is thought to be due to the increased consumption of alcoholic beverages and tobacco smoke in many areas of

the world (Rubin & Farber, 1998) and/or increasing stress (Ronson, 2006). A study of the genetic expression between adenocarcinoma and squamous cell carcinoma cell lines showed no gene was up or down regulated between the two. However, the microarray used had only limited numbers of genes related to oesophageal cancer (Kan *et al.*, 2001).

1.4.3. Small cell carcinoma

This may also be known as oat cell carcinoma, argyrophil cell carcinoma, neuroendocrine carcinoma or anaplastic carcinoma. First described by McKeown (1952) it is only seen in the bottom two thirds of the oesophagus. Only a small percentage of all oesophageal cancer cases present this type, once reviewed at 2.4 percent of all primary oesophageal carcinomas with this morphology (Day & Dixon, 1995). Histologically, they are renowned for a rosette like formulation, from both squamous and glandular tissue. Some people view these to be a metastasis from lung tissue (Briggs & Ibrahim, 1983)

1.4.4. Benign tumours

Benign tumours of the oesophagus are usually leiomyomas (benign smooth muscle tumours e.g. uterus and digestic tract) and seen in the smooth muscle of the oesophageal wall. However, lymphangiomas (benign angioma e.g. lymphatic system), lipomas (benign tumour of fat cells) and fibromas (benign fibrous tissue e.g. extracellular matrix) can also be present. A mass of vascular, fibrous and adipose tissue covered by an intact mucosa are seen in mucosal polyps and are often just cut out with endoscopy if they grow too big and impede bolus food from being swallowed (Cotran *et al*., 1999).

1.5. Diagnosis, Prognosis and Treatment of Oesophageal Cancer

1.5.1. Symptoms

Progressive weight loss in a short time is seen in up to 70 percent of the patients, mainly due to dysphagia (Ojala *et al.,* 1982; Galandiuk & Hermann, 1986). This primarily begins with difficultly swallowing bolus solids and can progress to liquids, owing to around two thirds of the lumen being obstructed. Little pain can be seen up to this stage; hence cancer can advance without the patient reporting the case. As the cancer increases and the lumen size diminishes then other than uncomfortable swallowing, pain can be associated with the dysphagia (called odynophagia) radiating in the back, arms, neck and jaw (Rubin & Farber, 1988). Pain can arise if the cancer spreads to the local lymph nodes, liver, lungs and pleura. Severe suffering as the disease develops could be due to tracheo-oesophageal fistulas (Braunward, 2001). These and cervical oesophageal tumours are thought to be related to the coughing and hoarseness seen in around a quarter of all cases. In between a third and a half of all cases, vomiting and regurgitation has been seen making the symptoms more aggravated and painful (Galandiuk & Hermann, 1986).

1.5.2. Diagnosis

Owing to the lack of symptoms at the early stages of oesophageal cancer development, a high index of suspicion is required to diagnose early. There are two primary techniques that can be used to diagnose oesophageal cancer: the oesophagoscopy or a barium swallow. Magnetic resonance imaging (MRI) and ultrasonography may also be used in diagnosis, although this is mainly restricted to just providing further information on the staging of oesophageal cancer. Because the lungs are the most common site of distant metastasis then a chest x-ray can be

performed (Morris *et al.,* 1998). The use of much of this equipment for diagnosis in the poor areas with endemic cancer of the oesophagus is limited due to lack of resources making early diagnosis problematic (section 2.1.3).

1.5.3. Staging

The staging of the tumour is of major importance to determine the prognosis and the direction of the treatment. A majority of cancers are staged by the tumour-node metastasis (TNM) status. This is split into three main sections as suggested by the three letters in the title: primary tumour (T), presence of tumour in regional lymph nodes (N) and distant metastasis (M). The primary tumour staging offers information on the progressive degree (1-4) of invasion of the tumour into the oesophageal wall. Regional lymph nodes refer to the nodal involvement. Distant metastasis represents if the metastasis has spread (Table 1.1)

Table 1.1 American Joint Commission on cancer TNM staging (AJCC) for oesophageal cancer (Heitmiller, 2001)

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor invades lamina propria or submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades adventitia
T4	Tumor invades adjacent structures
Regional lymph nodes (N)	
Cervical esophagus (cervical and supraclavicular nodes)	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
Thoracic esophagus (nodes in the thorax not those in cervical supraclavicular or abdominal areas)	
N0	No nodal involvement
N1	Nodal involvement
Distant metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No evidence of distant metastasis
M1	Distant metastasis present

Stage grouping				
Stage 0	Tis	N0	M0	
Stage I	T1	N0	M0	
Stage IIA		T2	N0	M0
	T3	N0	M0	
Stage IIB		T1	N1	M0
	T2	N1	M0	
Stage III		T3	N1	M0
	T4	Any N	M0	
Stage IV	Any T	Any N	M1	

Although CT and MRI scans are used for the staging of the primary tumour the gold standard is the endoscopic ultrasound (EUS).

1.5.4. Treatment

The type of treatment is dependent on the stage of the cancer itself. Surgery is the most common form of treatment for oesophageal cancer (2002) although this is only feasible in 40 percent of the cases due to poor patient recovery (Braunward, 2001). When a patient is not responding to other treatments then surgery might be the only option. A recent debate that took place at the Service de chirurgie digestive et generale, Lille, France argued with little conclusions of whether there was still a place for surgery in oesophageal cancer treatment due to the improvements in chemotherapy (Mariette, Piessen and Triboulet, 2007).

As an alternative to surgery, owing to a weakened physical state or advancement of disease in the patient, then radiation therapy is an option. Eighty percent of cancer sufferers that undergo radiational therapy seemed to be cured of dysphagia, although temporarily, and half of these cases may have tumour regrowth in six months (Hishikawa *et al.,* 1991).

If surgery is not possible then a blockage in the oesophagus may be removed using laser therapy. A neodymium:yttrium-aluminum-garnet (ND:YAG) laser degrades the tissue by using short, one second bursts of high intensity light (80-129 watts). Lumenal damage is rare. Repeated treatment sessions may be required, up to 6 times (Nava *et al.,* 1989).

In poor areas such as much of Transkei in South Africa treatment is restricted to surgery, due to the high costs of the methods above. Prevention, as in many cases of disease, would provide a much cheaper alternative to treatment.

1.6. Epidemiology of Squamous Cell Carcinomas of the Oesophagus

Previously 90 percent of all oesophageal cancer patients had squamous cell carcinoma, (Turnbull *et al.,* 1973) although this has changed in the last decade with the increased incidence of adenocarcinoma. Men are 4 to 6 times more likely to develop oesophageal cancer than women (Office for National Statistics, UK, Cancer Statistics registrations, 2004), and it is five times more common in afro-Americans (Ferlay, 1992). Most cases of squamous cell carcinomas occur in adults over the age of 60. In 2004 in the UK there is a yearly average of 7,400 cases (Office for National Statistics, UK, Cancer Statistics registrations, 2007).

Globally, there is huge variation in prevalence. Incidence is a lot higher in South Africa, China, Japan, Switzerland, Iran, France and Finland at 4 to 20 times that of other western countries (Franceschi *et al.,* 2000). Within these countries, regional incidence may also vary widely (Naidoo & Chetty, 1999). In most countries the incidence rates of oesophageal squamous cell carcinoma (OSCC) are around 2.5 to 5 per 100,000 for males and 1.5 to 2.5 for females (Office for National Statistics, UK, Cancer Statistics registrations, 2007), although this can be 100 fold higher in specific ethnic groups in certain countries. In Transkei this is around 357/100.000 males aged 35 to 64 and in Kazakhstan 547/100,000 males aged 35 - 64 (Parkin *et al.,* 2005).

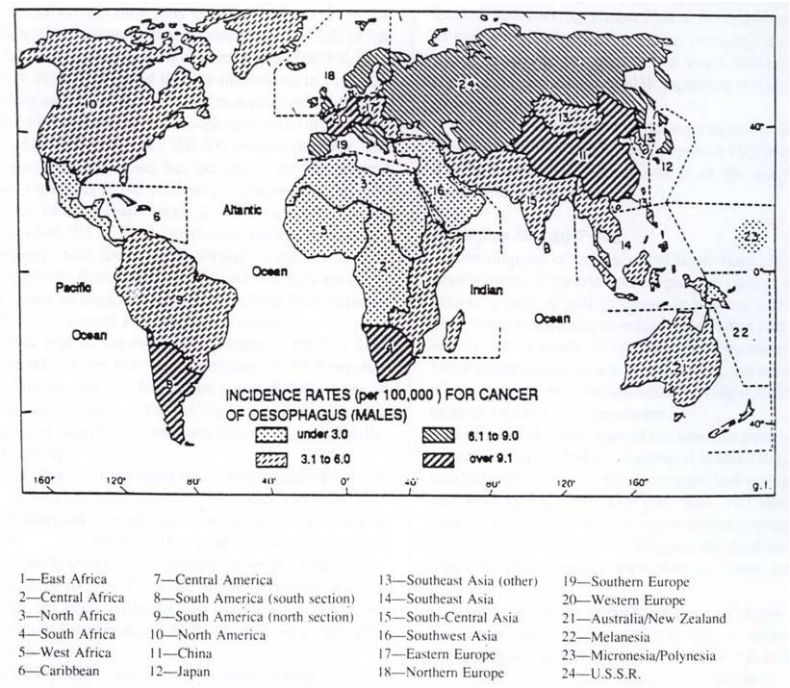


Figure 1.8: Incidence rates for oesophageal cancer in males around the world in 1988 (taken from Ghandirian *et al*., 1992)

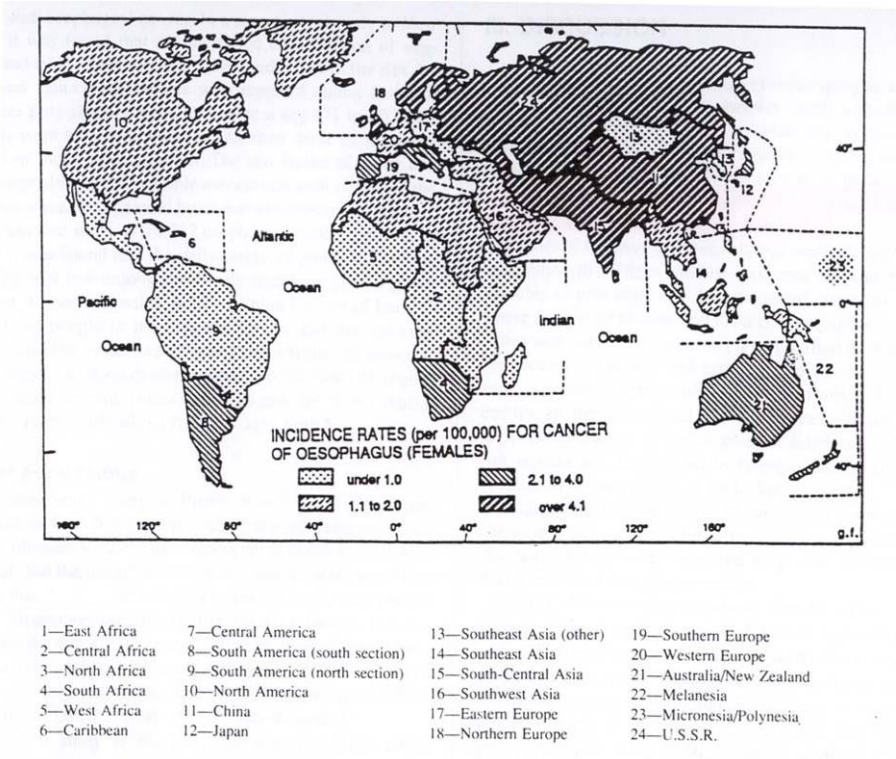


Figure 1.9: Incidence rates for oesophageal cancer in females around the world in 1988 (taken from Ghandirian *et al*., 1992)

1.6.1. Tobacco Smoke and Alcoholic Beverage Consumption

Substantial research points to tobacco smoke and alcohol consumption being the main factors leading to oesophageal cancer for both men and women (De Stefani *et al.,* 1999). Dose dependent studies of the effect of tobacco smoke and the development of squamous cell carcinoma have demonstrated a decrease in risk with smoking cessation (Choi & Kahyo, 1991). This has also proved true for smokeless tobacco products, such as snuff and chewing tobacco (Christen *et al.,* 1989). It has also been shown that a moderate intake of beer and spirits increases the risk of oesophageal cancer, although this has not been proven in the case of wine consumption (Gronbaek *et al.,* 1998). This could be owing to the fact that carcinogens such as nitrosamines and polycyclic hydrocarbons have been linked to alcoholic beverages (Cotran, *et al.*, 1999). The use of tobacco and alcohol together dramatically increases the risk, as shown by Castellsague *et al.* (1999). This group postulated that tobacco has a strong role both in the initiation and promotion of carcinogenesis, and alcohol just in promotion. A team from China recently examined questionnaires given by over 400 squamous cell carcinoma patients to conclude that increases in poor diet, smoking and alcohol consumption due to cultural changes can be strongly linked to huge increases in cancer (Wang *et al.,* 2007). This may partly explain tumour development in the western world but a number of papers have shown that in high incidence areas, such as Transkei, South Africa, they have little, if any, alcohol consumption related cancer (Sammon, 1992). A paper has shown that the white population of South Africa smokes more than the black population, but does not have the same incidence of cancer of the oesophagus (Rose, 1979). This questions the value of smoking being the single powerful carcinogen in this area, but is an additional predisposing factor. A positive relationship between smoking and the high incidence areas such as Transkei and India has been disputed elsewhere (Ghadirian *et al.,* 1992). Smoking has been postulated as being a carcinogenic factor in the Transkei population (Sammon & Alderson, 1998) and one of the factors of the theory developed in this thesis.

1.6.2. Dietary Effects

The most common and varied external environmental factors that could be the cause of, or connected to oesophageal cancer development and progression must be the ingestion of substances, and it is thought that one third of all cancers are related to what we eat (Naidoo & Chetty, 1999). Ghardirian (1992) in particular stated that populations with a high incidence of oesophageal cancer shared similar diets and dietary habits. A German cohort study of over 520,000 people showed oesophageal cancer was linked to socioeconomic status due to the patients poor diet (Nagel *et al*., 2007). This factor has a major importance on this project as a positive correlation between the consumption of maize and oesophageal cancer has been found (Sammon & Alderson 1998), discussed below (section 1.9).

Several epidemiological studies have stated the negative relationship with fruit and vegetable consumption and cancer of the oesophagus (Ghadirian, 1992; Gao, 1994). An association to increased dietary levels of protein, carotene, vitamins C and E, and riboflavin (vitamin B2) found in few meats, fruit and vegetables, and a decrease in oesophageal cancer risk is also well documented (Gao, 1994; Block *et al*., 1992; Launoy *et al.,* 1998). This relationship of high vitamin intake lowering squamous cell carcinoma oesophageal cancer risk has also been seen with the intake of supplements (Barone *et al.,* 1992). Studies in endemic areas of Linxian, North Central China, showed that residents have low blood concentration of beta-carotene, vitamin C and riboflavin (Munoz *et al.,* 1982). In some cases high intake of protein with fat, butter, eggs and starchy foods have been implicated with an oesophageal cancer increase (Franceschi *et al.,* 2000). A high intake of certain meats has been linked to squamous cell carcinoma (Brown *et al.,* 1998; Barone *et al.,* 1992; De Stefani *et al.,* 1999). The opposite of this has been shown with the intake of monounsaturated fatty acids, olive oils (Tzonou *et al.,* 1996) and vitamins (Franceschi *et al.,* 2000) in two Italian studies. One study has stated a strong inverse relationship between oesophageal cancer risk and consumption of all micronutrients of plant origin (Launoy *et al.,* 1998). Niacin, a vitamin heavily associated with poultry and fish, when deficient, can cause oesophageal lesions linked to cancer of the oesophagus (Franceschi *et al.,* 1990). A Chinese study by Gao (1994) showed that drinking green tea appears to reduce the

risk of oesophageal cancer, as it is known to contain polyphenols and other antioxidants.

It is not just the consumption of the foods but also how they are consumed that may be an underlying factor of oesophageal cancer. A Japanese study of 720 men and women showed that those most at risk from cancer of the oesophagus are those drinking scalding hot tea (Kinjo, 1998) shown again with hot soups and porridge (Gao *et al.,* 1994). N-nitroso compounds used to preserve vegetables and salted foods in Chinese high incidental areas were shown in one American study (Bogovski & Bogovski, 1981). Although a case control study from Washington suggested the opposite of this, and that they protected the aerodigestive tract (Rogers *et al.,* 1995). Physical irritants in the Caspian Littoral have been implicated with high risk areas such as scratchy breads often contaminated with extraneous seeds such as the *phalaris*. The *phalaris* seed surfaces contain a large amount of fine fibrous silica particles. These silica particles are also seen in human cancer patients and the gullets of cancer suffering chickens in China. A possible cause was that the particles are from millet bran provide an anchorage for the spread of the growth (O`Neill, 1980). In Transkei, people consume a daily diet of roughly ground maize that has been mixed with silica from the grind stone used. It was suggested "an irritant" like silica or even the roughly ground maize "may play a part, at least in determining the site of the cancer", hence the ingestion through the oesophagus (Rose, 1979).

1.6.3. Infectious Agents

Human papillomavirus (HPV), Herpes-simplex virus (Galloway & McDougall, 1990), cytomegalovirus and Epstein-Barr virus (Chang *et al.,* 1992) have all been implicated as a possible cause of oesophageal squamous cell carcinoma, although there is no direct proof of this. The data on whether HPV could be part of carcinogenesis seems conflicting (Sur & Cooper, 1998; Monsonego, 1995; Qi *et al.,* 2006). HPV may have some action on oesophageal cancer by inactivation of normal p53 function, by the binding of E6 protein to the p53 protein functioning as a tumour suppressor. This E6

protein has been shown to up regulate the VEGF gene (VEGF suggested tumour method explained in section 1.7.1.d) via its promoter region. It has been postulated in oral squamous cell carcinomas that the E6 protein in HPV could lead to p53 degradation (Min *et al.,* 1994; Furihata *et al.,* 1993). Murono *et al.* (2001) found the Epstein-Barr virus induces COX 2, an important protein relating to this study (section 1.7.1), and Jenson *et al.* (1997) found Epstein-Barr virus in small tissue tumours induced the epithelium growth factor receptor (EGFR). In the target population of this study there are mixed views of whether HPV is prevalent in the South African high oesophageal cancer areas. Cooper *et al.* (1995) found the presence of HPV genes in 52% of the DNA of 48 archival oesophageal carcinoma biopsies. Van Rensburg (1996), using the same techniques, found little relationship between HPV DNA and the oesophageal squamous cell carcinoma samples.

Helicobacter pylori is associated with gastric cancer and is classified as a class 1 carcinogen by the International Agency for Research on cancer (Jiang & Wong, 2003). Little proof has come to light as to whether *Helicobacter pylori* could be a factor in the oesophageal squamous cell carcinoma development, although a link may occur through gastric infections and reflux shown in adenocarcinoma tissue of the stomach (Wu et al., 1996). Calam suggested that the eradication of *H. pylori* can increase the likelihood of oesophagitis developing (1999) which itself has been linked to oesophageal cancer development (Rubin & Farber, 1994). An experiment carried out by Wu *et al.* showed that investigating 300 people with and without oesophageal squamous cell carcinoma concluded that *H. pylori* may be a factor in protecting against oesophageal squamous cell carcinoma although it is not known why (2006). Previously it was suggested that *H. Pylori* infection may induce atrophic gastritis, which results in a less acidic gastric reflux, and *H. Pylori* may also neutralise gastric acid by producing ammonia (Richter, Falk & Vaezi, 1998). One other paper has investigated the relationship between *H. pylori* infection and increasing risk of oesophageal squamous cell carcinoma showing a borderline positive result thought to be connected to increased intragastric nitrosation (Limburg *et al.,* 2000).

Gastric tissue in rats treated with *H. pylori* and bile, promoted COX 2 expression compared to controls (jiang & Wong, 2003) and increased levels of prostaglandin E_2, the product of COX 2 (Al-Marhoon *et al*., 2004). This would support the Sammon

theory explained in section 1.9. Although a study carried out by Sammon in 2003 stated the results were not significant in the sample population with only half of the samples registering *H. pylori* positive in the 30 samples tested.

There are a number of fungal and bacterial infections in the oesophagus that have links to the aetiology of oesophageal squamous cell carcinoma. Infections by *Candida* species in the oesophagus are by far the most common (Bartelsman & Tytgat, 1991), and in China this has been shown to correlate with a degree of dysplasia (Yang, 1980). On a number of occasions a pure culture of *Candida albicans* can be isolated from hyperplastic epithelium and carcinoma *in-situ* of the oesophagus (Yang, 1980). A bacterium, which is normally present in the upper alimentary tract, may convert nitrate into nitrite, which at high levels in saliva have been seen in patients with marked epithelial dysplasia and carcinoma (Linxian Research Team, 1978).

Fungal contamination of foodstuffs like maize, millet and other grains have been investigated as a possible cause, mainly in Africa (Marasas *et al.,* 2001) and China (Chang *et al.,* 1992). Common species like *Fusarium (i.e. Oxysporum)* and *Aspergillus (i.e. flavous)* are known to reduce nitrates to nitrites have also been shown to decompose proteins and increase the amounts of amines promoting nitrosamine formation in China (Yang, 1980). In one study, *Alternaria alternata and Penicillium cyclopium* fungal contaminated cornbreads were fed to rats resulting in the induction of oesophageal carcinomas (Zhen *et al.,* 1991). *Fusarium moniliforme* and *Aspergillus flavous* found in maize of Transkei releases the mycotoxins fumonisin B1 and aflatoxin B1, respectively. This has been shown to be closely related to oesophageal cancer in these areas (Marasas 1986). Aflatoxin B1 in the contaminated food supply in these areas is associated with G:C to T:A transversions leading to a serine substitution at residue 249 of the p53 gene in hepatocellular carcinoma (Harris, 1993). This research in South Africa is often discussed although little hard evidence of its mechanism to increase oesophageal cancer has been published. Isaacson in 2005 concluded in his review of the subject that more fungal testing of South Africas food should be taking place, “If nitrosamines are detected, their carcinogenic potential should be studied experimentally. Should these tests prove positive, it would be vital to break the Fusarium-nitrosamine-cancer chain”. It is still be discussed whether the increase in oesophageal cancer is more about maize consumption and not the increase

of mycotoxins found on the maize. The South African Medical Research Council has this subject as one of their research areas with little new information.

1.7. Molecular Pathogenesis

The pathogenesis of normal oesophageal tissue to carcinoma via squamous dysplasia is a multi-step process and is thought to be driven by multiple genetic events caused by continuous exposure to the carcinogens described in section 1.2 (Farber, 1984). These genetic changes could be due to oncogenes, the inactivation or mutation of tumour suppressor genes, enhanced intracellular transcription or the amplification of growth factors and their receptors. Once such changes have taken place then any of the following could take place: tumour suppression to a cell may be lost, increased cell proliferation, changes in cell death control, changes in cell differentiation, loss of cellular cohesion and finally the induction of metastasis mechanisms may occur. The molecular control of all of these pathways is being extensively researched to increase the understanding of pathogenesis.

1.7.1. Cyclooxygenase

COX is a haem-containing glycoprotein, also known as PG endoperoxide synthase, PG synthase or PG G/H synthase. There are two isoforms of COX, COX 1 and COX 2.

Although the two enzymes share 60% of the same amino acids, their functions are thought to be very different. COX 1 is known as a "house keeping gene" as it mediates physiological tissue homeostasis. The main study of COX 1's biological function is through its ability to synthesise cytoprotective prostaglandins in the healthy gastrointestinal tract (Flower, 2003), although other areas of its cellular

effects such as depression of platelet aggregation are still being researched (Mitchell *et al.,* 1993). COX 2 is known as the inducible enzyme, induced by many different stimuli such as Interleukins, hormones, oncogenes and growth factors (Tanabe & Tohnai, 2002). Due to huge increases in the expression of COX 2 and its production of Prostaglandin E2 (PGE_2) (the basis of this thesis) it has been linked to inflammation (Williams, 1999). However it is also involved in pain, fever, ovulation, bone formation and thought to be linked to Alzheimer's disease, because of the high concentration present (Murakami *et al*., 2001). Much of this is thought to be due to PGE_2 release (section 1.7.2). While Simmons and colleagues (1999) examined COX expression, they came across a COX 1 variant that they have dubbed COX 3, although little research has been done on its function in the body.

The actions of the COX enzymes have been investigated by the use of gene transfection and gene knockout in mice (Loftin, 2002) and also COX 1 and COX 2 specific inhibition. There are many drugs on the market now that are known to inhibit COX action, Aspirin being the most common. Aspirin is known as a non-steroidal anti-inflammatory drug (NSAID). It works by suppressing the prostanoid production of COX 2 that leads to an inflammation response. One of aspirin's side effects is the development of stomach ulcers. This is thought to come from the inhibition of the COX 1 enzyme suppressing its gastro-protective effect. This has led to commercial interest in the development of COX 2 inhibitors that can have a suppressive effect on inflammation reaction seen in rheumatoid arthritis, without the gastric side effects. It has been stated that this industry was worth in excess of US $ 4 billion in the year 2000 (Merrill Lynch, 2001). Those include drugs like Celecoxib (Celebrex, Pfizer), Rofecoxib (Vioxx, Merck), Valdecoxib (Bextra, Pfizer) and Etoricoxib (Arcoxia, Merck). The safety of these drugs has recently been questioned. The FDA in March 2005 released the following statement: "Based on emerging information, including preliminary reports from one of several long term National Institutes of Health (NIH) prevention studies, the risk of cardiovascular events (composite endpoint including MI, CVA and death) may be increased in patients receiving Celebrex. FDA will be analyzing all available information from these studies to determine whether additional regulatory action is needed". This caused the FDA to withdraw the release of these NSAIDs until further tests have been carried out. NICE also withdrew Rofecoxib in the UK. Therefore a majority of modern research carried out with COX 2 relates to

the therapeutic effect of its inhibition and side effects of decreasing PGE2 production that leads to inflammation.

1.7.1.a. COX 2 and Cancer

This thesis will be concentrating on the mechanisms of COX 2 and its relationship with cancer.

COX 2, as well as in many other tissues of the body, is found in the gastrointestinal system including the oesophagus. Within the cell, COX 2 functions in the endoplasmic reticulum and the nuclear envelope. Over expression of COX 2 was first studied in Colon cancer, therefore much of the research has taken place in this field (Kawai, 2002). Now a number of studies have demonstrated that COX 2 expression was elevated in carcinomas of not only the colon, but, also stomach, breast, lung, liver, pancreas and importantly the oesophagus (Yu, 2003; Miyashita *et al.,* 2006). By using COX 2 inhibitors, such as aspirin and ibuprofen, in patients with rheumatoid arthritis, it was discovered there was a fall in the number of colorectal cancers (Giovannucci *et al.,* 1995). This has led to the discovery that COX 2 inhibitors in different tissues, such as oesophagus (in both *in-vitro* and *in-vivo* studies), have shown to suppress cellular proliferation and induce apoptosis, therefore suppressing the important factors in cancer genesis (Farrow *et al.,* 1998). Various papers have shown how even occasional use of aspirin or other NSAIDs is inversely related to the risk of oesophageal cancer: the reduction of relative risk varies from 50% to 90% (Altorki, 2004; Funkhouser, 1995). Significant over-expression of COX 2 has been seen in cancer tissue. This has been studied and is thought to be related to the grade or differentiation of the tumour (Wolff *et al.,* 1998). This has also been shown in immunostaining of squamous cell carcinoma of the oesophagus (Zimmermann *et al.,* 1999). Li *et al.* (2000) reported that aspirin treatment resulted in significant growth inhibition of 10 oesophageal cell lines. Rubio (1984) previously showed that another NSAID, indomethacin, suppressed the development of oesophageal cancer induced in mice by *N*-nitrosomethylbenzylamine, a potent carcinogen. Ranka and his team (2006) at Norwich University Hospital have shown that NSAID treatment can help

protect the oesophagus from cancer by decreasing lower oesophageal sphincter relaxation.

There are many possible mechanisms of how increased COX 2 expression and protein could aid carcinogenesis. Due to the COX 2 enzyme having both oxygenase and peroxidase functions, one carcinogenic action could be to catalyse the conversion of pro-carcinogens to carcinogens. COX 2 activity is thought to be responsible for the conversion of the pro-carcinogen benzo(a)pyrene, present in tobacco smoke, into the carcinogen of mutagen benzo(a)pyrene diolepoxide (Kelley *et al.,* 1997). Ultraviolet B (UVB) light is a risk factor in skin cancer. Irradiation of human keratinocytes by 30mJ/cm2 UVB light results in a 6 fold increase in COX 2 levels (Buckman *et al.,* 1998).

1.7.1.b. COX and Apoptosis

Apoptosis is controlled cell death mediated by the cells to control tissue reconstruction and unwanted cells. Between this and cellular proliferation there is a fine balance that keeps the tissues at an optimum turn over. Should this balance be upset by increasing cellular proliferation or decreased apoptosis it leads to prolonged survival of abnormal cells and tumour growth, hence cancer. COX 2 has been associated with suppressing apoptosis causing this unbalance. COX 2 concentrations have been shown to have a positive correlation with the induction of the anti-apoptotic protein *Bcl-2* and reduce levels of *Bax*, a pro-apoptotic protein (Li, 2000). The suppression of these proteins, by treating with COX 2 inhibitors has been shown in a number of tissues, including oesophageal cancer cells (Weitzman & Gordon, 1990). There are reports that aspirin induces release of cytochrome C, a pro-apoptotic molecule, from mitochondria by increasing pore permeability (Zimmermann, 2000).

1.7.1.c. COX and Invasion and Metastasis

Invasion and metastasis are two common characteristics of cancer development. It has been suggested that COX 2 activates enzymes that digest the collagen matrix of the basement membrane, therefore allowing cells to break off from the main tumour and spread to the rest of the body (Tsujii *et al.,* 1997). However, some studies carried out in intestinal epithelial cells showed that elevated COX 2 protein levels are related to increased adhesion to extra-cellular matrix proteins. This can therefore aid cell survival due to stronger adhesion (Tsujii & DuBois, 1995). COX 2 over expression is also associated with the induction of CD44, a cell surface receptor implicated with invasions and metastasis (Dohadwala *et al.,* 2001). CD44 is expressed in many types of migratory cells and metastasis tumour cells. The inhibition of COX 2 in oral squamous cell carcinoma cell lines was shown to reduce the expression of CD44, hence the invasiveness of the cells (Kinugasa *et al.,* 2004). This is backed by *in-vivo* experiments that have shown COX 2 inhibitors to reduce the number and size of metastases in animal experiments (Tomozawa *et al.,* 1999).

1.7.1.d. COX and Angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is important in tumour genesis, and is required for tumours to grow. This is one of the most researched areas of COX 2 and its carcinogenic effects. COX 2 has been shown to regulate vascular endothelial growth factor (VEGF), claimed to be the most important growth factor in the proliferation of new vascular tissue (Gallo *et al.,* 2001). Using COX 2 knock out mice tumour growth, VEGF production and microvessel density were all reduced compared to control (Williams *et al*., 2000). This has also been shown in a phase II clinical study, where treatment of COX 2 inhibitors with patients that are suffering from lung cancer showed reduced circulating levels of VEGF (Ohm & Carbone, 2002). It was shown that in COX 2 knock out mice colon

tumours produced few tumours over 1mm thick, this is commonly known as the limit tumours can grow to without angiogenesis (Seno *et al.,* 2002). In *In-vitro* studies, hypoxia induces COX 2 that increases the VEGF level inducing angiogenesis. This may be the method used by tumours to grow when cells start loosing the ability to reach the oxygen due to tumour expansion (Liu *et al*., 2001), therefore COX 2 aids this process. Other pro-angiogenic growth factors in colon cancer have shown to be expressed following COX 2 expression. These include bFGF, bFGF-binding protein, TGF-β and PDGF (Shiff, 2003) although a small number of papers have been written on this. The angiogenesis that takes place from the presence of COX 2 is suggested to be a natural response that is required during blastocyst implantation and decidualisation, therefore a vascular environment for the nutritional requirements of an embryo (Williams *et al*., 1999). This theory is backed up by the fact that COX 2 deficient mice show multiple reproductive failures in early pregnancy, such as ovulation, fertilisation, implantation and decidualisation (Dinchuk *et al*., 1995). The COX 2 levels would indirectly stimulate natural vascular growth; although COX 2s over expression could push the progression of the tumour.

In February 2004 the FDA approved the use of Avastin (Genentech Inc) for colon cancer patients which it thought to work by reducing tumour stimulated angiogenesis by inhibiting VEGF. This is marketed in the UK by Roche and is called Bevacizumab and was given NICE appraisal in January 2007.

1.7.1.e. COX and Immuno Suppression

It is for the very same reasons of promoting embryo development through vascularisation that COX 2 and prostaglandin E2 (PGE_2), its product, are implicated with immuno-suppression. PGE_2 has been shown to inhibit the production of tumour necrosis factor and induces the production of interleukin-10, an immunosuppressive, therefore avoiding the attention of surveillance macrophages in the microenvironment (Kunkel *et al.,* 1986). COX 2 inhibitors have been shown to attenuate tumours through immuno-suppression *in vitro* (Stolina *et al*., 2000).

1.7.2. Prostaglandins

Much of the research on prostaglandins are aimed at the manipulation of COX 2 enzyme, through inhibiting COX 2 by chemical treatment or by using knock out mice. The main effects of COX 2 are thought to be due to the production of the prostaglandins and one in particular, prostaglandin E2 (PGE_2). PGE_2 seems to be the mediating factor causing the inflammation and induced effects. PGE_2 is the most common prostanoid studied and is critical for numerous biological processes, including inflammation, ovulation, implantation, angiogenesis, platelet aggregation and immunologic function (Morita, 2002). These effects are produced via three sequential enzyme reactions (Figure 1.10):

- The release of arachidonic acid (AA) (20 carbon unsaturated fatty acid distributed throughout the lipid bilayer of the cell) from membrane glycerophospholipids by phospholipase A_2 (PLA_2),
- Conversion of AA to the unstable intermediate prostanoid PGH_2 by cyclooxygenase (COX)
- Isomerisation of PGH_2 to PGE_2 by prostaglandin E synthase (PGES).

Arachidonic acid (AA) is derived from the essential polyunsaturated fatty acid, linoleic acid. This is commonly available in dietary fat. Other than the COX pathway, the AA can be metabolised by the lipoxygenase pathway and the cytochrome P-450 monooxygenase pathway.

Langenbach showed that the deficiency of COX 2 reduces the levels of PGE_2 production by approximately 75%, while the deficiency of COX 1 reduces PGE_2 level by 25 %, therefore PGE_2 production can still be taking place in the absence of COX 2 (Langenbach *et al.,* 1999).

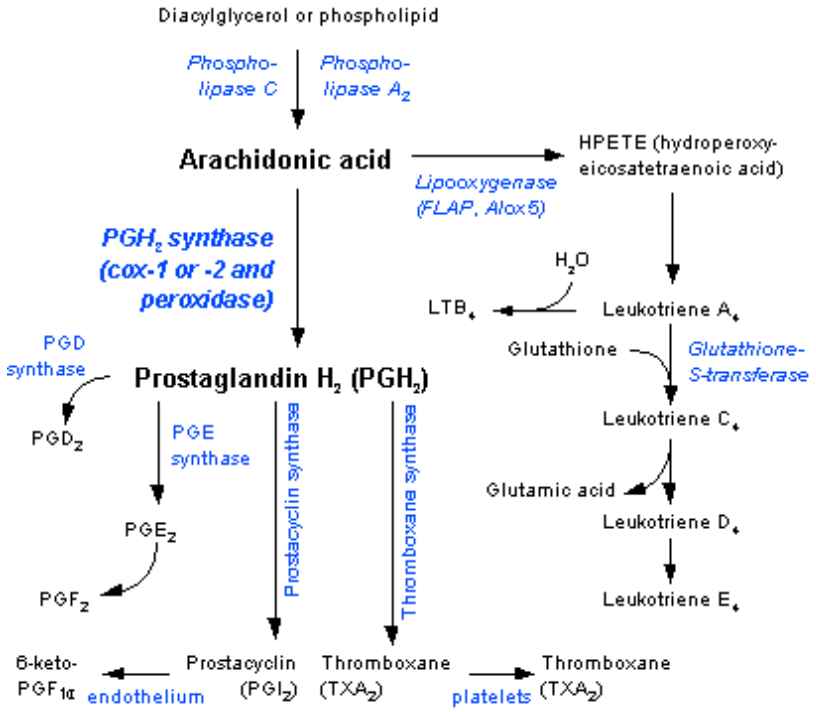


Figure 1.10: An overview of prostaglandin synthesis and metabolism (Permission granted by Dr J. F. D. Wolff, 2006)

All prostaglandins, including PGE_2, are thought to build up within the cytoplasmic compartment of the cell. Following enough synthesis, the prostaglandins efflux across the plasma membrane to enter the extracytoplasmic compartment, through simple diffusion (Schuster, 2002). The PGs can then be transported further a field by prostaglandin transporter proteins (Kanai *et al.,* 1995). They are hormone-like, but just induce responses via their own receptors (named TP,IP,EP,FP and DP) locally in a paracrine or autocrine fashion. Prostaglandins are thought to be produced in short bursts in response to specific stimuli. Following stimulation PGs can be detected within 10-30 seconds and their synthesis proceeds for 1-5 minutes, followed by rapid degradation (Klein, 1990). As prostaglandin has a short half-life and a huge response, this acts to restrict effects in the locality of its production. The most typical action of these PGs are relaxation and contraction of various types of smooth muscles. PGs regulate secretion and motility in the gastrointestinal tract as well as the transport of ions and water in the kidney (Narumiya *et al*., 1999). Plasma levels in humans are between 10-100 pg/ml (Leonhardt *et al,* 1997). PGE_2 (3.27 ng/min) and two of their

receptors, EP1 and EP3, have shown to be expressed moderately in both the oesophagus and the stomach (Sarosiek *et al*., 1994; Morimoto *et al.,* 1997). Its natural action here is thought to be the modulation of inflammatory stimuli, as PGE_2 levels appear to parallel the degree of tissue damage in acid damaged oesophagus in rabbit models. This is also seen in human patients suffering from oesophagus damaged by oesophagitis (Ottignon *et al.,* 1987). This natural regulation could be required due to pathological substances that could enter with food, or the suppression of immunological homeostasis in cases where foods may irritate the gastrointestinal system. It is suggested that the PGE_2 is trapped within the mucus layer and released under the impact of acid and pepsin (Jimenez *et al.,* 1997). One study stated that salivary PGE_2 concentration increased by 132% when control patients chewed on a tasteless parafilm (Sarosiek *et al.,* 2000).

It is established that PGE_2 suppresses the immune system. T-helper lymphocytes mediate delayed-type hypersensitivity reactions. PGE_2 inhibits T-cell proliferation, thought to be a cause of a number of different processes. These include inhibition of a required polyamine (i.e. spermine), inhibition of intracellular calcium release, and the decrease in protein tyrosine kinases (Choudhry *et al.,* 1999). This is thought to be a natural negative feedback response as in inflammation the mucosal T-cells up-regulate and express EP receptors, reducing the T-cell production (Cosme *et al.,* 2000). It has been shown that PGE_2 induces CD4+ and CD8+ thymocytes to undergo apoptosis, both *in-vitro* and *in-vivo* (Cosme *et al.,* 2000). It is through this suppression of the immune system in times of high PGE_2 production, that it is thought that tumour cells, would otherwise be destroyed and broken down, can continue to grow which increasing mutations and tumour size.

1.7.3. PGE2 and Carcinogenesis

Increases in PGE_2 have been shown in colorectal cancer tissue compared to normal colonic mucosa (Pugh & Thomas, 1994). An increase in tumour progression from the effects of PGE_2 have been documented before, where it was suggested that PGE_2

altered cell morphology and increases cell motility (Gately, 2000). Pharmacological inhibition of EP1 and genetic disruption of EP2, two receptors for PGE_2, both result in a significant reduction of aberrant crypt foci in colorectal cancer tissue (Murakami, 2002). It has been suggested that suppression of angiogenesis in polyps can be carried out by inhibiting the EP2 receptor, which suppresses the release of VEGF (Seno *et al*., 2002). Administration of PGE_2 to a growing prostate cell line has been shown to increase cellular proliferation (as measured by the cell number) (Tjandrawinata *et al.,* 1997).

1.7.4. p53 and Related Proteins

p53 is a transcription factor tumour suppressor gene, which stimulates DNA repair and regulates cell proliferation and apoptosis to prevent the division of potentially malignant cells. p53 gene mutations and changes are some of the most common aberrations that occur in 40-50 percent of oesophageal cancers. This was demonstrated by Hollstein and colleagues (1990) who showed that p53 mutations are part of the tumourgenic process of oesophageal cancer, and recently supported by Qi *et al*. (2006). The p53 gene activates the production of p21, which itself is an inhibitor of cyclin and cyclin dependent kinase complexes (Ng *et al.,* 1999). This is not expressed if the p53 is in a mutated form (El-Deiry *et al.,* 1993), which has been shown in squamous tumours leading to malignant phenotype. This can be reversed when wild type p53 is restored (Schantz, 1995). One paper also stated over expression of p21 protein in squamous cell carcinoma, was present, even independent of p53 mutation (Ng *et al.,* 1999). A gene that encodes for a p53 auto-regulatory feedback loop protein called murine double minute 2 (*mdm2*) oncogene has been found, that when over expressed stops the action of p53. This leads to malignancies in squamous carcinomas (Heinzel *et al.,* 1996). Ng *et al.* (1999) questions whether mdm2 was that important in carcinogenesis putting the weighting on p21, as only 20-30 percent of SCC cells expressed it. Wales and colleagues speculated in one paper, that there might be another tumour suppressor gene similar to p53 such as HIC1 that has a larger part in tumourigenesis, although this has not been shown in squamous cell

carcinoma (Wales *et al.,* 1995). The absence of p53, another apoptotic protein called *Bax*, and the sporadic expression of *Bcl-2* and *mdm-2*, was reported in a paper several years ago, suggesting the link between premalignant and malignant squamous lesions (Schoelch *et al.,* 1999). This was backed by a South African research group using oesophageal cancer models at the University of the Witwatersrand, Johannesburg (Sur *et al.,* 2003).

1.7.5. Growth Factors

Growth factors, as well as other cellular regulation factors, mainly promote proliferation of cells by intervening in the cell cycle. If cells are deprived of growth factor there will be a senescence and inhibition of growth. This is due to extracellular growth factors being specific to their receptors, leading to promotion of cell signal pathways to gene expression, notably *c-fos* and *c-jun*. This in turn allows cells to progress from a non-proliferative state called G_0 into the replicative cell cycle, G_1. The rest of the cell cycle is normally then driven by intracellular processes (Meyers, 1995).

1.7.5.a. Epidermal Growth Factor

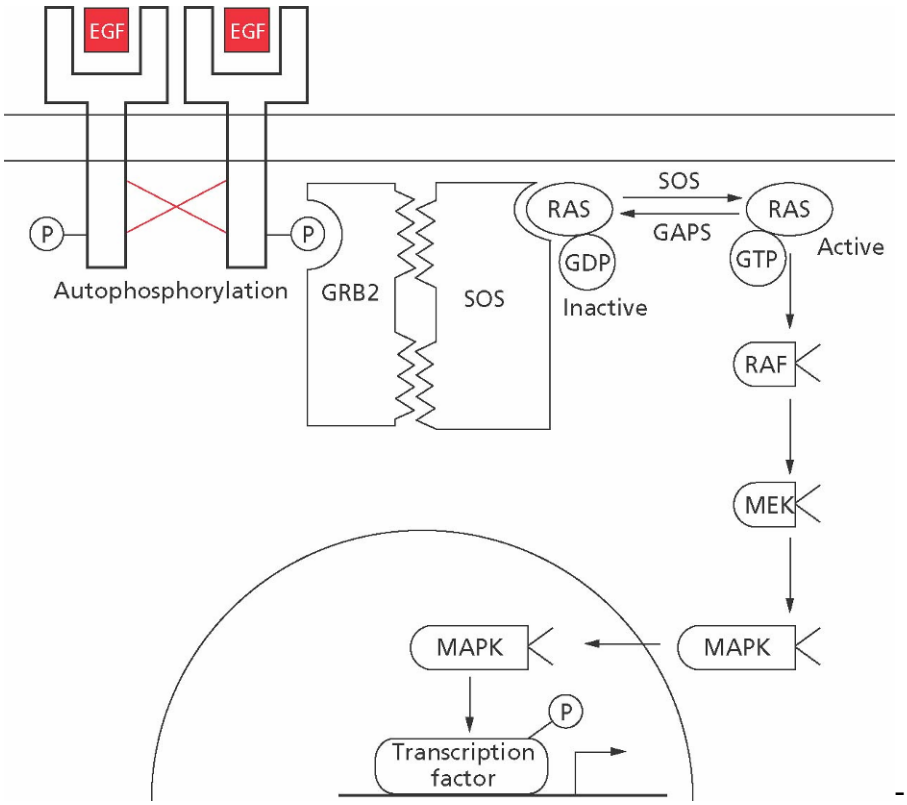


Figure 1.11: Key molecular pathways of cell cycle regulation. Growth factor signaling, such as that from EGF, results in the expression of target genes, including cyclin genes essential for cell cycle progression. (Pecorino, 2005)

Epidermal growth factor (EGF) is a protein (53 amino acids) with three disulphide bonds and two distinct foldings. EGF and its relating growth factors, such as transforming growth factor α (TGFα), amphiregulin and cripto have been extensively researched (Meyers, 1995). All over the body this growth factor is essential for stimulation the cell cycle, hence new tissue proliferation. Should this mechanism be over stimulated then this could increase the chances of cancer initiation. EGF is secreted with saliva into the gastrointestinal mucosa and oesophageal epithelium, attracting research in this area (O'Keefe *et al.,* 1974). In 1989 it was discovered that an EGF increase was closely correlated with progression in oesophageal squamous cell carcinomas and their prognosis, due to the raised levels from high EGF

expression in proportion to the amount of EGF receptors present (Ozawa *et al.,* 1989). This has been demonstrated in a number of papers (Cowley *et al.,* 1984; Banks-Schlegel & Quintero, 1986), although questioned in a number of others (Kamata *et al.* 1986; Barnes, 1982) with one stating that EGF is proliferative *in-vivo* but not *in-vitro* (O'Keefe *et al.,* 1974). Subcutaneous EGF has been shown to cause oesophageal epithelial hyperplasia in pigs and rats (Juhl, 1995). EGF treated vascular smooth muscle cells have been shown to induce COX 2 mRNA and the COX 2 protein (Pash & Bailey, 1988). This has also been reported with EGFR, a receptor commonly over expressed in oesophageal cancer (Mestre *et al.,* 1997). It has been demonstrated that EGFR is present in ten times the concentration in oesophageal squamous cell carcinoma than gastric cancer cells. EGFR is commonly over expressed in 71% of oesophageal squamous cell carcinomas (Stemmermann, 1994). It has been suggested that this up regulation of ligand and receptor found in carcinomas could be one of the major causes of epithelial cancers (Stemmermann, 1994). A team in Japan have examined a potential new drug called Gifitinib that inhibits the tyrosine kinase pathway down stream from the EGFR, inhibing the growth of squamous cell carcinoma cell lines (Teraishi *et al.,* 2005).

1.7.5.b. Hepatocyte Growth Factor (HGF)

Hepatocyte growth factor (HGF) is a 105 kDa protein known for its mitogenic action on hepatocytes (Takada, 1995). HGF, also known as scatter factor, is a multifunctional heterodimeric polypeptide that binds to the cMet tyrosine kinase receptor (Bottaro *et al.,* 1991). Some of these functions include the growth and scattering of various cell types (Bottaro et al 1991), promotion of angiogenesis (Rosen *et al.,* 1993) liver generation, and wound healing (Burr *et al*., 1998). HGF has been suggested to have an action in gestation, as high HGF has been found in embryonic tissues (Chan *et al.,* 1988). The HGF and its receptor function almost exclusively in close proximity to tissues. The HGF is released from the cells in a paracrine and autocrine method stimulating localised tissue only (Di Renzo *et al.,* 1991). The cMet receptor has been shown to be present in rabbit oesophageal epithelial cells, and HGF

stimulated restitution in these cells (Takahashi *et al.,* 1995). Jimenez *et al*. (1998) also showed how HGF can stimulate cell restitution in damaged rabbit oesophagus epithelium. Takada *et al.* (1995) stated that the average HGF levels in a normal sample were 80 ± 183 ng/100 mg protein from the normal oesophagus mucosa. They found mean levels of 600 ± 416ng/100mg protein in carcinoma tissues; a significant increase. It has been suggested that autocrine stimulation of the cMet receptor from HGF, induces amplification of cMet, therefore having positive feedback characteristic (Boccaccio *et al.,* 1994). It has been demonstrated in human gastrointestinal tumours that the cMet gene, coding for the HGF receptor, can be over expressed (Kuniyasu *et al*., 1992). Naughton *et al.* showed that HGF serum levels are increased in men with prostatic cancer, implying that it may be a potential tumour marker (Naughton, 2001). Prostaglandins have been shown to induce HGF expression in gastric fibroblasts *in-vitro* (Takahashi *et al.,* 1995). It was also demonstrated that HGF induces COX 2 expression in gastric epithelial cells *in-vitro* (Jones *et al.,* 1999). Baater *et al* carried on this research, to show how Celecoxib (a COX 2 inhibitor) significantly suppresses cMet mRNA increased in rat oesophageal ulcer healing by the treatment with acetic acid (Baater, 2002). Therefore, autocrine cMet stimulation could lead to constant epithelial stimulation aiding in carcinogenesis.

1.8. History Of The Epidemic Squamous Cell Carcinoma In South Africa

1.8.1. Transkei

Figure 1.12: This figure shows where Umtata, the Transkei capital, a 250km square region is in South Africa (World Atlas, 2002)

Transkei, the area shown surrounding the capital Umtata, is a rolling grassland area of South Africa covering about 16,000 square miles (Rose, 1982). This starts from the Drakensberg Mountains down to the Indian Ocean coastline, itself extending 250km. There is a subtropical climate with nearly all of the rain falling in the summer months of October to March (Sammon, 1999).

Transkei has a population of around two million people, who are predominantly Xhosa (the X in English is swapped for a C and a distinctive click sound). They owe their origin to the East African migration of Nguni people. Sotho speakers make up four percent and Zulu speakers make up the remaining one percent of the population with very few Caucasians. Migrant labour, subsistence farming and limited industry is the main employment. A number of men will work for one to two year spells for "white" South African farmers to support the family. A significant percentage of the Transkei populations live in poor conditions with many people to a hut, without sanitation or clean water.

Umtata is a large city towards the south of Transkei consisting of 100,000 people with a bustling town centre. The general hospital and the University of Transkei offer the samples and local scientific expertise for this project (Sammon, 1999).

1.8.2. Local Squamous Cell Carcinoma

In Transkei, oesophageal cancer is the most common malignancy encountered in men (Naidoo & Chetty 1999). An estimated 20 percent of men will be affected by the tumour before the age of 75 years, if other causes of death are excluded (Environmental Associations with oesophageal cancer in Transkei (E.A.O.C.T) 1979). In Transkei 357/100,000 males aged 35 to 64 suffer from oesophageal cancer (Parkin *et al*., 2005). It was virtually unknown before 1940 for an epidemic of oesophageal cancer in black South Africans, although there seemed to be massive increases after 1949. This could be linked to either poor diagnosis, or increased risk factors. A local study in Natal found an association between oesophageal cancer and socioeconomic status, associated with lower social status and less education (Van Rensburg *et al.,* 1985). This therefore makes a good sample population to study the causes of oesophageal cancer, since discovery of an obvious common predisposing factor should aid oesophageal cancer research worldwide. However, it seems unlikely that a single etiological factor can account for such ranges in incidence rates (Parkin *et al.,* 1988) and with the oesophagus mucosa being in direct contact with so many environmental factors, a plethora of factors could be the cause of pathogenesis.

Over the last century the spread of maize as the main crop over Africa has taken place, which along with bread, margarine, beans and pumpkin, forms the staple diet of Transkeians. Maize is a high yield crop, which grows easily in relatively poor soils. This diet is sometimes supplemented with meat and garden vegetables (Sammon, 1999). Maize is used in many forms to produce drinks and foods. Maize consumption has already been connected with high incidence areas of Africa for oesophageal cancer (Van Rensburg, 1987). All oesophageal cancer patients in significant papers

used both maize and beans, identifying the "at risk" group as being users of this staple diet (E.A.O.C.T, 1979; Marasas *et al.,* 1988).

Heartburn was seen in 60 percent of individuals in a study in Transkei people, who see this symptom to be normal following a meal and do not seek medical attention for it. Heartburn was commonly accompanied by a symptom strongly suggestive of gastro-oesophageal reflux, namely the regurgitation of fluid into the mouth following the ingestion of umqa wethanga and amarewu (maize based foods). When the patients were given aspirin the heartburn was abolished or alleviated (Sammon, 1994).

Oesophagitis (inflammation of the oesophagus) has been reported in the high oesophageal cancer incidence areas, seen in 84 percent of one sample population in Linxian, China (Munoz *et al.,* 1982). In Johannesburg, 28 children that had died between the ages of 2 and 18 years had their oesophagus examined, to find 15 had non-specific oesophagitis characterised by atypical basal cell hyperplasia (Hamilton & Issacson 1985). Rose found inflammation of the oesophagus in cases negative for oesophageal cancer in post mortems of Transkei people. However, there are no numbers in this paper to base any conclusions on (Rose, 1982).

Smoking tobacco and alcoholic beverage intake, as mentioned in section 1.6.1, is linked to cancer and is just as significant in Transkei, where home grown tobacco has been shown to have a higher mutagenic potency than commercial tobaccos (E.A.O.C.T, 1979). There are three arguments questioning whether tobacco could be the sole factor in oesophageal cancer.

- Smoking has been common for over a century (Van Rensburg, 1981), but the disease appears to be relatively new major cause of local death.
- Significant papers have shown a sizeable amount of non-smokers affected by oesophageal cancers (Sammon, 1992; Van Rensburg *et al.,* 1985).
- Van Rensburg shows that the total mean quantity of tobacco used in nearby Natal is considerably less than used in rich western communities where oesophageal cancer is rare (Van Rensburg, 1981).

In the endemic areas of Transkei, there has been no connection between oesophageal cancer and alcohol abuse (Rose, 1982; Sammon, 1992). A difference between high

and low incidence areas were that in high incidence areas a majority of the traditional beer produced used maize instead of malt (Bradshaw, *et al*., 1983). Therefore tobacco and alcohol use could be predisposing risk factors, although the evidence does not point to these being the sole factors of carcinogenesis.

1.9. The Sammon Theory

1.9.1. Basis of the Theory

Alistair M. Sammon, with the help of colleagues, over the last ten years of working in the U.K. and in Africa, postulated a theory on the carcinogenesis of squamous cell carcinoma oesophageal cancer in "black" South Africans, in 1998 (Sammon & Alderson).

He stipulated that there might be four factors associated with the endemic squamous cell carcinoma:

- Heavy dependence on a maize based diet over a very long term
- Slow rise in incidence
- Relative poverty
- Tobacco use at a high-risk level.

The first three factors are all related, as the slow rise in SSC over the last fifty years seems to have a relationship with the increase use of maize. This itself has become a primary component of the Transkeian diet, due to poverty in the area. The link between oesophageal cancer and tobacco use is well documented and is likely to be part of the carcinogenic process (Fig 1.13).

This theory is split into three parts:

- A high level of linoleic acid ingestion via maize is associated with increased prostaglandin E_2 production in the stomach. Other dietary factors increase this effect.
- PGE_2 inhibits gastric acid secretion and reduces the tone of the pyloric and lower oesophageal sphincters, resulting in a chronic alkali duodenogastro-oesophageal reflux (DGOR).
- Chronic low-acidity DGOR predisposes to squamous carcinogenesis.

1.9.2. Prostaglandin E_2 Production in the Stomach

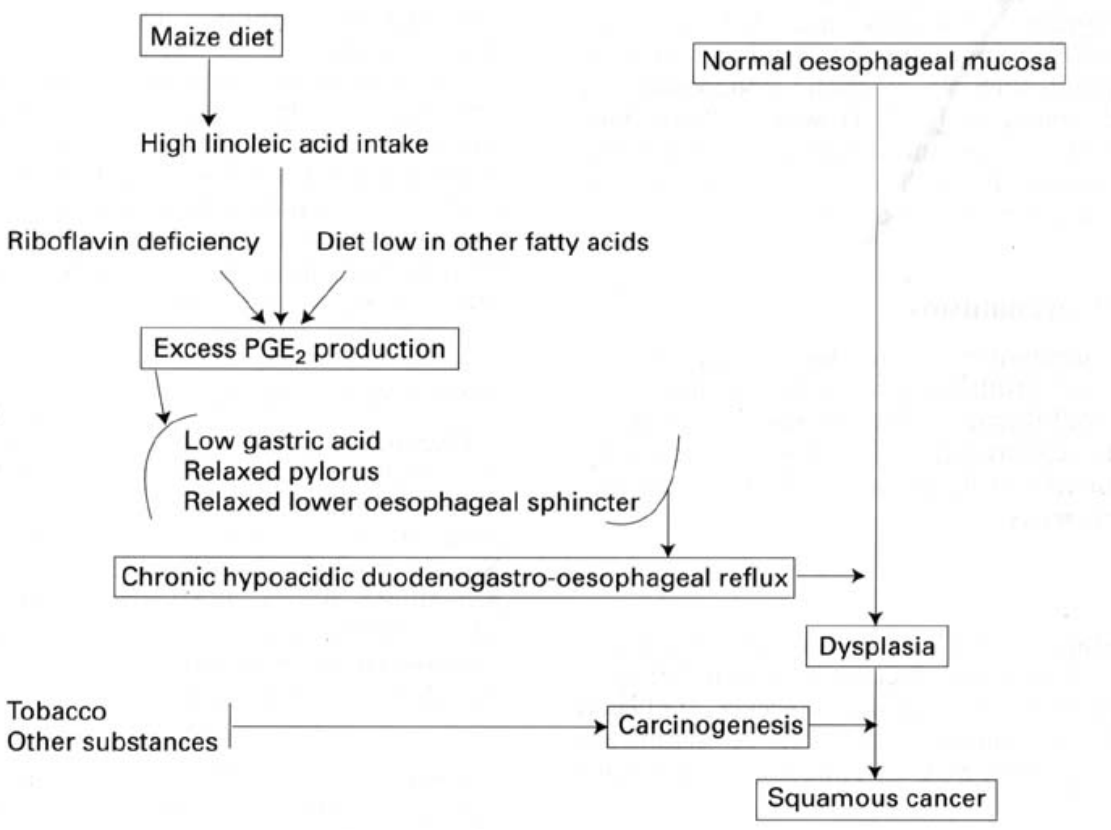


Figure 1.13: A diet based on maize with deficiency of other elements will lead to high prostaglandin E_2 production and a chronic low acid oesophageal reflux, with consequent predisposition to cancer (Sammon & Alderson, 1998)

Van Rensburg (1981) concluded that prolonged exposure to maize is greatly linked to oesophageal cancer. In a latter paper Van Rensburg *et al.* (1985) explains how the risk changes for various forms of maize ingested due to linoleic acid release in the stomach. Maize oil is rich in linoleic acid and very low in other essential fatty acids. Dupont (1990) stated that 59 % of corn oil is linoleic acid. Whole kernels are poorly digested releasing little oil in the stomach. Stamped maize has the oil-containing husk removed and provides no linoleic acid at all. These two are shown to be low risk for oesophageal cancer. Conversely, Maize meal provided all the linoleic acid to the stomach in an easily digestible form. Consumption of maize meal has been shown in Italy to be linked to high-risk areas (Rossi *et al.,* 1982). It has been proved that Linoleic acid is a prostaglandin (PG) E_2 precursor and when a high linoleic acid diet is fed to a rat, there is an increase in gastric prostaglandin E_2 (Schepp *et al.,* 1986). PGE_2 production is promoted by lack of dietary fatty acids which cause competitive inhibition (Raederstorff & Moser, 1992) and riboflavin deficiency, (Pelliccione *et al.,* 1985) which can be seen in the Transkeian diet. Riboflavin deficiency has also been implicated with oesophageal cell proliferation and dysplasia (Munoz *et al.,* 1987). Dietary linoleic acid in one study was shown to be an immunosuppressive, therefore linked to the PGE_2 immuno suppression discussed above (Bennett *et al*., 1987; Sammon, 1999). Sammon has suggested this may be the cause of another common disease in South African children that present a low immune system, called kwashiorkor (1999).

1.9.3. The Implication of Increased PGE_2 .

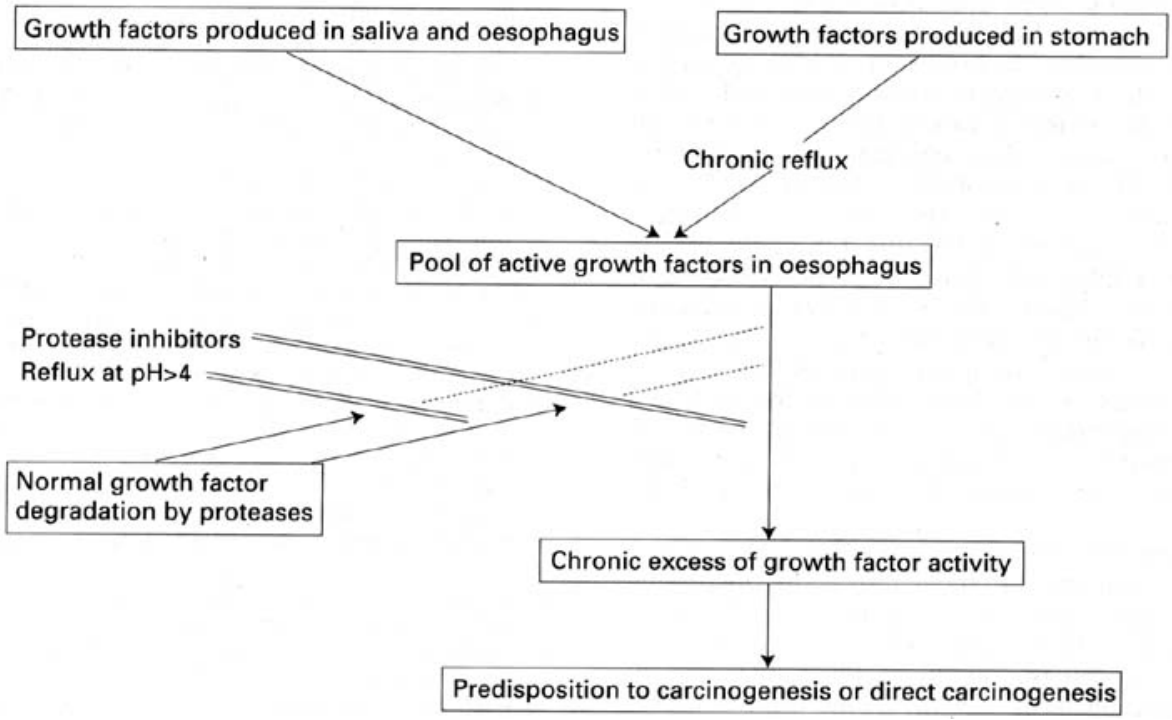


Figure 1.14: Reflux of a high pH and ingestion of protease inhibitors ensure excess growth factor activity in the oesophagus. (Sammon & Alderson 1998)

As stated before, heartburn is very common in the Transkei area, affecting 60 percent of the local healthy people following a meal (Sammon, 1994). Increased PGE_2 affects two things; it causes a reduction in the lower oesophageal and pyloric sphincter pressures (Milenov & Golenhofen, 1982; Hausken *et al.,* 1991), and reduces basal and stimulated gastric acid output (Schepp *et al.,* 1986). These could well be the underlying factors causing the heartburn via an increase in oesophageal reflux of gastric fluid of reduced acidity. Sammon, along with Iputo and their team (2003), measured the pH of fasting gastric fluid in the sample population. They discovered a

bi-modal distribution of pH peaks at 2 and 7. The lower pH group equates to a relatively normal pH distribution. The higher pH group was significantly associated with maize consumption.

1.9.4. Chronic low –acidity DGOR predisposes to squamous carcinogenesis

Various studies have shown that duodenal regurgitation into the oesophagus, in rats and partial gastrectomy patients can be the cause of squamous cell carcinoma (Seto *et al.,* 1991; La Vecchia *et al.,* 1994), which itself could be connected to the reflux of high pH into the oesophagus. PGE_2, which could be in the reflux, has been shown in rats to promote oesophageal carcinogenesis (Shimizu, 1986). It is this DGOR that could be the key to the carcinogenesis. In the gastrointestinal tract over expression of Epidermal growth factor (EGF) and transforming growth factor (TGF) α, which are normally products of the salivary glands, oesophageal mucosa and stomach, has been linked to proliferative drive and carcinogenesis (Yoshida *et al.,* 1990). These growth factors are partly or wholly inactivated by the enzymes trypsin (Marchbank *et al.,* 1995) and pepsin at a low pH (Playford *et al*., 1995). Therefore a DGOR of high pH gastrointestinal fluid could inactivate these enzymes, allowing the growth factors in the reflux and salivary growth factors to remain active. The growth factors may then cause carcinogenesis directly or via pre-disposition, which could be born from a proliferative drive. Beans and pumpkin are the staple diets of many Transkeians, these has been postulated to contain trypsin inhibitors, therefore, large amounts of growth factors are not naturally broken down, increasing this local proliferative drive (Bradbury *et al*., 1985). The vegetable *solanum nigrum* used in Africa has been shown to contain a pepsin inhibitor (Akhtar & Munir, 1989), also shown to be connected to cancer of the oesophagus (Sammon, 1992). It has been demonstrated that bile acids can induce COX 2 expression and PGE_2 production, therefore increasing any carcinogenic effect they may already have in the oesophagus (Li, 2000).

1.10. Aims and Objectives

1.10.1. Aim

The literature reviewed so far suggests that generally, oesophageal cancer may have many causes and contributing factors. However, the rise in incidence in Transkei in parallel with other factors suggests a particular mode of carcinogenesis in this region, as postulated by the Sammon theory.

The aim of this project is to investigate the Sammon theory (Sammon & Alderson, 1998) at a molecular level with the intension of providing evidence to support a link between diet and predisposition to cancer through PGE_2 and its molecular pathways in the population of Transkei, South Africa rather than the commonly researched Western World.[1]

[1] The Sammon theory (section 1.9) forms a basis of how epidemiological factors could lead to increased cancer progression, it is of major importance to update this research with an investigation of the target population and further investigate the carcinogenesis of this oesophageal cancer. This increases the breath of understanding of how the disease initiates and ideally its prevention. Treatment is expensive and labourous; making is difficalt to afford in Transkei a more cost effective approach needs to be investigated.

1.10.2. Objectives

- Observe and understand a rural South African population and evaluate any risk factors that may predispose oesophageal cancer and have not yet been tested in present theories.
- Extend the present theories of the development of oesophageal cancer in the target population and provide a novel understanding to how these factors affect the expansion of carcinogenesis in cells, at a molecular level.
- Discover whether gastric fluid from the sample population has a mitogenic potential *in-vitro* and investigate what may cause this effect, if it occurs.
- Develop an *in-vitro* model to manipulate proteins that may drive this mitogenic force in oesophageal squamous cell carcinoma.
- Investigate how specific dietary components in the sample population may modulate the increased proliferation of oesophageal cancer through the *in-vitro* model.
- Evaluate whether or not this research supports the Sammon theory of whether PGE_2 is the major factor in the development of oesophageal cancer, and therefore give evidence and understanding as to why this may be the case at a molecular level.
- Suggest cost effective methods to help the target population suffering from oesophageal cancer.

2. A Study of the Sample Population

2.1. A Study of the Sample Population

2.1.1. Introduction

An understanding of the target populations' difficulties at a local level was important due to the sample population inhabiting an area quite different to the UK in terms of social and financial status, environment, and health care. It was hoped that the questioning of local people would help the epidemiological aspect of the project. Hence a greater understanding of incidence and aetiology would facilitate theory development for the rest of the project. This is of generic importance for oesophageal cancer throughout the world, as from population to population the molecular biology changes may be very similar.

2.1.2. Methods

The methods used were general observation, questioning, note-taking, photos and my personal diary taken throughout the trip. The majority of the questions were asked of Mr Sammon, with whom I travelled. He had spent much of his time there for the last ten years. Some information has been asked of the local inhabitants, hospital staff, university staff and students. The following chapter is written from my personal experiences.

2.1.3. Results and Discussion: Experiences of Transkei and Local Questioning

The journey took us from Johannesburg airport, in the North of South Africa, on a drive to Umtata, Transkei in the south (Eastern Cape) of the country. This took two days in a car with an overnight stop off at Pietermaritzburg. This gave a chance to take in the rich culture of South Africa and gain a better picture of the land. During this journey, to and from Transkei, we visited hospitals and health centres that have been set up by Christian missionaries, again to gain a clearer understanding of the social situation of the South African population, hence, potential cancerous factors.

2.1.3.a. General Impressions of the High Incidence Areas

It has been widely published that Transkei is the worst affected area for oesophageal cancer in South Africa (Burrell, 1962; Rose, 1979; Rensburg, 1987). Sammon found that the disease mainly affected rural people, principally the poor who own and farm land, but not the very poorest that have no income. This was reported in previous papers. A case-control study by van Rensburg (1985) of Zulu men, showed that most affected were in a transitional state between tribal subsistence farming and Western living. Cancer of the oesophagus patients in Durban were found to have more schooling and speak English more often than lung cancer patients (Bradshaw, 1968). Rose (1979) has reported that semi-westernisation was an apparent risk factor in Transkei.

This raised three points to question:

- Was the risk factor related to items that someone with a small disposable income may be able to afford, that some one very poor could not?
- Is there is an occupation health risk?
- Do the people with a little education have more confidence to approach the often "politically white" medical centres to gain a diagnosis?

These three items have been woven into the findings and experiences below and will be commented on later.

According to the local medics it was commonly thought that the South East of Transkei, near Butterworth, has been more affected than the Northern part (Jaskiewicz *et al.,* 1987). This has been questioned because of the different recording methods taken of disease from different medical services, and the way they are recorded in different ways.

The Northern areas also seemed to have large "white" farming areas that employ the local "black" communities for labour. Perhaps in Southern regions that have not had to rely on this income due to the food availability of the coast, having greater independence from the "White" farmers. This increases the cultural gap between the two races. This highlights further questions, as when evaluating the areas of high oesophageal cancer incidence, are these a real representation of the high oesophageal cancer areas, or do people respond differently to medical research depending on their culture clashes. For example, are the local population going to trust and respond honestly with the often Caucasian sampling scientists, due to previous political mistrust? This presents a number of social-political issues that cannot all be addressed here due to the biological scientific nature of the project. Therefore it is to be made clear that all of the factors raised have been reviewed with social issues in mind.

It was asked if there were poverty stricken areas, or whether there were areas that people healthily lived in and due to "non-western influence" they are misinterpreted as poverty areas. The general response seemed to be that there was genuine poverty there. In Mr Sammons experience of people in the townships, they knew how to eat

properly, and wanted to eat properly, but did not have the money to buy fruit, vegetables or adequate protein sources.

People with a lack of income seemed more apparent when we travelled south, out of the city of Umtata on the few hours of journey towards to the coast. Many families seemed to have come to depend on the now dying trade of the tourists who travel to the coast. They were selling nuts, fruits and items made from shells and empty cans by the side of the road, and when the car was stopped, people seemed desperate to sell these items sometimes surrounding the car. This could have been a sign of poverty or a tourist sales technique. The array of foods on offer seemed limited and the people occupying the areas seemed more spread out, but still with limited land, due to a majority of surrounding area being owned by rich caucasian South Africans and used for farming.

This may suggest that the food available in these high incidence areas is limited to what the locals grow themselves. They also have limited access to health care. Nearer the coast, where the incidence levels decrease, there seemed to be more western development where tourists had once regularly visited in large numbers before the change in the political situation and public perception of South African areas being unsafe.

2.1.3.b. Landscape and Vegetation

Figure 2.1: Left: Photo taken from the road two hours drive north east of Umtata of the Drakensberg Mountains (Ukhahlamba - the Barrier of Spears). Right: Photo taken from the Umtata hospital over looking the valley of Umtata.

The area was visited in May, which is classed as winter. The weather was cloudy with mixed rain and sun. There were vast amounts of vegetation, but even in winter the plants were yellow and dried out from droughts of the rest of the year. The plants that did appear to be green are hardy vegetation that cannot be eaten. This may cause malnutrition problems if the population rely on the vegetation and self grown produce. Much of northern and central areas of South Africa seemed to be well farmed with plenty of produce like sugar beet. This is mainly owned by the caucasian farmers and many of the poorer "black" people from the townships have worked for them during certain crop picking and sowing seasons and then return to their family following the work. With so much political change, Mr Sammon with whom I travelled stated that many of these farms seem to be reducing in size and productivity, therefore having income issues on the workers that have learnt to rely on this finance.

Tarmac roads were used to drive south rather than the usual dust roads, which maybe financed by the richer farmers indirectly through there tax income, therefore it is in unfair to say these green fruitful vegetation, the roads on which we through, are typical of Northern South Africa where oesophageal cancer is not as prevalent as that found in Transkei. A study by Jaskiewicz and colleagues (1987) showed variation within the Transkei area, with a higher incidence in the south-western districts than

the north-eastern districts, but all had very high cases of oesophageal cancer (Makaula *et al*., 1995).

Driving south it was apparent that the areas were very dry and there were many signs of where locals have burnt massive areas of the dry vegetation to make way for new growth. Even though the plants, in the picture (Figure 2.1, Right) taken from the damp hills of Umtata, appear very green they are not a representation of the dry and dusty crop fields that can be seen in the distance and in the other picture taken north east of Umtata (Figure 2.1, Left). The lands can be dusty for around 6 months a year; this presents an environmental issue that may affect the oesophagus through irritation. When asking if there were many cases of hay fever, which has been implicated with PGE2, cases were not reported, as large amounts of pollen are not present.

2.1.3.c. Housing and Families

There was a huge variation in the social and economic status of the various populations in South Africa. Many of the townships have developed small shops and built their own economy through help from governmental schemes. This has increased income allowing families to produce different products to trade, increasing the variation of items like different food groups in poverty suffering areas. Many of the townships driven through represented small dusty busy villages with market areas of apparently healthy happy people rather than the often-perceived shantytowns.

The people that have a higher economic status tended to live in brick housing, very much like a basic three bedroom bungalow seen in the United Kingdom. Many of the people in the townships used to live in the traditional huts, but now live in more modern “tin houses”, so called because of the thin metals they are made from.

Figure 2.2: Left: Photo taken an hour drive south of Umtata but very typical of the hut housing that many townships are made up of. Right: Photo taken of the houses around the hospital of Umtata typical of the newer tin housing that is replacing the traditional huts.

There do not seem to be any essential health hazards of living in the housing and they appeared clean and dust free. Less family members live together in the houses than a few decades ago. Many have between two and five children per family. The houses are much more communal than expected in western society, where all the young brothers will share one part and the sisters another. There are many single parents for various reasons that including parents travelling to find work or death by diseases such as AIDS. Traditionally the women would have worked in the fields although nowadays there is a chance that in large populated areas they will have a paid job. Examples of this include local shop work, and employment in schooling and health centres.

Figure 2.3. Photo taken from the road in the north of South Africa of a tin house township, now typical round the developed cities with large populations like here near Johannesburg.

Up to a few decades ago there were no toilets and people used to use the fields. Little black pigs were plentiful and tidied up afterwards. Now there appears to be quite a number of pit toilets. The source of the bathing water would previously have come from the nearby rivers, but the cities for several years have had running water taps piped in. Traditionally the unmarried girls collected the water from the nearest river or lake. There seemed to be no tradition of treating or boiling the drinking water.

2.1.3.d. Health

There are about thirty rural hospitals in Transkei staffed by doctors, each with a system of surrounding health clinics run by nurses. This means that everyone in Transkei is less than two hours away from receiving medical attention. Some local people also use two kinds of traditional healers: Witchdoctors and Herbalists. The witchdoctors depend on a spiritual content to their approach, with divination by throwing bones, and casting spells and curses. Some witchdoctors use medicines, although it was not asked about their content. Herbalists treat with locally grown plants and have no significant spiritual content to their approach. Due to the small percentage of people using these methods for healing they do not appear to be the single significant cause of the oesophageal cancer epidemic.

When asked if the traditional cultures are scared of going to seek medical attention, the general response was no, but they do tend to only see the nurse when the symptoms are greatly developed. This presents a problem in oesophageal cancer due to the fact that symptoms tend to present at a late stage of tumour development, when swallowing is affected.

There are a number of prolific diseases more prevalent in South Africa than most western countries: Tuberculosis and AIDS are rife. According to the National Department of Health, 25.2% of the population in the Eastern Cape have both tuberculosis and AIDS together (The 1997/8 Annual Report; *Strides and Struggles in TB Control*, DOH, RSA).

There are a moderate amount of sexually transmitted diseases. Malnutrition is also common. There are lots of gut parasites – roundworm particularly. Hepatitis is common as shown by the majority of people having antibodies to Hep A and many with antibodies to Hep B (Sammon – Personal communication). Following a review of the papers there does not seem to be a link between the presence of Hepatitis and oesophageal cancer.

Ikhelebha is an acute inflammation and ulceration of the lower lip seen in the sample population. This has been linked to a time of the year when vegetables are scarce leading to riboflavin deficiency (Jackson, 1952).

As PGE_2 is linked to giving birth by modulating contractions and baby development then it was asked if they have many problems with child development and there did not seem to be any more reported than the rest of the world.

When asked if there were any factors that are exacerbated by poverty the only answer was that of health caused by malnutrition and increased cases of tuberculosis (TB).

2.1.3.e. Exposure Risk Factors

There was a lot of exposure to smoke in most traditional Transkei huts. These huts had a fire in the middle of the floor but no chimney, the smoke just dissipated up through the roof. Some unpublished work carried out by Mr Sammon looked at the relationship between open fires and oesophageal cancer, but he failed to find any correlation (Sammon – personal communication).

Relating to occupational threats, a high amount of silicosis and TB has been seen due to many of the Transkeians having worked in mines. Again there are many farming jobs where there is high contact with open fires.

A large number of the local community standing around in the town appear to smoke, although this could be a poor representation of the community as a whole. Sammon stated that around 72% of the patents with cancer of the oesophagus in the Transkei area smoked tobacco, compared to 53% without oesophageal cancer who did not smoke (Sammon, 1992). Many of the educated students and staff at the university said they did not smoke. Therefore smoking appears to be a significant local risk factor for oesophageal cancer. A high percentage of the cancer suffers do not smoke, questioning whether this is the single most important factor in carcinogenesis, although not ruling out a multi-factorial approach.

2.1.3.f. Food

Mr Sammon provided much of the following information of the food groups and their descriptions.

Many of the foods consumed in the locality contain maize meal in one form or another. This appeared to be a staple diet since the 1940s and several cooking methods have been developed to exploit maize for recipes, meals and drinks.

Many of the most common meals are explained below.

The three main foods consumed:

- Maize – This is eaten as whole kernels, stamped or ground.
- Lima Beans
- Flat white pumpkin and Usolontsi – Locally grown pumpkins

Other foods

- Umqa Wethanga – Maize meal and white pumpkin cooked together and eaten in the form of a mash. *
- Amarewu – Non- alcoholic drink made by fermenting maize flour and wheat flour *
- Imithwane – Relish made from green pumpkin leaves
- Animal fats – Saved following slaughter
- Sour Porridge – Maize meal porridge left to naturally sour or soured with herbs
- Wild Vegetables and herbs – African spinach (*amananthus*), Nettles (*Chenopodium*), Irwabe, Umsobo (*solanum*).
- Xhosa beer – Traditionally made from millet, but now made with maize.
- Meats – Cattle, Goats and sheep depending on the wealth of the family

Commercial food items that are slowly becoming popular:

- Bread
- Holsum – A commercial cooking fat made from 95% fish oil
- Sunflower Oil
- Margarine

Items that are no longer being extensively used:

- Sorghum
- millet

* Mr Sammon said these are being commonly blamed for heartburn.

Self-induced vomiting (cleansing as it was known) and regurgitation following a meal was said to take place more than once a week, this seemed common in much of South Africa.

The regular food habits for the majority seemed to consist of maize porridge in the morning and stamped maize and beans in the evening. Maize beer is the usual alcoholic drink, but this is not generally consumed in large amounts.

The foods often seemed spicy and some of the teas I was offered seemed to contain high amounts of cinnamon, which can cause a burning sensation on the throat. Although none of these meals gave an impression that they would cause physical irritation, the plants *solanum nigrum* and *chenopodium album* are both used in cooking and have been previously related to oesophageal cancer (Sammon, 1992).

2.1.4. Further Discussion

Although there seem to be a number of issues that may well effect carcinogenesis of the oesophagus in the local population, there were few new factors that could be identified. This may be due to the short stay during my visit, problems with not being able to speak Xhosa, and the number people not speaking clear English. Some locals may mistrust the "white" population because of local politics (personal stories of violence were shared), and many scientists use these target populations as a study, taking samples but never returning with further help and findings. It was apparent that in the main population there was a lack of understanding of health issues and understanding of disease, similar to the lack of education found in western poverty areas. The human resources that could help educate people about detection of early symptoms are stretched and are busy educating people about more common killers such as AIDS and its prevention. One of the main issues to prevent the development of cancer in a patient is catching the disease before it metastasizes. Therefore it is important to diagnosis cancer before it develops too far, but locally many patients only seek medical attention when the symptoms are advanced making cancer

treatment and prevention almost impossible, and the prevalence increase. In addition, local medical services are stretched; as trained staff gain experience many move on to areas where they can receive increased wages abroad or in the cities like Durban, Cape Town or Johannesburg, sending money home.

One issue was concerning the population with a small income, rather than none, that seem to be affected by oesophageal cancer more. There does not seem to be any obvious occupational health risks and it was mentioned that people with all education levels use the medical facilities available. However, many health centres can be up to two hours away which may take all day to reach if you do not have transport, so this may not always be used if people fear they will loose their income for the day as a result. The most evident reason for increased prevalence in this subset of people may be linked to individuals being able to justify spending the extra income on smoking, presenting an additional risk for cancer development.

There did not seem to be a "single" social factor that could be an obvious cause of cancer other than the lack of dietary variation. This may be due to lack of money to buy the right tools for irrigation systems like those found in the green farms in northern areas of South Africa. This leaves large farming areas dry, cracked and damaged such that few plants that can grow to support the large populations found in many areas. Therefore, many of the foods available in such areas were reliable, moderately fast growing crops such as maize. There is an obvious concern in the area about lack of varied diet especially for their children, but little money to do anything about it. This, along with lack of education about the spread of disease, prevalent smoking and common bile regurgitation seem to be obvious cancer predisposing factors to be investigated further. Although these areas have never been rich, the problems may have worsened with changes in politics, decreases in tourism and an increasing population. There are occupational risks stated above though they would only affect a small number of people. There are many common diseases in these areas but little research shown any relationships between oesophageal cancer and these diseases, such as Tuberculosis and Hepatitis other than the predicted decreases in the immune system having a knock-on effect.

There is a great deal of information to be collected and case studies to be carried out to discover the connection between the components of the poverty areas, and its

malnutrition, and the relationship with oesophageal cancer. For the purpose of this project this information was used to understand the history of the samples and suggest further work, but could not directly shed light on the aims and objectives of this project other than it is likely that we are dealing with a multifactoral affect resulting from these often poverty stricken factors.

3. The Expanded Sammon Theory and its Molecular Implications

3.1. Introduction

It was important at this stage to collate the present peer reviewed research to investigate the process of molecular carcinogenesis of oesophageal squamous cell carcinoma in the target population. This was to bind the knowledge of the previous peer reviewed research in chapter 1, and the observations of the population studied in chapter 2. Figure 3.1 was therefore composed, along with the relevant references to provide my interpretation of the Sammon theory (Sammon & Alderson, 1998) explained in section 1.9. Figure 3.2 then looks further into the cellular interaction of some of these components of interest in this project and how they may be driving this tumourigenesis.

The experimental work of this thesis was based on testing individual areas of this predicted molecular carcinogenesis of oesophageal cancer shown in Figure 3.1. A large amount of the time was spent on the development and understanding of these theories, for example transposing environmental factors in this target population to molecular factors that could push the progression of cancer from hyperplasia to a pre-malignant state. This was not just through literature review but through oral communication of scientists at conferences, student think-tanks and feedback of presentation of the work. This was important to solidify, develop and peer review the theories.

Due to the novel aspect of this research some components of the theory have been developed using research papers of other tissue types and populations. Therefore the references have been colour coded to show what has been peer reviewed in the target population and tissues, and what has not.

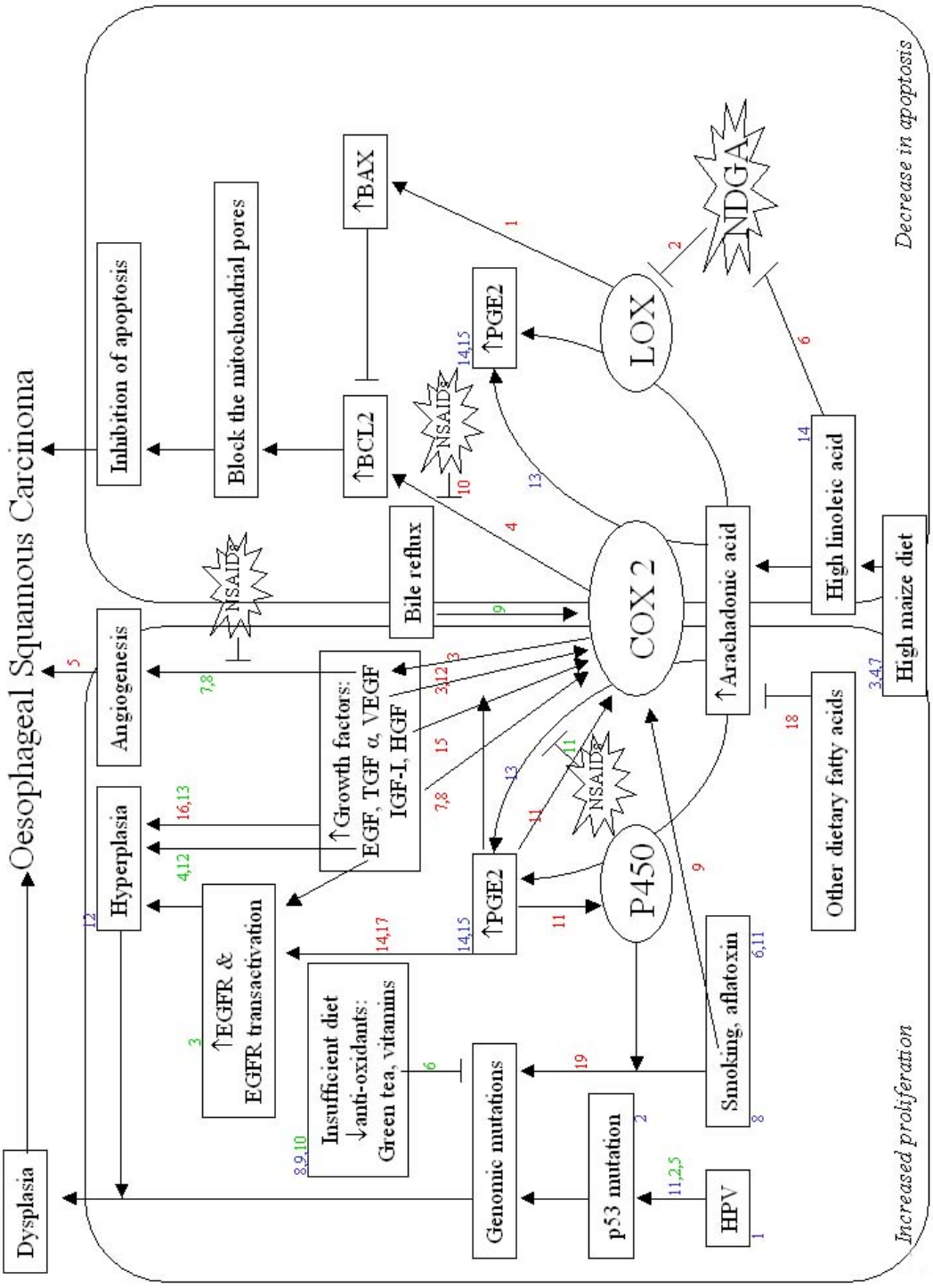


Figure 3.1: Developed theory of the carcinogenesis of oesophageal squamous cell carcinoma in the target population with the knowledge of the previous peer reviewed research in chapter 1, and the observations of the population studied in chapter 2.

Abbreviations

COX 2 – Cyclooxygenase 2, PGE2 – Prostaglandin E2, LOX – Lypoxygenase, NDGA - Nordihydroguaiaretic acid, HPV – Human Papilloma virus, NSAID – Non steriodal anti-inflammatory drugs.

References

Shown in relation to Oesophageal cancer, not necessarily in South Africa:

1. Takada *et al.*, 1995
2. Sur and cooper, 1998
3. Ozawa *et al*, 1989
4. Juhl *et al*., 1995
5. Ono *et al.*, 1994
6. Muller *et al*, 1997
7. Inoue *et al*, 1997
8. Koide *et al*., 1999
9. Zhang *et al.*, 1998
10. Gao *et al.*, 1994
11. Farrow *et al.*, 1998
12. Jankowski *et al.*, 1992
13. Jimenez *et al.*, 1998

Shown in other tissues:

1. Tang *et al*., 1996 (mammary)
2. Goodman *et al.*, 1994 (colon)
3. Murphy and Fitzgerald 2001 (colon)
4. Liu *et al*., 2001 (mammary)
5. Folkman, 1990
6. Rouzer *et al.*, 1990 (leukocytes)
7. Perkins and Kniss, 1997 (WISH cells)
8. Diaz *et al.*, 1998 (fibroblasts)
9. Kelley *et al*., 1997 (Oral)
10. Liu *et al.*, 1998 (prostate carcinoma)
11. Brueggemeier *et al.*, 1999 (breast)
12. Gallo *et al*., 2002 (Head and Neck Squamous Cell Carcinoma)
13. Fosslien, 2000
14. Pai *et al.*, 2002 (colon)
15. Jones *et al*., 1999 (colon)
16. Schmassmann *et al.*, 1997 (gastric)
17. Buchanan *et al*., 2003 (colorectal carcinoma)
18. Raedersdorf and Moser, 1992 (Kidney and Liver)
19. Mace et al., 1997 (liver)

High incidence in oesophageal cancer in South Africa:

1. Williamson *et al.*, 2002
2. Vos *et al.*, 2002
3. Van Rensburg *et al.*, 1985
4. Van Rensburg *et al.*, 1981
5. Cooper *et al.*, 1995
6. Rheeder *et al.*, 1992
7. Rose, 1982
8. Van Rensburg, 1987
9. Dayne and Munoz, 1982
10. Matsha *et al.*, 2002
11. Matsha *et al.*, 2002
12. Hamilton and Isaacson, 1985
13. Grant *et al.*, 1988
14. Sammon, 1994
15. Sammon and Morgan, 2002

3.1.1. Development of the Sammon Theory

Over the first twelve months of the project the theory pictured in Figure 3.1 was developed, by reviewing available literature (section 1.7), local work (section 1.8 and 1.9) and studying the target population (Chapter 2). This shows the complexity of the Sammon theory, when a number of carcinogenetic factors that have been peer reviewed are added. To simplify Figure 3.1 it has been split into two sections, increased proliferation on the left-hand side and decreased apoptosis on the right-hand side, two of the most common characteristics of cancer tissue. This thesis investigates proliferation caused by increased dietary PGE_2, therefore the molecular components that drive this proliferation have been further reviewed and their connections linked in Figure 3.2. The production of PGE_2 can take place via three enzymic pathways: Cyclooxygenase (COX), Lypoxygenase and the p450 enzymes. A majority of the research published in this area has investigated COX 2. COX 2 has become an exciting and popular enzyme to research, not only due to its suppression leading to tumour suppression, but the large financial gains manipulation can bring (section 1.6.1).

Stated in the Sammon theory one action of PGE_2 maybe to relax the oesopho-gastric sphincter, allowing gastric reflux (Figure 1.13). The oesophageal damage seems to mainly arise from the squamous tissue, progressing to squamous cell carcinoma. Once the hypo-acidic reflux has washed through the oesophagus the Sammon theory predicts that the increase in pH is not optimum for the action of the natural enzymes that breakdown the growth factors present, allowing them the drive further proliferation. Due to the very short half life of growth factors this is unlikely. Also repeated growth factor stimulus in normal cells would cause receptor desensitisation through changes in the G protein response. It may be postulated that this reflux promotes further growth factor release, stimulation or activation, in other ways increasing the overall proliferation of the oesophageal tissue. It is probable that the PGE_2 and growth factor interaction is essential in the carcinogenesis of squamous cell carcinoma in this target population, by researching the expression of these growth factors following treatment of the predicted components of the gastric reflux could help investigate the Sammon Theory.

Following the visit to South Africa it is obvious that there are factors that could promote oesophageal squamous cell carcinoma carcinogenesis. These may include the side effects of diseases like AIDS, Hepatitis B, gut parasites, HPV and tuberculosis perhaps by the inhibition of the immune system suppressing cancer protection. Many cancer researchers in South Africa believe that aflatoxin from mouldy maize is the leading carcinogen, and as reviewed in the introduction this research is still primitive but may be an inclusive factor. It is clear that there is not a single major factor that could suggest the development of oesophageal cancer.

The hyper proliferation that maybe driven directly or indirectly by the PGE_2 would provide the right environment for initiation of carcinogenesis to develop and the factors mentioned above may progress this carcinogenesis further. Hence, decreasing the rate of hyper proliferation may reduce the incidences of oesophageal cancer in the target population. This provided the first area of investigation.

The gastric fluid samples were tested for PGE_2 content and mitogenic activity on oesophageal cell lines, should the PGE_2 content be high and cellular proliferation increased, then time could be spent on researching any proliferative proteins involved. The obvious proliferative proteins are growth factors (section 1.6.10), therefore

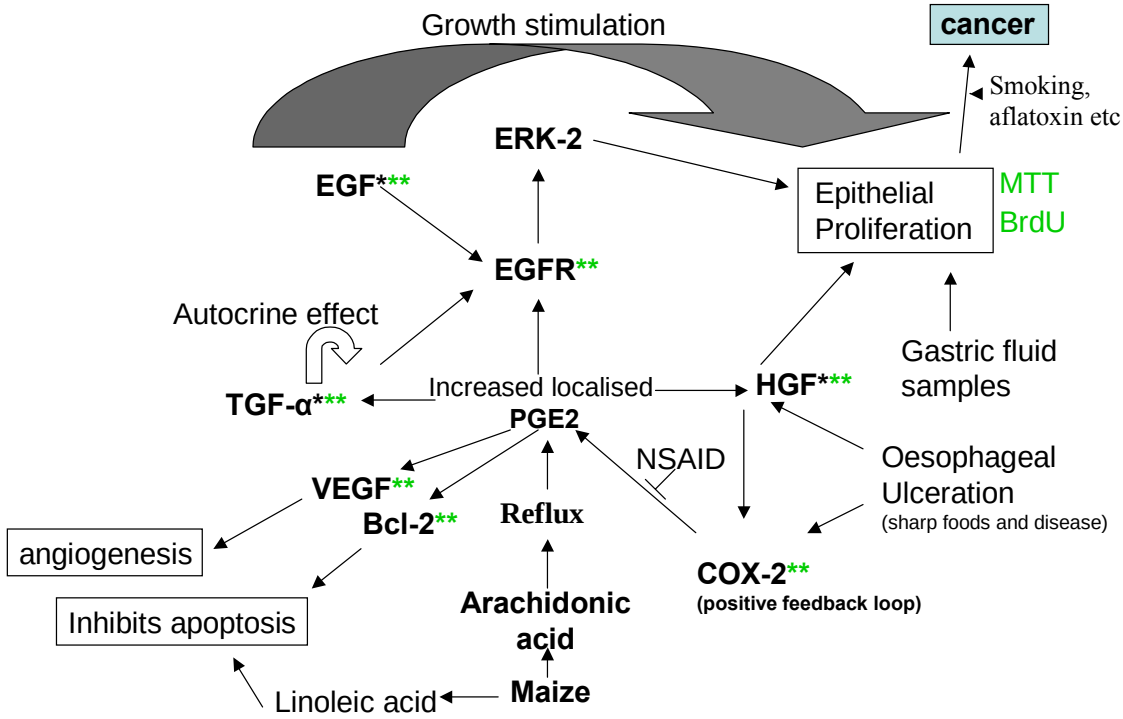
developing the theory shown in Figure 3.2, based on the interaction of growth factors and PGE_2, the product of COX 2.

3.1.2. The Cellular Components and their Predicted Interactions in the Development of Oesophageal Carcinogenesis Examined in this Research

From Figure 3.1 factors were selected and investigated in order to develop a molecular basis for the hyperplasia in the Sammon theory. In Figure 3.1 all the components between the high maize diet box and the increased PGE_2 box have been investigated and peer reviewed in the target population. Therefore it was important to carry out some fundamental research studying any proliferative affects of PGE_2. If PGE_2 has a proliferative effect on the oesophageal cells, then the cause of this cellular drive, and potential therapies could be investigated.

It is commonly known that growth factors drive cellular proliferation. A large number of studies have addressed the subject of growth factors in oesophageal tissue, and investigated the effects of EGF (Juhl *et al.,* 1995; Jankowski *et al.,*1992; Jimenez *et al.,* 1998, Fosslien, 2000; Pai *et al.,* 2002). A study by Abiru and colleagues (2002) showed how non-steroidal anti-inflammatory drugs (NSAID) such as aspirin, inhibit the invasiveness of HGF. Jones *et al*. (1999) showed that HGF triggers activation of the COX 2 gene in rat gastric epithelial cells, through activation of the ERK 2 signalling pathway. Gastrin, a component of gastric fluid, was shown to increase the expression of COX 2 and HGF in gastric tissue (Konturek *et al*., 2003). In oesophageal tissue it was shown that inhibition of COX 2 suppressed ulcer repair by inhibition of ulceration triggered HGF receptor induction (Baater *et al*., 2002). Therefore HGF seems a key growth factor to research in the current project. By measuring the HGF expression and protein content in the oesophageal cell lines then the effect that treatment of these with PGE_2 has may add additional value to this investigation (see chapter 7). Both EGFR and VEGF are two of the major factors in the development of carcinogenesis (sections 1.7.5.a and 1.7.1.d). This has provoked companies such as Merck to develop anti-colonic cancer drugs that interact and stop

the action of these growth factors. Bevacizumab (successfully known as Avastin in the USA) is an antibody based VEGF inhibitor and Cetuximab is an antibody based EGFR inhibitor. EGFR is commonly over-expressed in oesophageal cancer (Mestre *et al.*, 1997). It has been claimed that VEGF is the most important growth factor in the proliferation of new vascular tissue (Gallo *et al.*, 2001). A study into the effect of Hypoxia discovered that induced COX 2 increased VEGF levels that induced angiogenesis (Liu *et al.*, 2001).



•*Reduced growth factor breakdown due to high pH reflux lowering the action of proteases
•**Test for expression via RT-PCR
→ Arrows represent a positive/stimulatory effect

Figure 3.2: Part of the molecular carcinogenesis of oesophageal cancer to be investigated in this research.

This postulated carcinogenesis opens many areas of research to help answer why PGE_2 produced due to a high maize diet may have a proliferative effect on the oesophageal tissue and its links to cancer in the target population. The following chapters will deal with small areas of this theory.

4. Gastric Fluid Sample Collection From the Sample Population of Transkei

4.1. Sample Collection

4.1.1. Introduction

Once there was an understanding of the sample population and theory development, then samples could be collected to investigate the molecular aspects of the dietary effect on the oesophageal cancer.

4.1.2. Methods / Results: Ethical Approval and Sample Collection

Ethical approval was agreed by the Research Ethics & Biosafety Committee, University of Transkei for the taking of the samples for oesophageal cancer related investigations within Transkei. The ethical committee cleared the use of these samples in UK under the agreement that the samples are not used for any other work (refer to appendix B for agreed ethical approval by the Research Ethics & Biosafety Committee, study information sheet and patient agreement form).

The samples for investigation were gastric fluid samples taken by Prof. Iputo and his team from visitors to a health centre in Umtata, Transkei, South Africa, under ethical conditions. Patients were excluded who were smokers, had symptoms of upper gastrointestinal disease or were receiving non-steroidal anti-inflammatory drugs. An explanation of the research and the use of samples were given to the sample donors in their own language (Xhosa). The patient forms were then explained to the patient and a signature or fingerprint, depending on their literacy, was given to show understanding and agreement to the use and collecting of gastric fluid samples. A

dietary study using these samples was performed which indicated that maize was a large component of their daily diet (Sammon *et al.,* 2004).

A hollow rubber medical tube was closed at one end, with small perforations in the bottom of tubing, and was connected to a syringe at the other end. The tube was swallowed and some gastric fluid was sucked back up the tube using the action of the syringe before the tube was removed. The samples were transported in liquid nitrogen and stored at –40^{o}C. The samples were kept in 2ml eppendorf tubes, wrapped in parafilm and placed in two seal-it bags. These were packed in dry ice in a domestic cooler bag, and placed in a domestic food cooler box. The samples were driven for 36 hours, picking up fresh dry ice after 24 hours, to an airport. The samples were loaded in baggage and travelled on an aeroplane for 10 hours. It was noted that the sample cooler box was found opened on the baggage carousel at London without the packing ice bars from the top. These travelled for a further two hours until reaching a –80 freezer for permanent storage.

As the samples were also taken for ongoing investigations undertaken by the University of Transkei the sample number was restricted to the samples that they had more than 1ml, allowing further studies to carry on in Umtata. By carrying out preliminary studies with the small but significant number of samples in the UK the concept was if there was time then return and increase research progression by teaching other students cell biology and molecular laboratory techniques that they could use on the rest of the samples. This dictated the sample number to 24 samples. The analysis of whether this sample size was enough is evaluated in the following chapter.

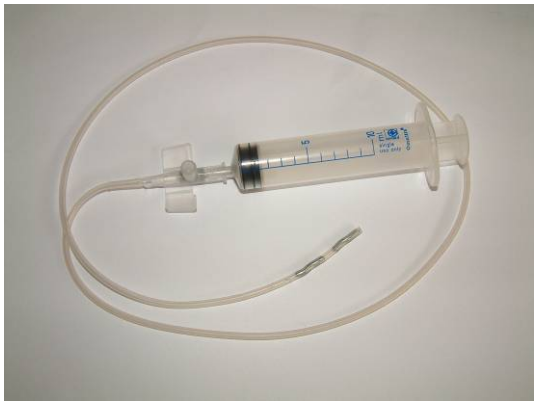


Figure 4.1: An example of the syringe and tubing used to take the gastric fluid samples.

4.1.3. Discussion

There are several factors, which may affect the investigation of gastric fluid samples. Although the samples were kept on ice or dry ice throughout the journey from Umtata to Johannesburg, by the time the airport was reached much of the dry ice had melted relying on the ice blocks to keep the sample cool. Although guaranteed by the check in-staff that the samples were to be kept as “fragile”, the sample were opened in London and arrived at room temperature. This may well have had a detrimental effect on the samples as the proteins in these samples will denature at higher temperatures. This means that the content of the samples may not be a true representation of the composition of gastric fluid in the sample population. Further testing was required to test the detrimental effect that this could have on the samples; this is described in the next chapter (Section 5.2.1).

4.2. Setting Up a Cell Culture Lab in South Africa.

One aim of the visit to South Africa was to work with the University of Transkei to enable them to continue investigations along a similar line to the work carried out in England using cell culture techniques. At the University of Transkei I was based at the Biology Laboratories where someone had previously set up a cell culture lab, but it was not known if it had actually been used. To enable this facility to be ready for use, the following was carried out:

- The lab was washed and cleaned with solvents.
- Windows were air sealed.
- Filed protocols and information sheets on equipment based on those used in the Cranfield laboratories were introduced
- The equipment was tested and a list of additional required equipment was produced
- Suppliers were contacted to provide catalogue information on products, costing and delivery methods.
- Reagents already available were organised and the required health and safety documentation produced.

Figure 4.2: Photos taken of the University of Transkei cell culture laboratory when ready for use.

The laboratory was clean and ready for molecular work. As no cell lines were found, the concept was, should available funds become available, I would return with donated cell lines and teach other students any molecular methods carried out on the samples. After some unsuccessful applications for grants this did not take place.

5. The Measurement of PGE_2 Concentration in Gastric Fluid Samples

5.1. Introduction

Due to the large number of risk factors shown in Figure 3.1, it is important to investigate these factors one at a time. Specific to this population, the Sammon theory is based on the increased production of PGE_2 following increased ingestion of maize. Therefore the *in-vitro* investigations were initiated to develop the molecular aspect of the theory.

As discussed in the introduction (section 1.6.8), increases in PGE_2 have been implicated in the increased cellular proliferation of colorectal tissue as well as affecting other tumorigenic factors. Therefore, PGE_2 concentration measurements of the gastric fluid samples were the initial route of investigation. As the journey of the samples may have had a detrimental effect on the PGE_2 concentration, it was important to study this possible affect by replicating the conditions endured by the samples during the journey, using substitute saliva samples.

Statistical analysis was carried out on the power of the sample number and whether there was confidently enough for the studies. This was achieved by analysing the PGE_2 concentrations of the samples as the statistical variable, as this was the basis of the research using these samples.

Throughout this chapter, increases in cell number or proliferation will be referred to as cell growth. This does not refer to the size of the cells but to cell number. Also the phrase “western diet” is referring to a population that ingests a full varied diet of all food groups including various fatty acids, where there is a lower prevalence of squamous cell oesophageal cancer.

5.2. Method

For measuring PGE_2 concentration, a competitive ELISA was used in a kit format produced by R&D Systems (Oxon, UK). Unless stated the component composition was not disclosed by the manufacturer. Duplicate samples were diluted 10-fold in the assay buffer provided in the kit. Eight PGE_2 standards were produced at, 5000, 2500, 1250, 625, 312, 156, 78 and 39 pg/ml.

The 96 well-plate was supplied pre-coated with goat anti-mouse polyclonal antibody (R&D systems). For each well 100µl standards and samples was added. In to these wells 50µl PGE_2 antibody conjugated to alkaline phosphatase was added, with 50µl anti-PGE_2 mouse monoclonal antibody, all provided in the kit. The plate was incubated for two hours at room temperature and washed three times with the wash buffer provided in the kit (composition not given). Of the buffered p-nitrophenyl phosphate, 200µl was added to each well and incubated for one hour at room temperature. The optical density was then read at 405nm using an ELISA plate reader (MRX, Dynex technologies, Worthing UK).

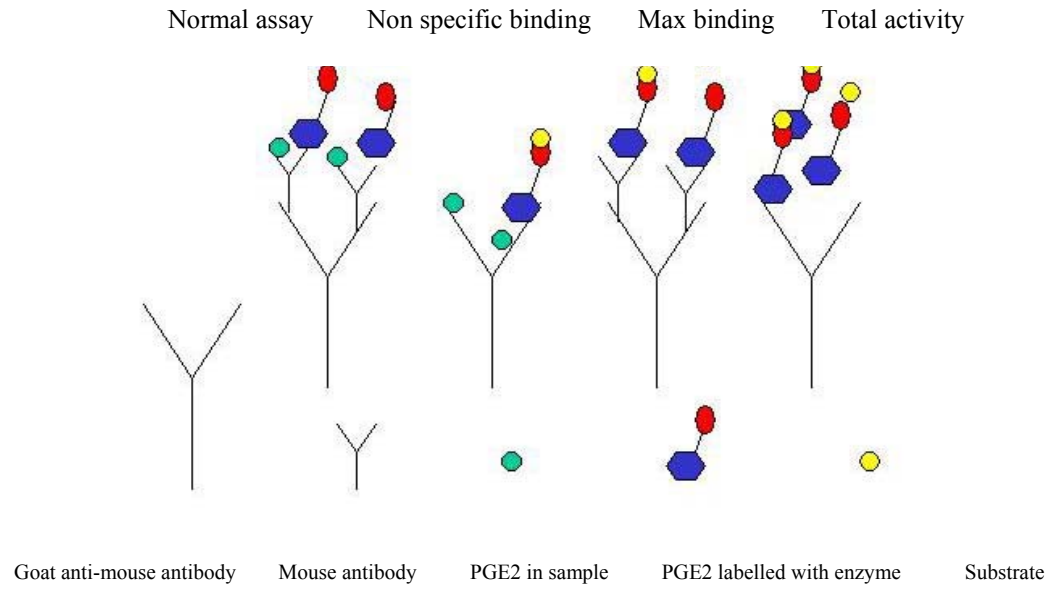


Figure 5.1. Binding scenarios of the competitive ELISA used and the various controls. The mouse antibody binds to the PGE_2 goat anti-mouse antibody absorbed to the

plate, to amplify the signal. The PGE_2 in the sample and the PGE_2 bound to the alkaline phosphate competitively bind to the goat antibody. When the p-nitrophenyl phosphate substrate was added to the buffered solution this gave a coloured reaction relating to the amount of the enzyme-bound PGE_2 present. This signal is inversely proportional to the amount of PGE_2 in the sample.

The average non-specific binding control (assay minus antibody), minus optical density average from each sample was calculated. This was divided by the maximum binding average (everything but sample) multiplied by 100 to achieve the percentage of maximum response.

5.2.1. Measuring PGE_2 Concentration Changes in the Samples Following the Journey from Umtata, South Africa to Bedford, Great Britain.

An evaluation of what affect the three day transportation would have had on the PGE_2 concentration of the gastric fluid samples. Table 5.1 shows the conditions under which the samples traveled and the conditions used the reproduce this journey.

5.2.1.a. Substitute Sample Preparation

Substitute samples were created by chewing on parafilm, to induce saliva, and spitting into a 15ml tube. This was then aliquoted into four 2 ml tubes and labeled 1-4. Tubes labeled 1 & 2 were spiked with 0.005 μg/ml of PGE_2 and 3 & 4 were untreated. Using the PGE_2 ELISA kit (Figure 5.1) these samples was measured for PGE_2 concentration while fresh. All the samples were placed at -80^{o}C, similar to that of the gastric fluid samples. Samples 1 and 3 were left here while samples 2 and 4 underwent the treatment replicating the conditions endured by the samples during the journey, explained in Table 5.1. After the 'Journey conditions' were simulated the samples were measured for PGE_2 content again.

5.2.1.b. Information asked and answered by specialists about conditions the samples would have experienced on the airplane

To reproduce the flight journey, information was required on the conditions the samples would have undergone. This includes the expected conditions experienced in an aeroplane flight. To gather this information questions were asked of two specialists in aircraft design (see appendix D for full questions and answers):

- Professor John Fielding, Head of Aerospace Engineering Group, School of Engineering, Cranfield University.
- Dr Craig Lawson, Airframe Systems Design, Aerospace Engineering Group, Power, Propulsion and Aerospace Engineering Dept., School of Engineering, Cranfield University

The results from these were used to produce the methods for the following section.

5.2.1.c. The conditions the samples experienced and the methods used to replicate these conditions.

Table 5.1: The conditions the samples experienced and the methods used to replicate these conditions. The conditions with a * are following the advice of the aircraft design specialist.

The Samples Journey from Umtata, South Africa to Bedford, UK	***The Methods Used to Reproduce these Conditions***
The samples were wrapped in parafilm, placed in 2 seal-it bags. These were placed on dry-ice and placed in a domestic cooler bag and placed in a cooler box – 20-23^{o}C for 24 hours	The samples were wrapped in parafilm, placed in 2 seal-it bags. These were placed on dry-ice and placed in a domestic cooler bag and placed in a cooler box – 20-25^{o}C for 24 hours

The dry-ice was replaced after 24 hours and travelled for another 24 hours at around 20-23^{o}C	The dry-ice was replaced after 24 hours, and left for 24 hours at 21^{o}C.
The samples sat at the airport for 3 hours before the flight	The samples sat at 21^{o}C for 3 hours.
On the plane for 11 hours	The samples sat in a cool greenhouse at 15^{o}C for 11 hours*
The samples could have been opened at customs and spent 30 minutes open on the carousel	The samples were unpacked and left open in 21^{o}C for 45 minutes.
The samples travelled for 3 hours packed up in a car and then placed in -80^{o}C	The samples were packed up and stored at 21^{o}C for 3 hours and placed in -80^{o}C.

5.2.2. Analysis of the Sample Size

The analysis of sample number to predict valid conclusions required statistical analysis undertaken on the PGE_2 concentrations of the samples. This was carried out using a statistical computer package called Statistica 7 (Statsoft Ltd, Bedford). A two –tailed (one sample mean) t-test was carried out on the PGE_2 concentration results shown in Figure 5.3. This was chosen so either a positive or negative deviation from the hypothesized value could be declared significant. By analysing changes in sample distribution the power of the sample results was provided. This information was used to provide the required sample number confidently to a selective level.

5.3. Results

5.3.1. The PGE_2 Concentrations of the Gastric Fluid Samples

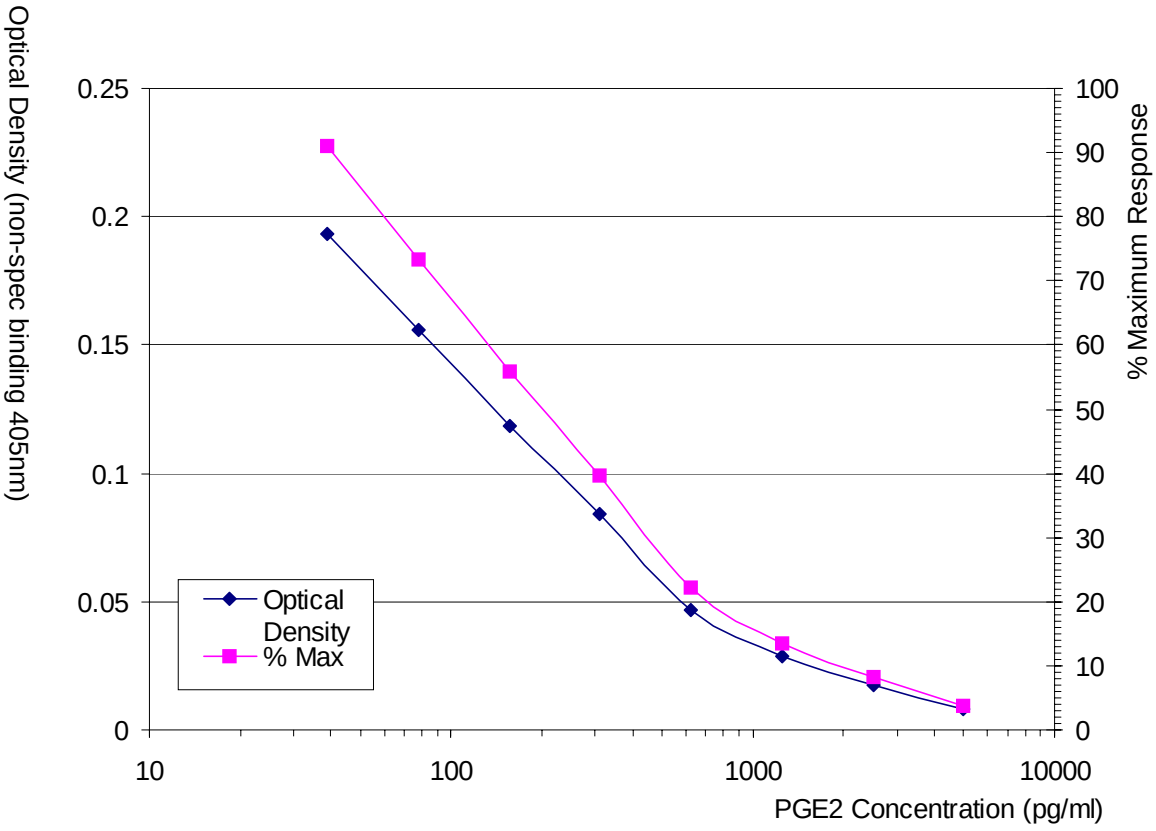


Figure 5.2: Typical standard curve taken from the average of two replicated wells for each standard, corrected by subtracting the non-specific binding results. From this the concentrations of the samples can be deduced from the optical density values.

Using the standard curve in Figure 5.2 from the results given by the PGE_2 ELISA kit the sample concentrations were calculated and are given in Figure 5.3.

When the samples were first taken, the research team at the University of Transkei measured the PGE_2 concentration of the samples by the same method (denoted 'Original Concentration'). Both of these results were compared to an average salivary

PGE_2 sample taken from a control group who consumed a regular western diet (Sammon and Morgan, 2002).

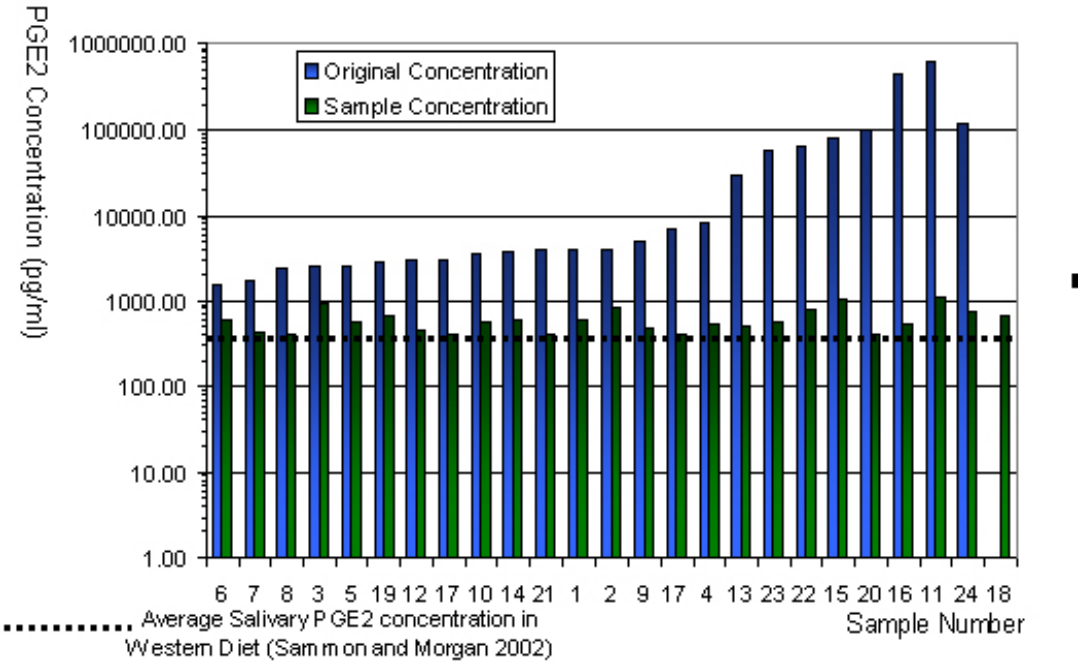


Figure 5.3: PGE_2 concentrations in gastric fluid samples taken from 24 individuals of the Transkei population, comparing measurements shortly after sampling in South Africa (Blue) and later taken following traveling 48 hours from South Africa at room temperature packed in bubble wrap and a domestic freezer box, followed by storage at -80°C prior to analysis (Green).

Figure 5.3 shows that the PGE_2 concentration of the samples after transportation and storage is much lower than the original PGE_2 concentration, for all cases. The average PGE_2 concentration of the samples before the transportation was 65571.67 pg/ml and 606.8 pg/ml after. However, they are still all above the average salivary PGE_2 concentration from a sample population from the UK who consume a regular western diet. This suggests an increased PGE_2 concentration in this population.

5.3.2. The Detrimental Effect of Sample Transportation from Transkei, South Africa to Bedford.

Explained by the methods in section 5.2.1.c an experiment was undertaken to reproduce any detrimental effect the journey from South Africa to Bedford would have on the samples. This was carried out by measuring changes in the PGE_2 concentration before and after travel conditions (Figure 5.4) and by looking at the effect this has on cellular growth changes explained in the chapter 6.

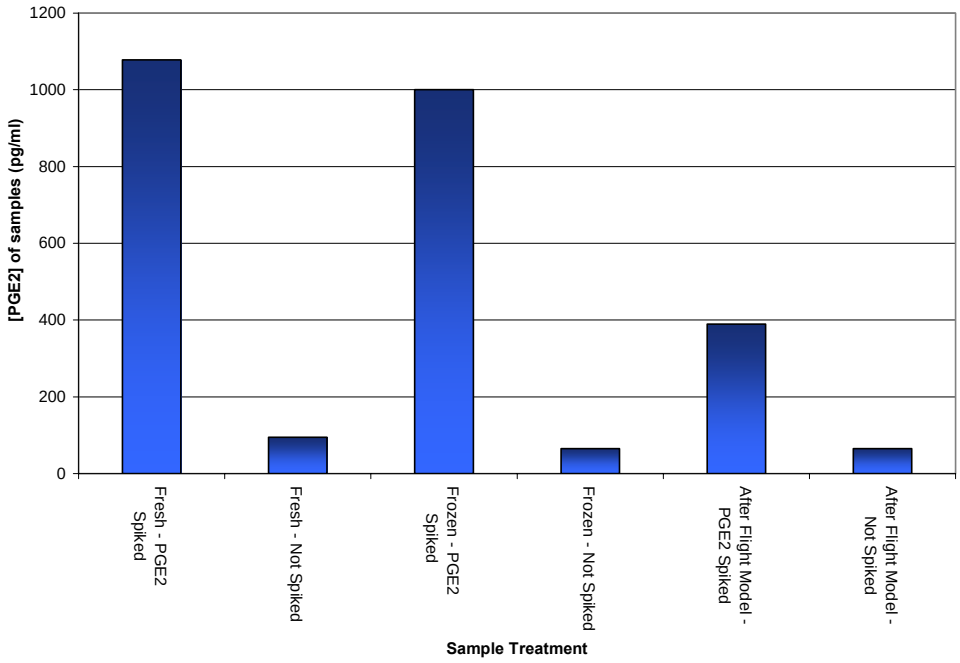


Figure 5.4: PGE_2 Concentrations in PGE_2 spiked and unspiked saliva before and after a journey conditions model using four repeats in a PGE_2 ELISA Kit. Fresh refers to the PGE_2 content straight after preparation. Frozen refers to the samples that where kept at -80^{o}C throughout the replicated sample journey. After flight model refers to the PGE_2 content of samples that were treated with a similar detrimental method of that of the original gastric samples.

It is clear from Figure 5.4 that the PGE_2 content within the samples has decreased significantly following the reproduced journey. Smaller amounts of PGE_2 seem to be more resilient to the detrimental effects of the journey.

5.3.3. Statistical Analysis of the Sample Size

Using Statistica 7 (Statsoft, Bedford) a two-tailed t-test was carried out on the PGE_2 concentrations of the gastric fluid samples, these were analysed to measure the power of the sample size[2]. The statistical workings of this are in the appendix F.

The statistical parameters used in this t-test:

- Null Hypothesized Mean (Mu0) = 312
- True Population Mean (Mu) = 606.8
- Population standard deviation = 196.06
- Confidence interval = 95%
- Power Goal = 90%

Therefore estimated sample size = 7

[2] Under the direction of Charles Marshell, Cranfield Uinversity Statstition and checked by Dr Heather Whittaker, Lecturer/Research fellow, Dept. of Statistics, Open University, Milton Keynes.

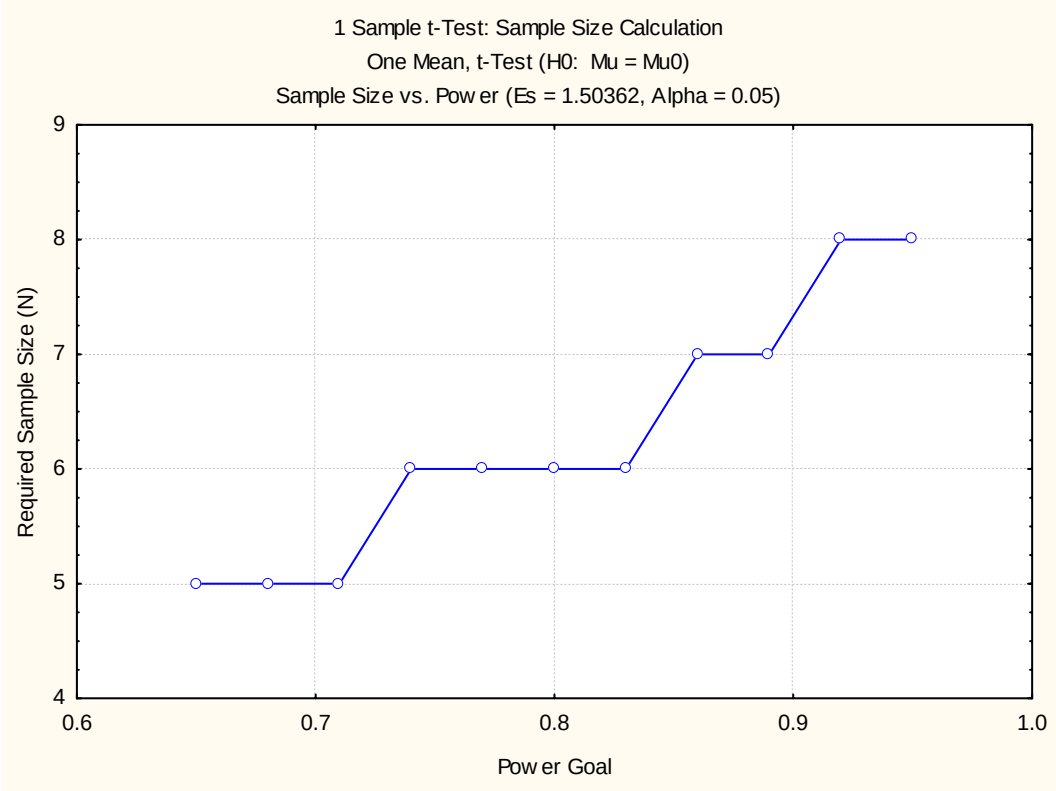


Figure 5.5: The sample size required of the gastric fluid samples to achieve a specific power goal.

Given the confidence level of 95% and a power level of 95% it can be stated that the 24 samples used in this study are enough samples to research the PGE_2 effects of the samples.

5.4. Discussion

This chapter has provided information on the three important factors concerning the required validity of the gastric fluid samples for this investigation allowing research to progress:

- Whether the gastric fluid samples contain the postulated high PGE_2 content, therefore the right variable for this study.
- The detrimental effect of transportation.
- Whether the sample size is adequately significant to form valid conclusions.

The results shown in Figure 5.3 suggest that the target population has a higher content of PGE_2 in its gastric fluid compared to a "western" population, assuming salivary PGE_2 concentration is similar to gastric fluid PGE_2 concentrations, as discussed below.

Shown in Figure 5.3, following transportation, all samples appear to have similar PGE_2 concentrations, compared to the large changes in PGE_2 concentrations seen for some samples before transportation. Figure 5.4 also shows a similar pattern. Compared to before and after the transportation method, the concentrations appear to fall to a certain concentration. It may be that a percentage of the PGE_2 proteins are more stable, perhaps bound to their transporter proteins (MRP4, ABCC4) (Ruis *et al.,* 2005). This requires a deeper investigation, and presents a very interesting possibility. This would have implications with the PGE_2 activity *in-vivo*. Klein (1990) stated that following stimulation PGs can be detected within 10-30 seconds and their synthesis proceeds for 1-5 minutes, followed by rapid degradation. It may be suggested that repeated reflux or localised PGE_2 release in the oesophagus would be required to provide enough time for the PGE_2 to have a direct effect on the squamous cells, due to protective mechanisms (further explained in chapter 6). The more stable protein transporter bound PGE_2 proteins proposed would change this pattern. PGE_2 in gastric reflux could wash around the oesophagus for longer periods of time. When a bolus of food removes the protective mucus layer this could allow a greater chance for PGE_2 squamous cellular binding. However, should the PGE_2 be bound to a stabilising protein this may not necessarily affect the stimulatory PGE_2 action, as long as the receptor epitope is free for receptor binding leading to secondary responses. Primarily, this basal level of PGE_2 was thought to be due to regular release from cellular production but the gastric fluid samples will not have any live protein producing cells., supporting PGE_2 stability.

A literature search on prostaglandin stabilisation found that Aoyama *et al.* (1990) discovered that the half life of prostaglandin I_2 (similar to PGE_2) was stabilised by apolipoprotein A-I (Apo A-I). Apo A-I is a major apolipoprotein of high density lipoprotein. Apolipoprotein synthesis in the intestine is regulated principally by the different fat contents of the diet and is implicated in heart disease, by being associated with the metabolism of cholesterol fats (Eichner *et al,* 2002). Apo A-I bound proteins were stabilised for up to 90 minutes compared to one to six minutes without. The PGE_2 and prostaglandin I_2 have very similar structures, produced by from Prostaglandin H_2 both produced by COX 2 (Figure 1.9), therefore both could have stabilising proteins. Eichner *et al*. (2002) state there is a relationship between apolipoproteins and fat in diet, although this needs more work. Should the high maize diet be producing these fats, then this could drive the PGE_2 effect further.

The aims of this thesis are to work on samples from the Transkei population. A similar study comparing samples of a population ingesting a 'western' diet would offer further insights into why oesophageal cancer is higher in the target population.

No studies investigating 'normal' PGE_2 gastric fluid concentration have been found. However, a study by Sammon (2002) did compare salivary PGE_2 in a UK population, and the same Transkeian population investigated by the current study. Sammon found content between 733 and 2357 pg/ml in the Transkeiain population with an average of just 312.5 pg/ml (94.5 pg/ml in the author's saliva shown in Figure 5.4) in the British participants. This suggests that there could be a similar difference in PGE_2 concentration between the two populations' gastric fluids, if not greater; this is likely to be due to diet. Combining Sammons results, and those in this study would suggest that the PGE_2 concentrations are higher in this sample population than a population ingesting a "western diet".

It was important to quantify the PGE_2 in these gastric samples to provide confirmation that PGE_2, a suggested proliferative component, had not been destroyed by transportation. The study on the effect the journey had on the samples showed the PGE_2 would have decreased over this time period. Figure 5.4 shows that the PGE_2 in the spiked saliva samples decreased to around half that of the samples that had been kept in the freezer. This explains why there is such a drop in PGE_2 concentration of the same samples shown in Figure 5.4 before and after the transportation. This

however, does not invalidate the samples as the PGE_2 levels are still higher than that expected in a subject consuming a low maize diet. These samples are important to show the mitogenic effect they may have on oesophageal cells. From these results it can be suggested that the gastric fluid samples could contain around double the amount of PGE_2 and its affects had the samples been kept frozen

Statistical analysis of the results took place to justify whether enough samples had been tested to gain a high enough confidence level. These results, using the two-tailed t-test, concluded that sufficient results were present.

In summary, should the above results be a true representation that the PGE_2 gastric fluid content is higher in the Transkei population (Figure 5.3) than that of a "western diet", and the Sammon theory (see Introduction: section 1.9) be true in that increased PGE_2 levels in the gastric fluid relax the smooth muscle controlling the gastric sphincter (Sammon, 1998), then these results would support the theory as to why a large number of the sample population suffer from constant regurgitation and sickness (referred to as 'cleansing' locally). This 'cleansing' typically follows a maize meal, which forms the major proportion of the target population's diet. The high gastric PGE_2 concentration and daily regurgitation into the oesophagus would result in frequent high concentrations of PGE_2 in the oesophagus, especially following every meal of food containing high amounts of the PGE_2 precursor, linoleic acid, such as maize.

These assumptions suggested the subsequent investigation into the effect this increased PGE_2 concentration may have on oesophageal cells. This may support the link between increased PGE_2 concentrations in the stomachs of the Transkei population, with a high maize diet, and high rates of cancer. The next step was therefore to look at the proliferative changes to squamous oesophageal cell lines as a result of increased PGE_2 concentration and the gastric fluid samples. This can be used to investigate changes in the cellular growth and hyperplasia, and as a precursor of oesophageal cancer in this population.

Summary of results supporting suggested theory:

- Despite the effects of transportation in decreasing PGE_2 concentration, reasons for their validity have been outlined.
- The sample number was significant.
- The results suggest that PGE_2 levels are higher in the present gastric fluid samples from the target population than previously recorded PGE_2 levels in samples taken from a population on a "western diet".
- Stable PGE_2 levels may suggest that a protein present has a protective effect allowing longer periods of stimulation.
- These results with studies described in Chapter 2, would suggest that these high PGE_2 levels in the gastric fluid would reflux into the oesophagus, washing over the squamous cells following every regular maize meal.

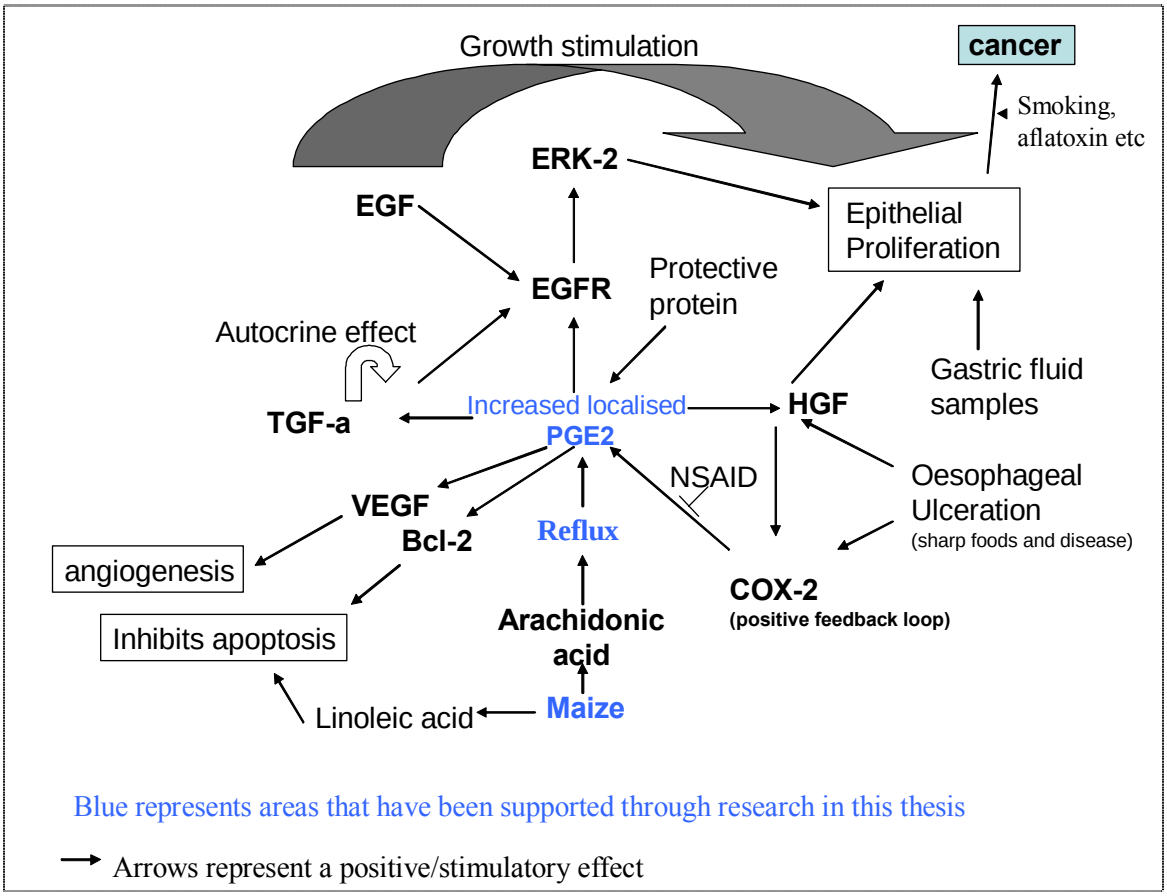


Figure 5.6: The Sammon Pink theory and how the research to this point supports it.

6. The Development of an *In-vitro* Model to Investigate the Effect of PGE_2 on Oesophageal Cells.

6.1. Introduction

Prior to studying the effects that the individual components of the gastric samples may have on cells, an investigation of the samples as a whole and whether they have a mitogenic effect (an increase in cellular proliferation), was undertaken. This approach is beneficial, as we know that the causes of cancer are multi-factorial, so we can take a multi-factorial approach to investigating cellular effects prior to analysis of individual components. As the main element that is postulated to have the mitogenic effect is PGE_2 (section 1.7.7), then the treatment of the cellular model with various concentrations of PGE_2 should also provide information on what could be the underlying pathway leading to increased cellular proliferation as a precursor of oesophageal carcinogenesis.

An *in-vitro* model was developed to look at the effects of modulation of PGE_2 concentration and the gastric fluid samples on cellular proliferation and the expression of proteins, which function downstream of PGE_2. This would provide a molecular basis for the study.

In chapter 5 a study took place to replicate the affect transportation had on the PGE_2 content of the samples travelling from Transkei, South Africa to Bedford, UK. Using the *in-vitro* model in this chapter the affect the transportation had on stimulatory growth effect of the samples was studied.

6.2. Methods and Materials

Following optimisation of methods on untreated cells, viable cell counts, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cell proliferation assay and a colourimetric BrdU incorporating assay were used to investigate the mitogenicity of gastric fluid samples on cells. These were chosen as they are 'gold standard methods' of evaluating cell growth without use of radioactive substances, for which facilities were not available.

6.2.1. Cellular Models

Two oesophageal cell lines were investigated, both grown in flasks at 37°C in 5% v/v CO_2.

The Het1A cell line was purchased prepared from the American Type Culture Collection (ATCC) (Washington, USA) provided with the following information not carried out in this study.

"This originated from a 25 year old black male's squamous epithelium of the oesophagus following autopsy in 1986. Epithelial outgrowths were sub-cultured and then transfected by strontium phosphate co-precipitation with plasmid pRSV-T consisting of the RSV-LTR promoter and the sequence encoding the simian virus 40 large T-antigen. Following 6-8 months of growth a colony was isolated which has remained non-tumourigenic in athymic, nude mice for more than 12 months. Karotyping of the cells showed that they are hypo-diploid (34-40 chromosomes). Het1a is stimulated by Ca^{2+}, and inhibited by transforming growth factor-beta 1, and transforming growth factor-beta 2 (Stoner *et al*., 1991)".

The Oe21 cell line was purchased prepared from the European collection of cell cultures (ECACC) (Porton Down, Wiltshire, UK) provided with the following information.

"These are known as JROECL21, was taken from a 74 year old Caucasian male patient's squamous cell carcinoma of mid oesophagus in 1993. The tumour was pathologically staged at IIA (Union Internationale Contre le Cancer (UICC)) and showed moderate differentiation. The cell line was established from the mincing and digesting the tumour samples in Dulbecco's modified Eagle medium (DMEM). These were left to grow in tissue culture Petri dishes and collagen-coated standard Petri dishes containing DMEM with incubation at 37^{o}C in a humidified incubator containing 5% v/v carbon dioxide in air. When these cells had grown sufficiently they were transferred selectively to fresh culture vessels using Dispase. When injected into nude mice, the Oe21 cells gave a tumour growth in less than 3 weeks. The Oe21 cells were characterized as grossly aneuploid and had only a small response to Tumour growth factor β1 (TGF-β1), which would normally inhibit cell growth (Rockett *et al.* 1997)".

See the appendix E for the cell culture methods.

6.2.2. Cell Proliferation Assays Used with the Cellular Model

6.2.2.a. PGE2 Mitogenicity – Viable Cell Counts

For each observation, with regard to PGE_2 concentration and time-point of extraction, two T25 flasks were seeded with each cell line at 2.5 x 10^5 in 6ml of media. The media for the Het1a cell line was DMEM, and RPMI 1640 with bicarbonate for the Oe21 cell line. These included the addition of 50 ml foetal calf serum (FCS), 500µl Penicillin (5 units/ml), Streptomycin (0.005mg/ml) and 1ml Amphotericin (250µg/ml). All cell culture reagents were obtained from Sigma-Aldrich (Dorset) unless otherwise stated. The medium was then spiked with PGE_2 (Sigma-Aldrich).

The median PGE_2 concentration of the gastric fluid samples found in Figure 5.3 was 3.9 μg/ml therefore, 0, 0.0005, 0.005, 0.05, 0.5 and 5 μg/ml were used to treat the cells. Following 24, 48, 72 and 96 hours of cell growth the cells were removed from the flasks with cell scrapers (Nunc, Fisher, Loughborough, UK) and centrifuged in 15 ml tubes at 176 x g for 10 minutes at 4°C. The cell pellets were collected and re-suspended in 0.5 ml of PBS. Using a haemocytometer, the cells were counted using trypan blue exclusion and a mean cell concentration was calculated (see appendix G-1).

6.2.2.b. PGE_2 Mitogenicity - MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Cell Proliferation Assay

The MTT cell proliferation assay measures the viability of cell populations and their proliferation rate. The yellow tetrazolium salt MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes contained in the mitochondria, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilised and quantified by spectrophotometric means. This offers an alternative to looking at the proliferation of the cell lines through counting (Mosmann 1983).

Two 96 well cell culture plates (Nunc) were seeded with Het1a and Oe21 cell lines at 6 x 10^4/ml with 200μl media in each well. After 48 hours of growth at 37°C, when the cells were at the desired concentration of about 50% confluence, the media was replaced with fresh media, with or without the desired concentration of PGE_2.

Every test was repeated in four wells to allow calculation of a mean. Four test lanes and six sample lanes were set up:

Test Lanes:

- N = Cells with no treatment and with MTT assay.
- NO = Cells with no treatment and no MTT assay.
- BC = No cells with MTT assay.
- NA = No cells and no assay.

Sample Lanes:

- A = 0 μg/ml PGE_2 treated cells (negative control)
- B = 0.0005 μg/ml PGE_2 treated cells
- C = 0.005 μg/ml PGE_2 treated cells
- D = 0.05 μg/ml PGE_2 treated cells
- E = 0.5 μg/ml PGE_2 treated cells
- F = 5 μg/ml PGE_2 treated cells.

These were grown for a further 48 hours and then tested for proliferation: The plate was centrifuged for 15 minutes at 70 x g and 150 μl of the supernatant from each well was removed. The MTT solution (1mg MTT powder (Sigma-Aldrich) in 1ml of PBS, filtered). Added to each well was 50 μl MTT solution and incubated at room temperature for two hours. Vigorously mixed in each well with a pipette was 100 μl of acidified Isopropanol (100μl concentrated HCl in 100ml Isopropanol). The absorbance of each well was then read in a plate reader (MRX, Dynex Technologies, Worthing, UK) at A_{550}.

6.2.2.c. PGE2 Mitogenicity - BrdU Colorimetric Cell Proliferation ELISA Assay

This is considered the gold standard method of non-radioactive assays for the measurement of cell proliferation (Rubmann *et al.*, 1993). Cells are cultured in the presence of the respective test substance for a standard period of time in 96 well plates. Cells are then incubated with BrdU (Roche, Lewes, UK) (5-bromo-2'-deoxyuridine in PBS). BrdU is a pyrimidine analogue that is incorporated in place of thymidine into the DNA of proliferating cells. The DNA is then denatured to allow access to the incorporated BrdU for detection by an antibody. This anti-BrdU monoclonal mouse antibody is conjugated with peroxidase, so with the addition of tetramethyl-benzidine (TMB) substrate, the reaction product can be quantified by measurement of the absorbance because of the colourimetric reaction. The developed colour, and therefore the absorbance values, directly correlate to the amount of DNA synthesis, thus to the number of proliferating cells in the wells (Porstmann *et al.*, 1985).

Two 96 well tissue grade cell culture plates (Nunc) were seeded with Het1a and Oe21 cell lines at 6 x 10^4/ml in 200µl media. The media, into which they were seeded, also contained 0, 0.0005, 0.005, 0.05, 0.5 or 5 µg/ml PGE_2. There were two controls: a blank, which did not contain cells and a background control that did not contain the BrdU. After 72 hours of growth at 37^{o}C, the original medium was replaced with 20 µl/well of medium containing the 20µM BrdU label. This was incubated for a further 24 hours at 37^{o}C. The BrdU media was removed and 200µl/well of denaturation solution in the kit (Composition not disclosed by the manufacturer), was added for 30 minutes at room temperature. This was removed by tapping the inverted plate and 100µl/well 1:100 anti-BrdU antibody was added and incubated for two hours at room temperature. The wells were washed three times with the wash solution, provided in the kit, and 100µl/well TMB substrate was added and incubated for 30 minutes. This caused a colourimetric change, and the reaction was stopped by adding 25µl/well 1M H_2SO_4 and placed on a shaker for 1 minute. The plate was read at 450nm on a plate reader (MRX, Dynex Technologies, Worthing, UK).

6.2.2.d. Serum Free Media and its Effect on Cellular Proliferation

Due to the stimulatory effect of the foetal calf serum used in media, the effect that this had on the proliferative drive of the model was investigated. By doing this, it would be easier to elucidate the response directly caused by PGE_2 or gastric samples. The cell counts were repeated with their specific media without foetal calf serum.

6.2.3. Gastric Fluid Sample Mitogenicity

The gastric fluid samples were tested for mitogenicity, using the MTT assay (section 6.2.2.b) and the BrdU assay (section 6.2.2.c). The methods for these are the same as the PGE_2 investigation mentioned above (section 6.2.2) but using gastric fluid samples instead of the PGE_2.

6.2.3.a. Setting Up the Cellular Model to Use with Gastric Fluid Samples

For each sample tested a duplicate of four wells was used so a mean could be calculated.

Each cell-line was seeded in a 96 well cell culture plate at 6×10^4 cells in 200 µl of media for each assay and left to grow for two days. The wells were then treated with 50 µl gastric fluid sample mixed with 50µl media. For controls, the sample was exchanged for an equal volume PBS. Following this treatment the MTT and BrdU assays were carried out on these plates by methods explained above (section 6.2.2.b & 6.2.2.c).

6.2.3.b. Non-Protein Gastric Fluid Sample Mitogenic Effects on Cellular Proliferation

To investigate non-protein constituent effects of the gastric fluid samples some were heat-treated to denature the proteins. The samples were placed in 0.5ml snap top tubes, wrapped in Para-film and placed in a water bath for ten minutes. The water bath was set at 80^{o}C, temperatures (above this temperature congealed the samples). These samples were then used in the MTT and BrdU assay to investigate changes in mitogenicity.

6.2.4. A Model to Investigate the Detrimental Effect the Transportation Would Have Had on the Mitogenicity of the Samples

A model was created to investigate the detrimental effect that the transportation may have had on the proliferative drive of the gastric fluid samples. Saliva samples treated and untreated were tested using the MTT assay (section 6.2.2.b). Treatment refers to additional spiking with PGE2 and various transport methods. The untreated samples, hence the samples that did not go through the replicated transportation method, remained at -80^{o}C (explained in section 5.3.2).

6.3. Results

6.3.1. PGE2 Mitogenicity – Viable Cell Counts

The following graphs represent the data of the cell count results of two different cell lines over four days treated with different concentrations of PGE_2.

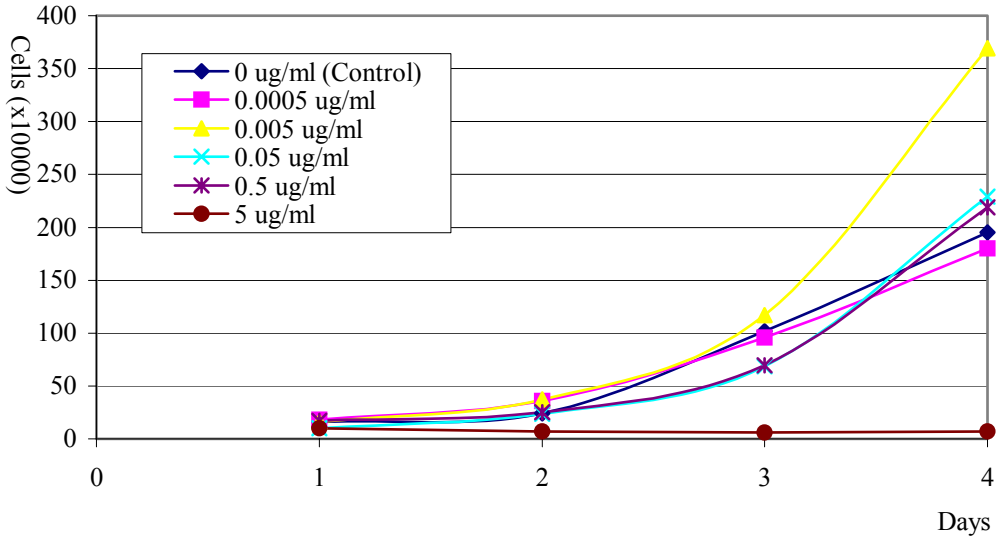


Figure 6.1: Relationship between cell proliferation and PGE_2 treatment in Het1a cells over four days. Each point is the mean of two cell counts.

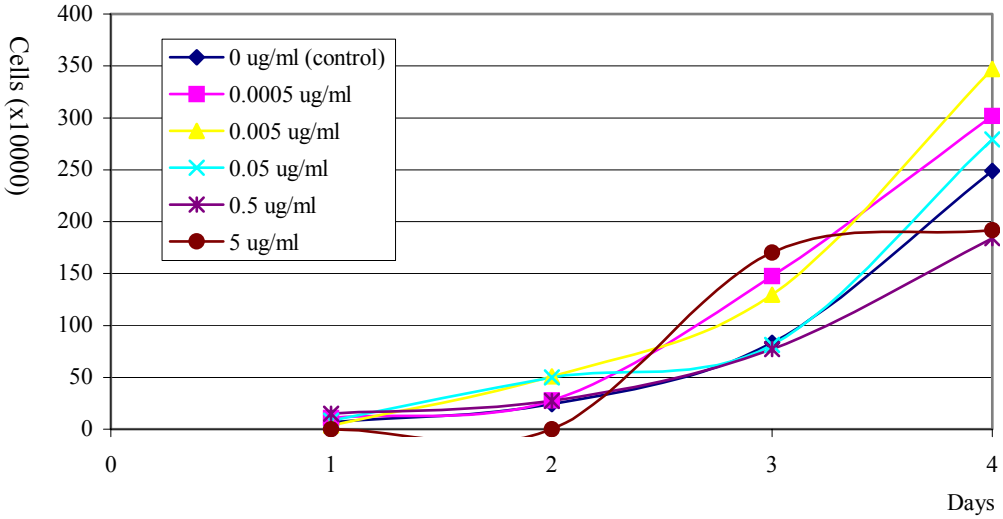


Figure 6.2: Relationship between cell proliferation and PGE_2 treatment in Oe21 cells over four days. Each point is the mean of two cell counts.

Figures 6.1 and 6.2 show that the different PGE_2 concentration treatments do not fit a simple linear relationship with increasing PGE_2 concentration and increased rate of cellular proliferation. The treatment of PGE_2 on the Het1a cell line, shown in Figure 6.1, at the lowest and highest concentrations of 0.0005 and 5 μg/ml, respectively, inhibited growth more than the control,. The 5 μg/ml treatment resulted in very little growth compared to the mid concentrations of 0.005, 0.05 and 0.5 μg/ml.. The cells treated with 0.005 μg/ml have grown almost twice as much; this increased growth is also seen with the Oe21 cell line, shown in Figure 6.2. Although in the Oe21 cell flasks the inhibitory effect has come from the two higher concentrations of 0.5 and 5 μg/ml. For both cell lines after 4 days there is a normally distributed growth curve. Note that the max concentration has the highest growth at three days in Oe21, which is different to non-stimulatory growth effect seen with the Het1a line.

6.3.2. PGE_2 Mitogenicity – MTT assay

The following graphs represent the data of the MTT assays of two different cell lines with different concentrations of PGE_2.

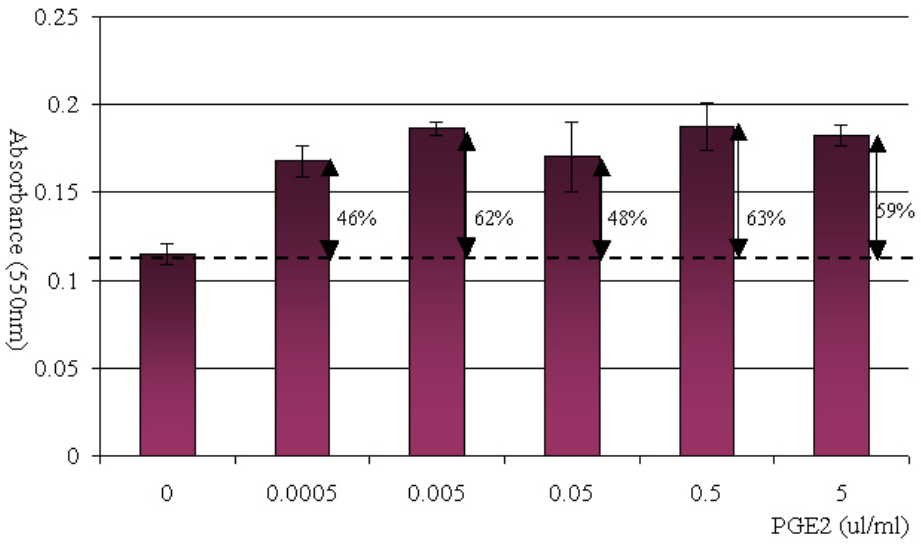


Figure 6.3: The mean of four sets of MTT optical density readings following the treatment of different PGE_2 concentrations on the Het1a cell line after 48 hours. The arrows represent the mean percentage increase in optical density compared to the control of 0 μg/ml PGE_2. The error bars relate to the standard deviation of the mean.

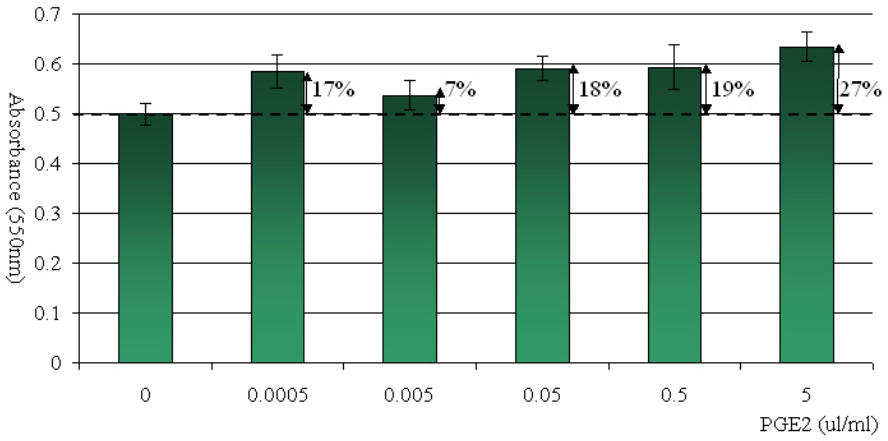


Figure 6.4: Graph representing the mean of four sets of MTT optical density readings following the treatment of different PGE_2 concentrations on the Oe21 cell line after 48 hours. The arrows represent the percentage increase in optical density compared to the control of 0 μg/ml PGE_2. The error bars relate to the standard deviation of the mean.

Figures 6.3 and 6.4 show that there is an increase in the overall number of viable cells when treated with PGE_2. As shown with the cell counts (Figures 6.1 & 6.2) there is a greater maximal response from the Het1a cell line than the Oe21 cell line. The Het1a cell counts in Figure 6.3 show the largest increase in cell number following 0.005μg/ml PGE_2 treatment. There is an increase in response to the PGE_2 treatment in the Oe21 cell line, although this is less than in Het1a.

6.3.3. PGE_2 Mitogenicity – BrdU U proliferation assay

The following graphs represent the data of the BrdU proliferation assay of two different cell lines with the treatment of different concentrations of PGE_2.

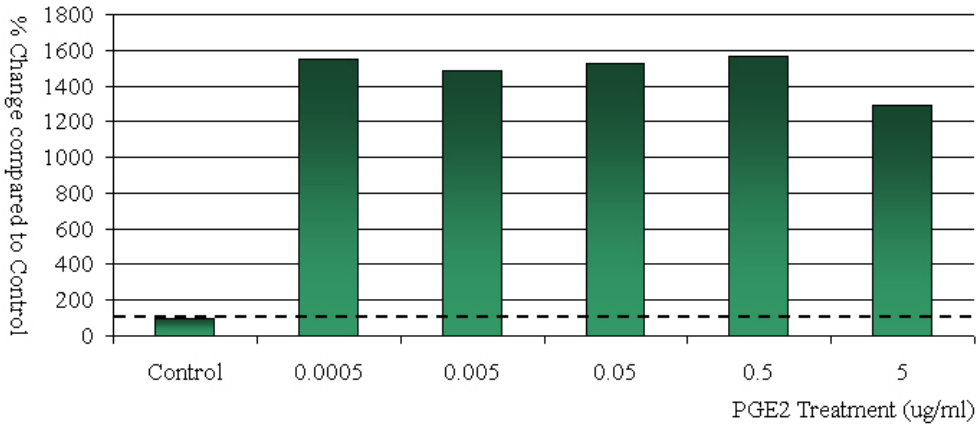


Figure 6.5: The mean of four sets of BrdU optical density readings then converted to a percentage change compared to the non-treatment control representing 100%. This follows the treatment of different PGE_2 concentrations on the Het1a cell line. The error bars relate to the standard deviation of the mean, these are too small to see.

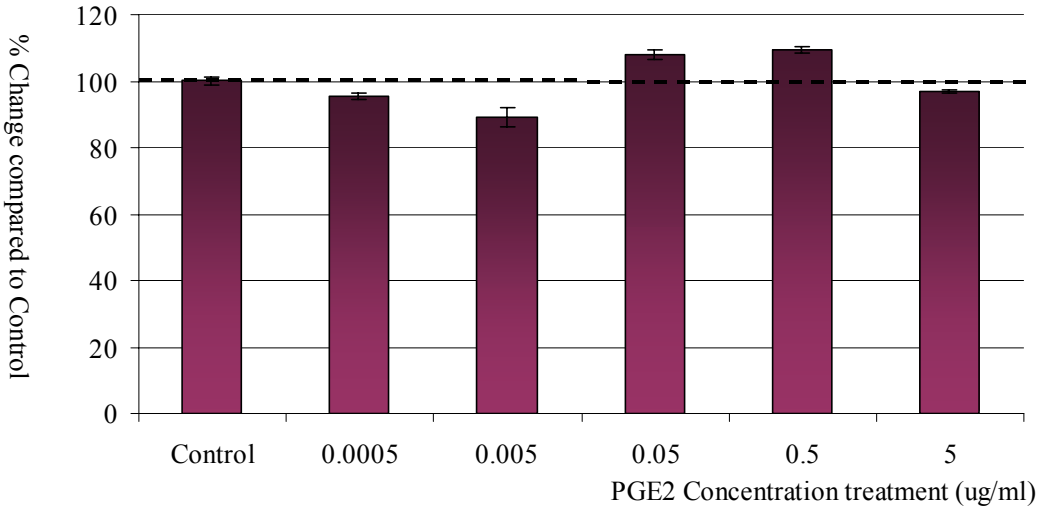


Figure 6.6: The mean of four sets of BrdU optical density readings then converted to a percentage change compared to the non-treatment control representing 100%. This follows the treatment of different PGE_2 concentrations on the Oe21 cell line. The standard deviation of the mean was calculated but was so small that the error bars hardly show.

Figure 6.5 shows there was a large increase in response to the PGE_2 treatment in the Het1a cell line. With the Oe21 cell line counts in Figure 6.6 there is an increase in cell proliferation following 0.05 – 0.5 μg/ml treatment, showing the same normally distributed results as the viable cell counts in section 6.3.1.

6.3.4. Gastric Fluid Sample Mitogenicity

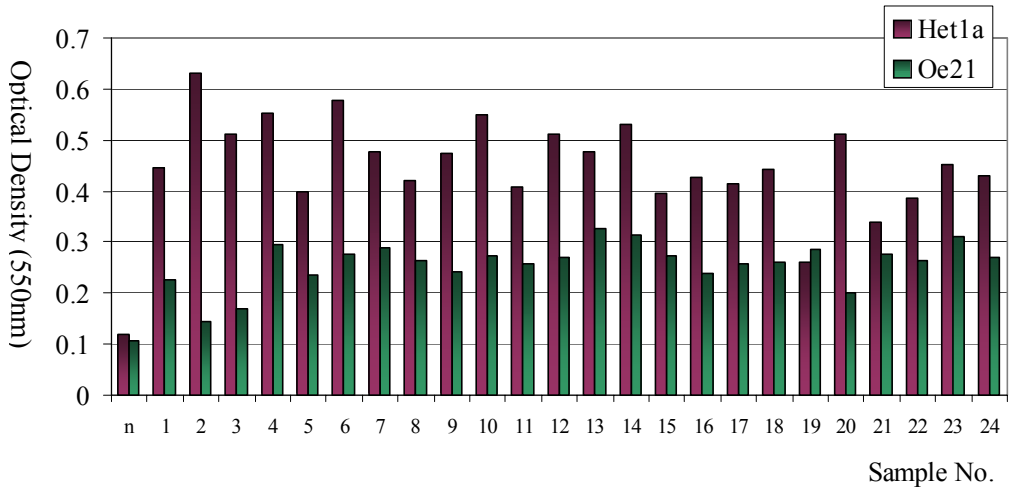


Figure 6.7: The mean of two sets of MTT proliferation assay optical density readings following the treatment of the gastric samples straight on the Het1a and Oe21 cell lines. N represents cells with no treatment of any kind other than a control solution PBS.

Figure 6.7 shows that all of the gastric fluid samples have increased the optical density, hence cellular proliferation, of the assay compared to the untreated cells.

6.3.5. Heat Treated Samples

To determine whether non-protein components of the gastric fluid sample could be producing the mitogenic effect, some samples were heat treated to denature the proteins in the sample. This was to test the liability of the mitogenicity of the gastric fluid samples without the growth factors, PGE_2 and other proteins, which may influence cell growth.

Only a small subset of gastric fluid samples were tested due to the small volumes of the other samples. These were chosen due to the large differences in PGE_2 concentration found in them. Sample numbers 4, 7, 8, 13 and 18 were selected for this part of the investigation, and their effects on the two cell lines seen in Figures 6.8 and 6.9.

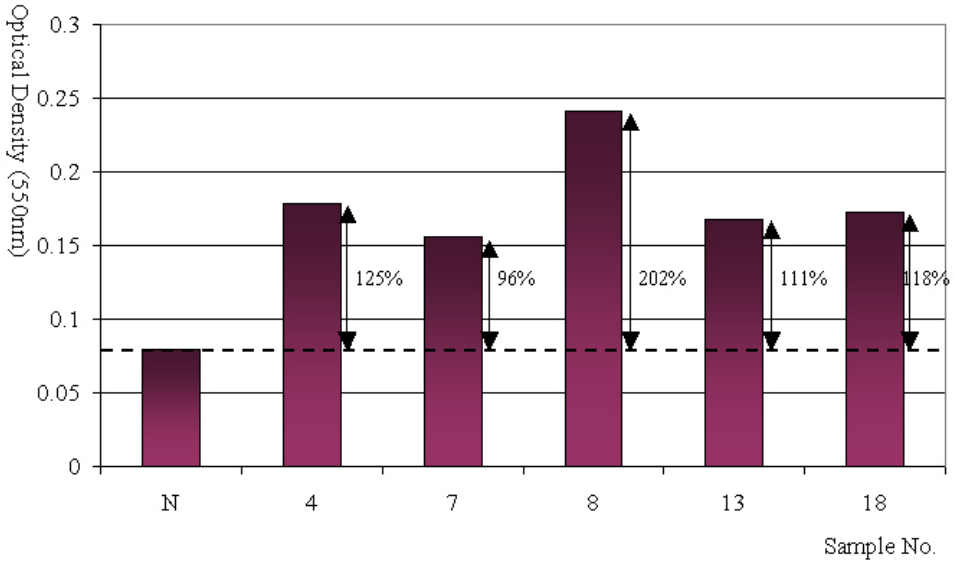


Figure 6.8: The mean of duplicate MTT assays following heat treatment of a selection of gastric samples on the Het1a cell line. The arrows represent the percentage increase in optical density compared to cells without gastric fluid treatment (N).

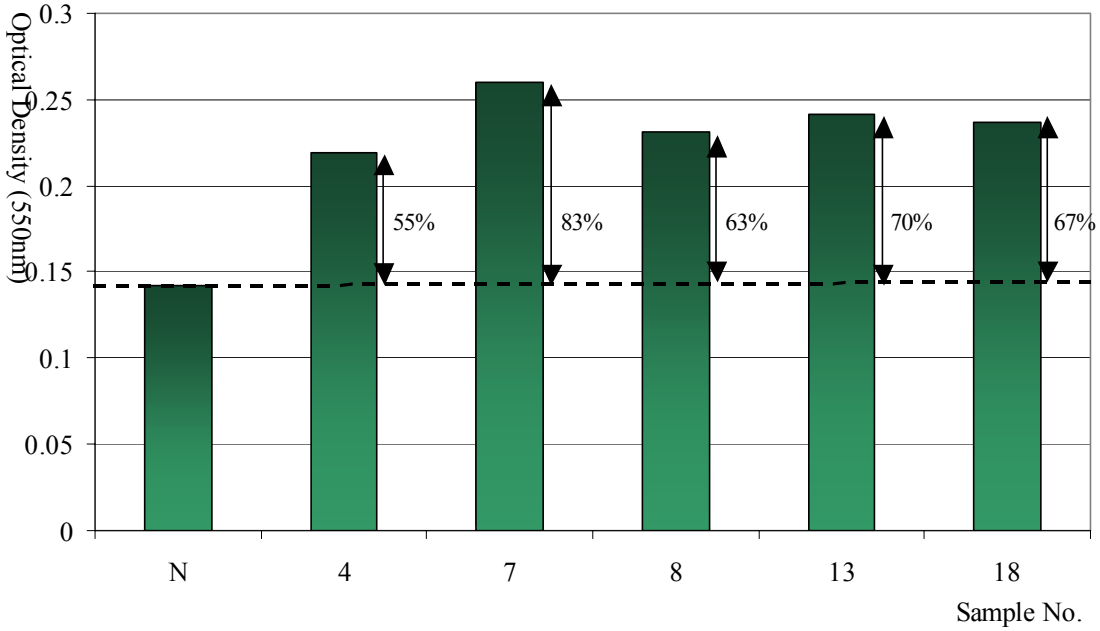


Figure 6.9: The mean of duplicate MTT assays following the heat treatment of a selection of gastric samples on the Oe21 cell line. The arrows represent the percentage increase in optical density compared to cells without gastric fluid treatment (N).

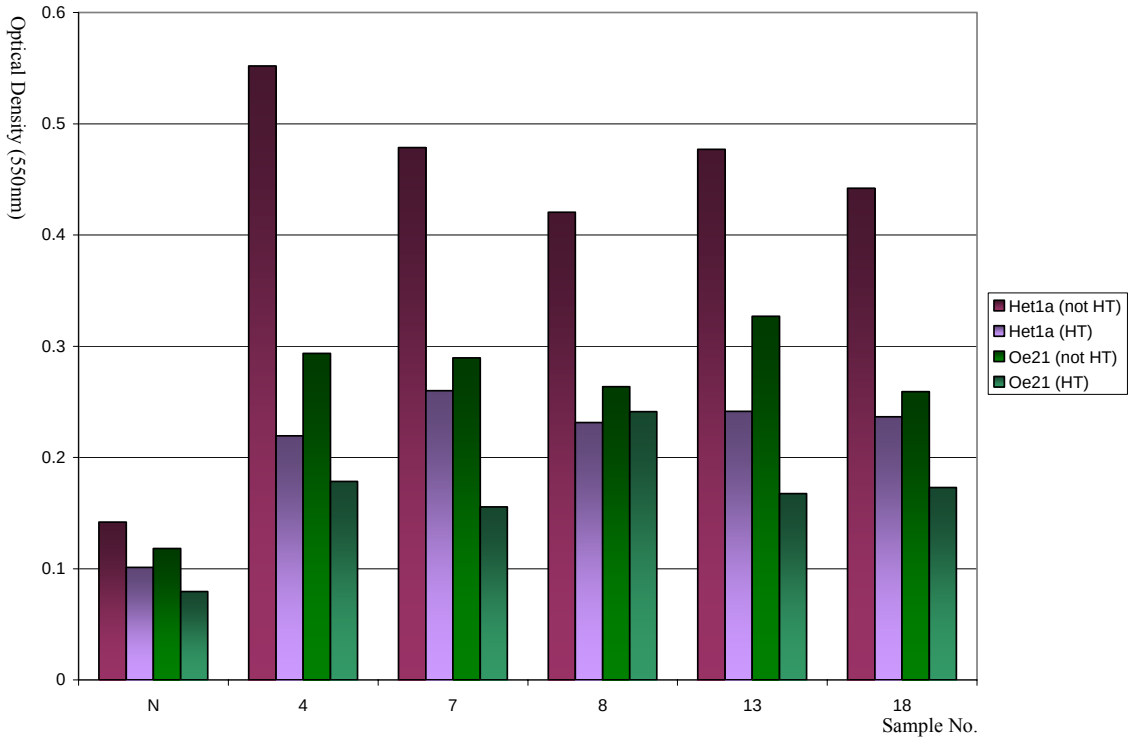


Figure 6.10: The mean of duplicates of MTT assays following the treatment of a selection of heat and non-heat treated gastric samples on the Het1a and Oe21 cell line compared to the control (N), which is heat treated media without adding a gastric fluid sample.

Figure 6.10 shows that in each instance the proliferative effect of gastric fluid has been reduced by heat treatment. There is an obvious difference in cell proliferation levels following treatment with and without heat treated gastric fluid samples. By heat treating the samples to denature the action of any proteins then there is a great reduction in cell proliferation in a majority of the samples compared to the normal.

6.3.6. Investigation of the Effect of Gastric Sample Treatment on Cells Using the BrdU Assay

As the results were very different between the MTT assay (Figures 6.3 and 6.4) and the BrdU assay (Figures 6.5 and 6.6), it was important to test the gastric fluid samples with the BrdU assay and check that the proliferation increase is seen with both methods.

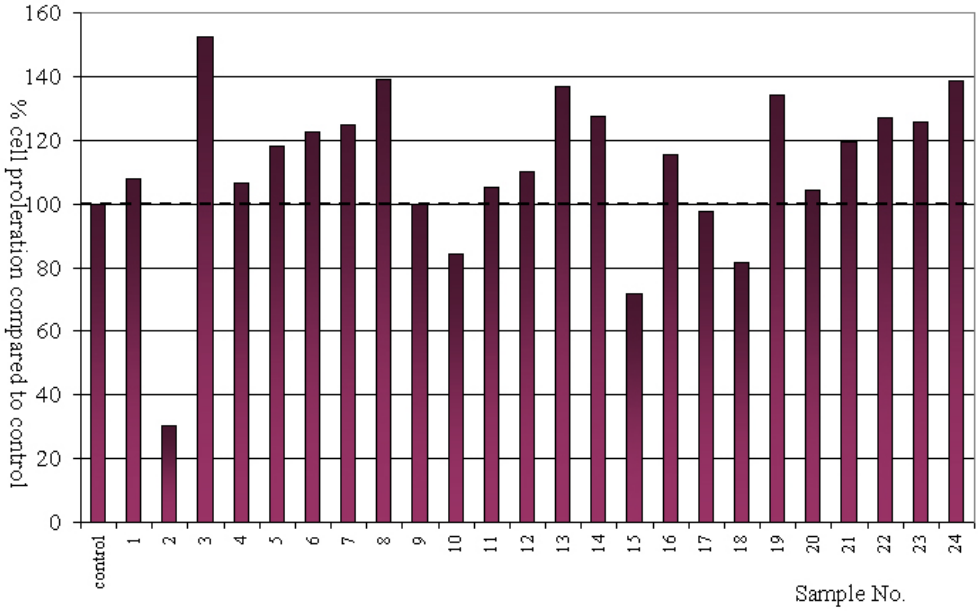


Figure 6.11: The mean of duplicates of the BrdU assays converted to percentage change compared to control following the treatment of gastric fluid samples on the Het1a cell line. The dashed line representing the control average.

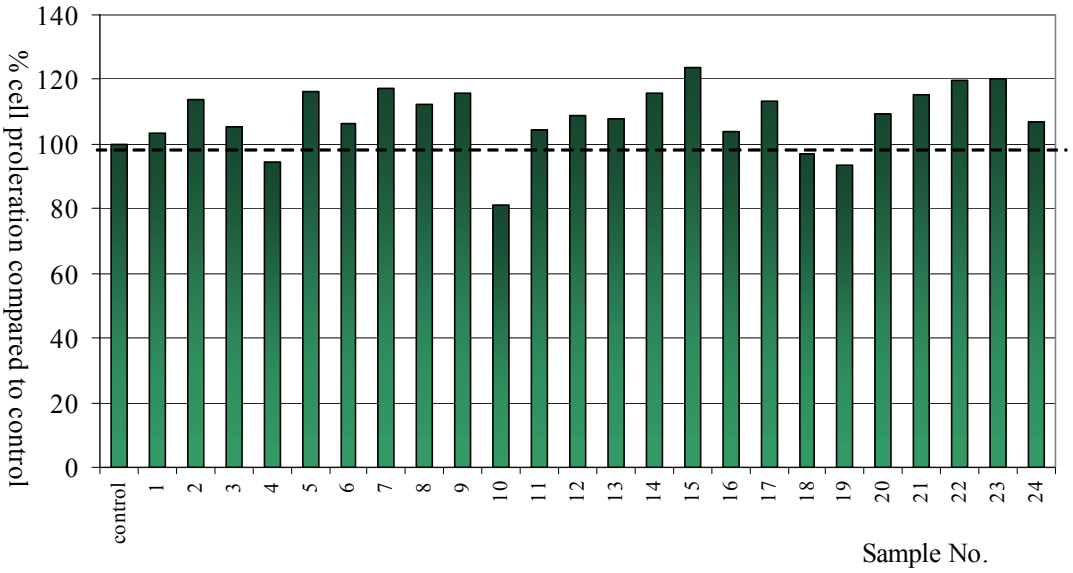


Figure 6.12: The mean of duplicates of the BrdU assays converted to percentage change compared to control following the treatment of gastric fluid samples on the Oe21 cell line.

When the two cell lines were treated with the gastric fluid samples a majority exhibit increased cell proliferation amounts.

6.3.7. The Detrimental Effect the Long Journey and its Condition May Have Had on the Gastric Fluid Samples

These are the graphical representations of the data of the MTT assay treated with dummy gastric fluid samples (0.005 μg/ml PGE_2 spiked saliva samples, explained in section 5.2.1). These samples were treated with the same detrimental travel conditions as the gastric fluid samples went through. These were described in the previous chapter (section 5.2.1.c).

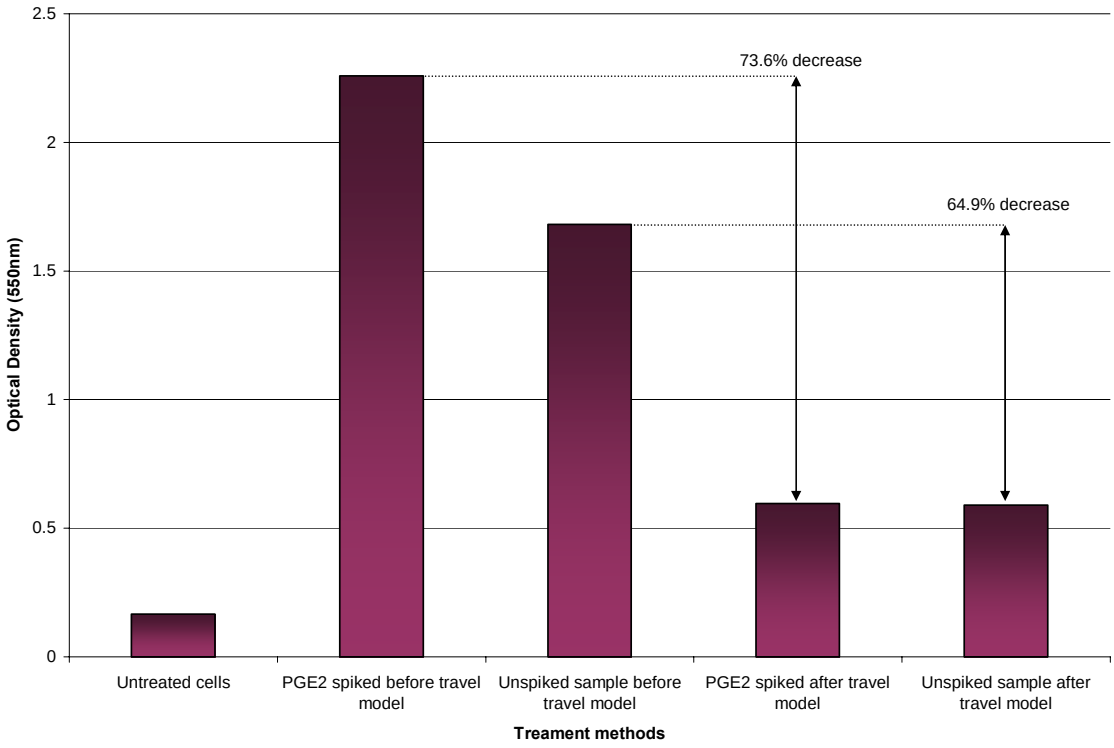


Figure 6.13: MTT Assay Optical Density Measurements after treatment with PGE_2 spiked saliva samples treated and untreated by different environmental conditions (section 5.2.1.c) on Het1a Cell line

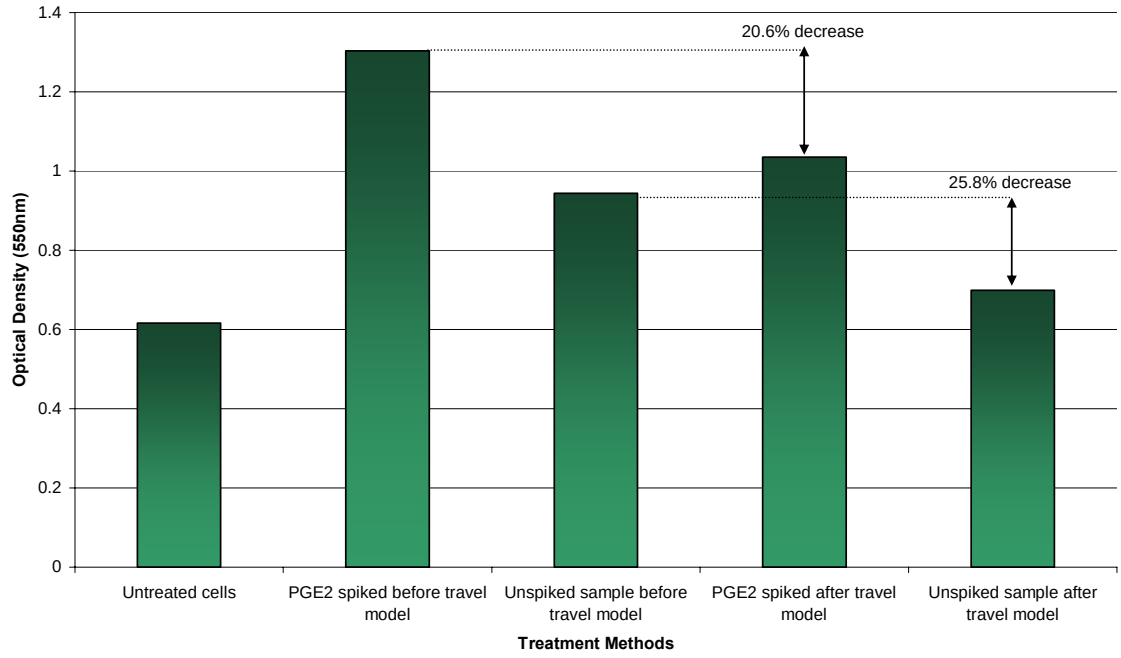


Figure 6.14: MTT Assay Optical Density Measurements after treatment with PGE_2 spiked saliva samples treated and untreated by different environmental conditions (section 5.2.1.c) on Oe21 Cell line

Figures 6.13 and 6.14 show that the conditions that the samples experienced between their transportation from Transkei, South Africa to Bedford, United Kingdom would have had a detrimental effect on their ability to promote cellular proliferation.

6.4. Discussion

The studies carried out in this chapter have used cell lines as an *in-vitro* model to see what effect PGE_2 has on their proliferative drive. The PGE_2 concentrations were measured in gastric fluid samples and they were tested on the cellular model for their

mitogenic activity. Heat treatment of the samples and investigation of their cell growth rates was investigated along with the effect the journey had on these samples.

Shown in Figure 6.1 het1a cells grew as normal, doubling daily. A similar result was produced under different concentrations of PGE_2 for the first two days. The highest concentration clearly had an inhibitory effect on the cells, which showed little growth over the entire four day period when treated with 5 μg/ml PGE_2. As PGE_2 is naturally found in contact with the majority of cells in the body, this may suggest that the high amounts of PGE_2 may lead to a enzymic negative feedback system through natural growth mechanisms (Morita, 2002). After four days there was little significant change in the growth rates between 0.0005, 0.05 and 0.5 μg/ml PGE_2 treatment, but the cells treated with 0.005 μg/ml had the maximum effect, nearly doubling the cell number compared to the control with PGE_2 treatment. This sigmoidal normal distribution shown in Figures 6.1 and 6.2, is also common with growth factor concentration treatment and cell growth. PGE_2 stimulation of growth factor expression may suggest one of the many functions of PGE_2, hence, increasing cellular proliferation. The stimulation of growth seen by the control sample, with no additional PGE_2, may be caused by the growth factors found in the serum of the media. This would suggest that greater concentrations of PGE_2 might suppress these growth factors via a negative feedback mechanism, reducing the cell numbers compared to that found by the no PGE_2 treatment model.

As might be expected with a characteristically altered cancer cell line, the PGE_2 treatment on the Oe21 cell line is very different from that found in the Het1a cell line. The growth characteristics of the Oe21 carcinoma cell line are likely to be less controlled. There is a smaller difference between the treatments of the various PGE_2 concentrations with the Oe21 cell line, than with the Het1a cell line, shown in Figure 6.2 compared to Figure 6.1. The 5 μg/ml treatment of the Oe21 cell line after three days, led to the maximal growth of all the concentrations. This peak changed over another 24 hours, as the growth rate for the two larger concentrations of PGE_2 are below that of the control after four days. This may suggest that there is a time delay, and all cells have to reach a certain concentration before the negative feedback system takes place in this cell line. The smallest of the three PGE_2 concentration treatments resulted in a larger number of cells present than the control, with the maximum

growth concentration again at 0.005 μg/ml. A similar rate of cellular increase is also seen with the Het1a cell counts and its PGE_2 concentration treatment (Figure 6.1). This also suggests a negative feedback system and maybe a relationship with growth factors.

It seems that there needs to be enough cells present in the assay for the PGE_2 to show changes in response compared to that of the control, as there is little change after 24 hours (Figure 6.1). This could suggest that the PGE_2 works on a component in the cells that has to be at a high enough concentration for stimulation; for example, growth factors and their receptors that can stimulate their own cells and the surrounding cells (autocrine and paracrine reaction). It is likely that in this model more than 24 hours are required for the cells to make contact with the growth surface and recover from stress of splitting with the protease. Also the cells have to be at a high enough concentration to make the assays record a significant difference. The Figures 6.1 and 6.2 support the suggestion that PGE_2 stimulates and inhibits the growth of oesophageal cells at various concentrations. This has been suggested before in mouse osteoblastic cells although the authors did not offer specific explanation (Suda *et al*., 2000).

As stated in the discussion of the results above, the stimulatory and inhibitory growth patterns caused by different concentrations of PGE_2 are very similar to that found with growth factor characteristics. Further research has to be carried out on PGE_2 and its effects on the expression of growth factors. It has been suggested in a number of cases that growth factors have an important mechanism in the hyper-proliferation of oesophageal tissue for the mechanism of healing (Juhl *et al.,* 1995; Jimenez *et al*., 1998; Baatar *et al.,* 2002). As stated in the Introduction (section 1.6), PGE_2 plays an important role in the mechanism of healing, therefore the relationship between hyper-proliferation and PGE_2 makes sense. Baatar *et al.*(2002) showed in rats that the healing of ulceration in the oesophagus can be delayed by treating the rat with selective COX 2 blockers, hence suppressing the PGE_2 production. They have also shown that this healing was suppressed by inhibiting the HGF receptor. The early response genes such as *myc, fos* or *jun* should be investigated to support the connection of PGE_2 and stimulation of growth factor, as the presence of these genes suggests growth factor stimulation (Almendral *et al.,* 1988). The cyclins and cyclin-

dependent kinases could also be investigated as these are expressed after a longer period than the early response genes (2-3 hours), following cell growth stimulation by growth factors (Sherr, 1993). Takeno *et al*. (2002) supported the view that measurements of cyclin B1 had prognostic value in oesophageal squamous cell carcinoma patients, due to its elevated levels relating to poor prognosis. Should PGE_2 increase cyclin B1, maybe through growth factor manipulation, then this could open up areas of investigation to explain these increased levels. It has been shown that PGE_2 induces cellular proliferation in prostate cells, mediated through the protein kinase A and *c-fos* mechanisms, hence, regulatory proteins implicated with growth factors (Chen and Hughes-Fulford, 2000). PGE_2 was also shown to induce *c-fos* and *c-jun* expressions via the EP1 subtype of PGE receptor in mouse osteoblastic cells (Suda *et al*., 2000). All these studies support the connection of PGE_2 causing a hyper-proliferative response via growth factors. This is also backed up by the knowledge that tyrosine kinases play an important role in the signal transduction pathways that are initiated due to growth factor stimuli, for example, in the induction of EGF receptor. In 1997 research showed that the tyrosine kinase protein was being over expressed in human oesophageal cancer (Nemoto *et al.,* 1997). The investigation of the interaction of PGE_2 with these growth factors could help provide information on this.

As seen with the cell counts, the MTT cell proliferation assay results (Figures 6.3 & 6.4) also show an increase in cell growth stimulation following all concentrations of PGE_2 treatment. This growth is much smaller with the Oe21 than the Het1a cell line, and in this model 0.005 μg/ml has not produced the highest growth stimulation as found in the previous cell number method (Figures 6.1 and 6.2). The differences between both the assays will be discussed later in this section.

All concentrations of PGE_2 have a greater stimulatory effect on the Het1a cell line than on the Oe21 cell line. This could be owing to changes into the overall characteristics of the cell lines. The Het1a cell line may have a greater turn over rate than the Oe21 cell line, therefore when stimulated they appear to increase faster. It would seem more likely that Oe21 cell cycle characteristics have changed over the period of tumourigenesis.

Carcinogenesis is a huge multifactoral process causing many changes in natural cellular processes. It could be expected that the method that favours PGE_2 to drive cellular proliferation in the Het1a cell line may be changed in the mutated, uncontrolled, Oe21 cell line. For example, the PGE_2 may stimulate the cell cycle to progress through growth factor activation, receptor activation or through a secondary mediator, lifting the natural cell cycle blocks i.e. p53 (Hollstein *et al.,* 1990) leading to cellular proliferation (see Figure 6.15 for what a mutation in p53 could effect). Should the Oe21 cell line have a mutated p53 protein, then the cell cycle may not allow continuation of growth if the p53 is blocking this growth pathway, but natural control of apoptosis would be suppressed therefore cells stimulated via other pathways may keep the cells growing and never be inhibited. Should the PGE_2 have an overall stimulatory effect on the oesophageal cell lines then changes in the cell cycle between the two cell lines could suggest an area to investigate. This may provide a further area of study.

There are many proteins that balance the cell cycle and apoptosis mechanism and fit the pattern found in the results e.g. *p53*, RAS, RAF, BCL-2, BAX etc. Investigation into cell cycle controlled feedback loops and their relating families may give a better insight into the process of carcinogenesis driving squamous cell carcinoma. Rather than speculating on the many examples here a deeper level of investigation is required, into the basic affects of PGE_2 on cell growth than has been possible in this study.

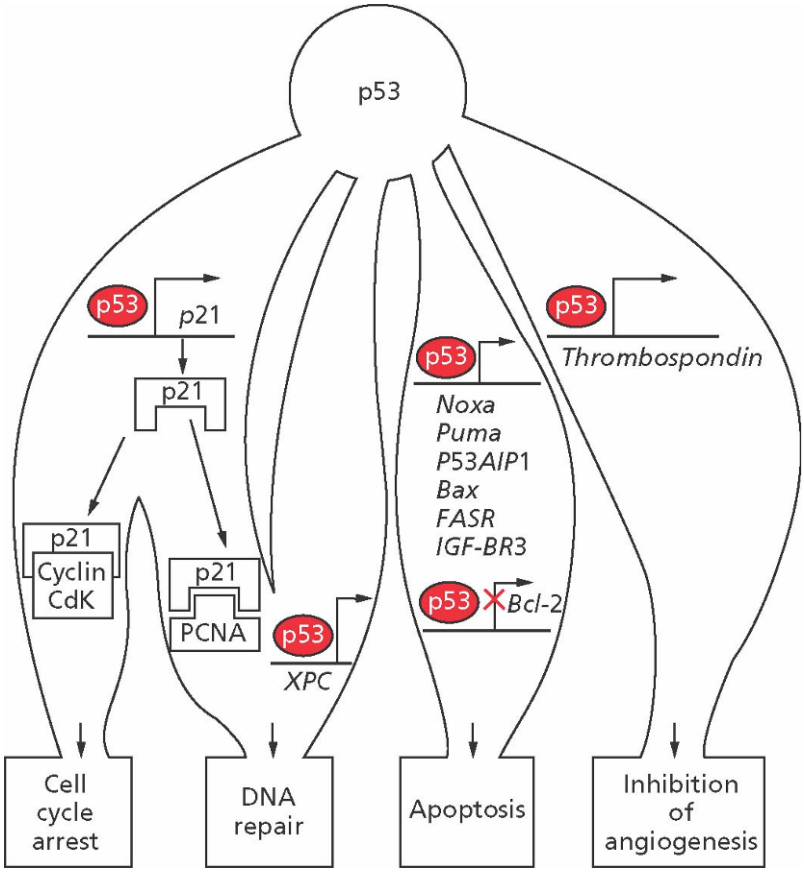


Figure 6.15: Examples of how a mutation in p53 could have an effect on other balances within the life of a cell (Pecorino, 2005)

Figures 6.5 and 6.6 show the BrdU assay results for PGE_2 treatment on cells. The pattern for the BrdU assay is similar to the MTT assay (Figures 6.3 and 6.4) as the het1a has a higher rate of growth compared to the much lower growth rate with the Oe21 cells. This growth also seems to be similar to the growth pattern seen from the cell counts for Oe21 cell line, with the lower and higher concentrations of PGE_2 having an inhibitory effect, but the middle concentrations having a small stimulatory effect. The cells here are also treated over four days like those examined using the cell count method, compared to two days of the MTT assay. These changes between Het1a and Oe21 maybe due to PGE_2 having a greater effect on the mitotic process of Het1a, that may be unstable or inhibited in the Oe21 cell line. Investigation here could be undertaken on the cell cycle movement from G2 to M and its checkpoint proteins such as cyclins, having an effect on the amount of DNA production perhaps forming multiple nucleus development.

Qureshi *et al.,* (1997) looked directly at the Het1a Cell line and postulated that EGF is an initiation factor and IGF is a progression factor (explained in section 1.3.1), due to their synergistic ability to promote cell growth in the Het1a cell line. Negishi *et al.* (1995) have explained that that PGE_2 exerts its functions by working on the PGE_2 receptors coupled with G proteins, also implicated with natural growth factor action. These activate or inhibit a growth factor pathway protein, inositol trisphosphate, that could cause an influx of calcium (Ca^{2+}). Research at the University of Cambridge showed that inositol trisphosphate, an important secondary mediator in the EGF receptor, controls Ca^{2+} channels allowing Ca^{2+} to have an effect on transcription factors to stimulate the cell cycle (Berridge, 1993). As stated in section 6.2.1 the Het1a cell line growth is stimulated by increases in local Ca^{2+}, which may explain the reasons for the increased growth of the Het1a cell line compared to Oe21 cell line. Changes in the presence of Ca^{2+} have been implicated in the interaction of EGF receptor although the mechanism has not been explained (Li & Villalobo, 2002).

All these results support the theory that PGE_2 has a stimulatory effect on the proliferation of the oesophageal cell line; no previous research has revealed this relationship. The BrdU and the MTT assay are regarded as the non-radioactive gold standards along with flow-cytometry to measure proliferation; their results slightly differ, but suggest the same concept that at a specific concentration of PGE_2 there is a stimulatory effect on the oesophageal cells. The viable cell counts gave a clear representation of the number of viable cells present, therefore a good representation of the cellular proliferation that has taken place over the number of days that the cells were analysed. All methods have suggested that at concentrations around 0.005 – 0.05 μg/ml there was a stimulatory effect on the growth of cell numbers of both cell lines.

Treatment of both of the cell lines' gastric fluid samples using the MTT assay gave an increased optical density reading compared with the control (Figure 6.7). Overall, the increases in growth were greater in the Het1a cell line than the Oe21 cell line, which is similar to the results from the cells treated with PGE_2. A majority of the Oe21 gastric fluid treated cells gave double the response compared to the control, and the Het1a cells an even greater response of between four to five times the control. This would suggest that the gastric fluid samples have the potential to cause increased oesophageal cellular proliferation using the same mechanism as PGE_2.

These results have been achieved using PGE_2 concentrations close to that found in the samples after there transportation (section 5.2.1). Therefore, in testing the samples these could be compared to the PGE_2 treated results. However, in the original gastric fluid samples the PGE_2 would have been much higher (quantified in Figure 5.3). As the higher PGE_2 concentrations in the cell model had a suppressive effect on growth then a much higher concentration found in the original samples would have been expected to an adverse effect and suppress any localised PGE_2 promoting growth. This does not seem to be the case, There may be a number of explanations for this. The oesophagus is lined by protective mucus that may not let all of this external local PGE_2 protein through to the proliferative layer of the basal squamous tissue or decreases its absorption rate to this area. The PGE_2 may only have a minimal effect on the terminally differentiated outermost cellular layers. Therefore, a much larger concentration may be required to have to the same effect that has been displayed in this *in-vitro* model. With the *in-vitro* model there will be constant stimulation from the introduced PGE_2, although, in the sample population we have predicted that this PGE_2 comes with and following a meal, hence, there is not likely to be constant stimulation. Due to the nature of these abrasive foods and the suggested fungal infection of the maize then localised damage may induce the local PGE_2 to aid healing through inflammation. The abrasive nature of these foods may also help by opening up patches of the squamous layer exposing basal areas to this increased PGE_2, increasing the localised concentrations in to one area perhaps in one of the narrowing sections found in the oesophagus due to other organs externally pushing in.

By heat treating the samples it has been assumed that this would have eliminated most of the activity of proteins in the sample. As expected, the growth of both sample-treated cell lines was reduced compared to the non heat-treated samples (Figure 6.10). There are a great number of proteins that could have a multi-factor stimulatory effect on the cell lines, including cytokines and growth factors. The reduced growth of the heat treated samples compared to the non heat-treated samples indicate that the gastric fluid still contains proteins that have not been denatured. This growth may be caused by the non-protein matter i.e. the pH, the bile salts etc. having a stimulatory effect on the cells, but it does suggest that proteins are a large factor in the proliferative drive by the gastric fluid. It is important at this stage that the results are not misinterpreted as suggesting that the PGE_2 is the stimulatory component of the gastric fluid, as this

fluid will have a number of different elements that could affect the cellular growth. It has been suggested in two papers that bile acids have a positive effect on the growth of intestinal cells through the increased expression of COX 2 (Zhang *et al.,* 2000; Myung-Whun Sung *et al*., 2003).

An investigation on the influence of gastrin, a hormonal component of gastric fluid, was shown to stimulate the epithelial cell proliferation in the oesophagus of rats (Van Nieuwenhove *et al*., 1998). In gastric epithelial tissue it was shown that HGF expression was increased in cells when treated with gastrin (Konturek *et al.,* 2003). It is not expected that the growth factors are still present in the gastric fluid due to their small half-life; therefore should the stimulation of growth be the result of growth factors then it seems likely that the cells are themselves expressing or secreting these growth factors through autocrine and paracrine stimulation, maybe through undenatured gastrin.

The BrdU assay results (Figure 6.11 and 6.12) suggests that a majority of the gastric fluid samples have a stimulatory effect on the growth of the cells, therefore supporting the idea that the gastric fluid can increase cellular proliferation.

As stated in the introduction (section 1.3.2), it has been shown that gastric fluid reflux is the predisposition factor of adenocarcinoma of the oesophagus (Heath *et al.,* 2000), but this has been proved not to be the case with squamous cell carcinoma (Altorki *et al.*, 2003). However, this theory has not been investigated with high concentrations of gastric PGE_2. This current study suggests that the PGE_2 content of the gastric fluid reflux has a proliferative drive, which itself is recognised as a predisposition to cancer through hyperplasia. This supports the theory that a high PGE_2 substrate diet could be the major cause of oesophageal cancer in the sample population.

It is clear from Figure 5.4 in the previous chapter that the transportation of the samples had a detrimental effect on the PGE_2 levels in the samples. This also had a detrimental effect on the growth stimulating potential of the samples shown by Figures 6.13 and 6.14. The transportation did have a detrimental effect although it did not invalidate the samples. According to the results in Figure 5.4, the PGE_2 levels may have been decreased by over half. However, Figures 6.13 and 6.14 show that the samples still had a proliferative effect on the oesophageal cell lines, much higher than

the control in the Het1a cell line. It can not be stated that the much higher concentrations in the original samples would have had a much greater stimulatory effect due to its higher PGE_2 concentration. As explained at the start of this discussion the PGE_2 concentrations have a sigmoidal growth curve with higher concentrations having a suppressive effect. The higher PGE_2 concentration treated *in-vivo* could have a greater mitogenic effect. Higher localised PGE_2 may suppress the closing of the gastric sphincter for longer periods of time and be in higher than normal concentrations having a greater chance of reaching the squamous cells.

The saliva samples used in the transportation investigation differ to the gastric samples by a few components. The main difference is the pH of the fluids. It is common knowledge that the fairly neutral saliva (pH 6.4-7), soaked in a bolus of swallowed food, is a stimulus for promoting release of acidic gastric juice, therefore this pH change between the two fluids can have a large effect on the efficacy of enzymic reactions. Although the buffered media (pH 7.2-7.6) that the cells grow in would neutralise much of this pH change between the two. Saliva contains a small amount of calcium (1.2-2.8 mMol/L) that may stimulate Het1a cell growth (Qureshi *et al*., 1997) although this would have little effect due to the calcium content already in the media having an even effect on both sample treatments on cells. Gastric fluid may have an increased growth stimulatory effect due to gastrin content and bile salts explained in few paragraphs previous. Overall the basic proliferative drive between the two samples would be minimal although this can not be stated without testing fresh gastric fluid. For the purpose of this study the use of saliva in place of gastric fluid was sufficient to use as a model.

In conclusion, this research shows that PGE_2 increases oesophageal cell proliferation at particular concentrations and that the proteins in the high PGE_2 containing gastric fluid samples from the sample population also increase oesophageal cell proliferation, suggesting a pre-disposition to cancer. This suggests a link between the two observations. It is postulated that this proliferative drive is carried out via the interaction of growth factors or their multi-compartmental mechanism, and the importance of testing this has been discussed. It has not been ruled out that another component of the gastric fluid could have the increased mitogenic effect, although the evidence supporting PGE_2 in this research is strong.

Summary of results supporting this theory:

- The results suggest that at optimum concentrations there is an increase in the proliferative drive from PGE_2 on the oesophageal cells.
- The growth is suppressed at very high concentrations, suggesting a negative feedback mechanism. This system common to growth factors, may propose the PGE_2 mitogenic mechanism seen in natural tissue healing.
- Changes between the growth patterns for the normal and squamous cell carcinoma cell lines suggest a cell cycle control protein difference between the two may help to indicate what causes this PGE_2 stimulated proliferation increase, for example a commonly studied protein such as p53.
- Much higher PGE_2 concentrations are seen in gastric fluid samples than are seen to suppress cell growth in the *in-vitro* model. Due to the oesophagus being mucus covered, larger concentrations maybe required to affect the squamous tissue and not affect the terminally differentiated outermost cellular layers.
- The high PGE_2 containing gastric fluid samples had a mitogenic effect on the cell lines. After the gastric fluid was heat-treated the mitogenic effect was largely reduced, suggesting that protein components, for example, PGE_2, and not bile salts etc., in the samples, have the largest effect on the proliferative drive.
- The transportation of the samples did have a detrimental effect although they still valid in this study.
- This suggests that the high amounts of PGE_2 produced by the maize diet, found in the gastric fluid, is reflux into the oesophagus. It is the PGE_2, and not general bile salts, pH change (suggested in the Sammon theory) in the gastric fluids that have a proliferative drive on the localised squamous tissue, maybe through stimulation of common growth factors. Due to the mucus barrier, large concentrations are needed to have an effect. This is enhanced by localised infection and damage caused by disease and sharp foods respectively.

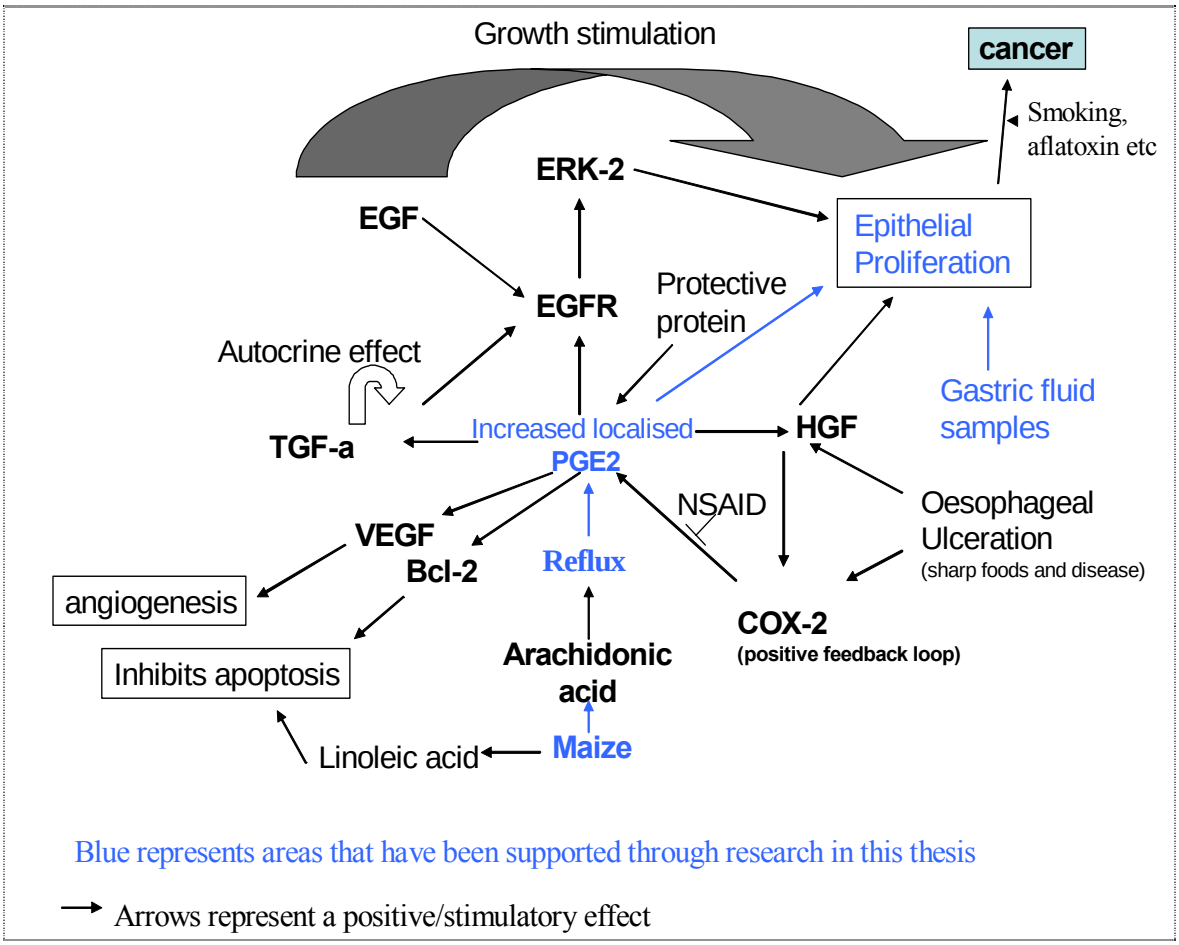


Figure 6.13. The Sammon Pink theory and how the research to this point supports it.

7. Assessing the Effect of PGE_2 Treatment on HGF, VEGF, EGFR and COX 2 Expression

7.1. Introduction

Once the basis of the molecular model had been shown in the previous chapters, a more focussed molecular investigation could take place. Following the theory of maize induced carcinogenesis, developed from Sammons work described in the chapter 3 (Figure 3.1) and subsequent studies documented in chapter 6, this chapter focuses on the growth pattern and expression of growth factors. It investigates whether PGE_2 has an effect on the expression of three important proteins: One implicated with epithelial growth promotion, Hepatocyte Growth Factor (HGF) (section 1.7.5), and two implicated with the tumourigenic potential of PGE_2: Epithelium Growth Factor Receptor (EGFR) (section 1.6.10.a) and Vascular Endothelial Growth Factor (VEGF) (section 1.6.5). The research reviewed in Chapter 1 showed how COX 2 inhibitors can suppress growth promoting carcinogenesis (section 1.6.2), although the mechanism is debatable, therefore it was important to examine how expression changes are to be stimulated by PGE_2. The results in chapter 6 have illustrated that PGE_2 increases proliferation of oesophageal cell lines, therefore should the expression of COX 2 increase; this could suggest a positive feedback mechanism, inducing hyper-proliferation of the oesophagus.

7.2. Methods

7.2.1. Western Blotting to Detect HGF

7.2.1.a. Sample Preparation

T75 flasks (Nunc) were seeded with 1ml 50 x 10^4/ml of Het1a and Oe21 cells and grown for 48 hours. The medium was removed from the flask and the cells were washed with 10 ml 2% protease inhibitor (Sigma) /PBS solution (PI/PBS). Using a cell scraper (Nunc) and 10 ml PI/PBS solution, the cells were scraped from the T75 and transferred to a 15 ml centrifuge tube. The tube was centrifuged at 1200 x g for 10 minutes at 4^{o}C. The supernatant was discarded and the cells were re-suspended in 1.5 ml of PI/PBS. The cells were transferred to an eppendorf tube and spun at 20,000 x g for 15 minutes at 4^{o}C. The supernatant was discarded and the cells were re-suspended in 1.5 ml CHAPS lysis buffer (appendix L). The cell solution was snap frozen and stored at –80^{o}C. When required the cell solution was thawed and incubated on ice for one hour. This was centrifuged at 20,000 x g for 30 minutes at 4^{o}C. The supernatant/protein was placed in a clean tube, labelled and stored at –80^{o}C. The protein concentration was measured using the Bradford Assay.

7.2.1.b. Western Blotting Method

The 1000 mg/ml protein samples (12 µl) were boiled for five minutes with 3 µl SDS running buffer (appendix L) to denature the protein to single polypeptide strands. These were spun briefly to collect the tube contents and placed on ice for two minutes. The samples were loaded into a SDS stacking gel (appendix L) alongside a molecular weight ladder (Santa Cruz Biotech inc, Wiltshire, UK), and run through a 8% w/v SDS resolving gel (appendix L) in an electrophoresis chamber in running buffer (appendix L) at a constant 125 Volts for 90 minutes. An anti-electric vinyl

membrane was placed on a clean blotter (Hoefer, San Francisco, USA) with a hole the same size as the SDS gel. On this four layers of filter paper (Whatman, Maidstone, UK) were stacked, pre-soaked in transfer buffer (appendix L). A PVDF micro-porous membrane (Millipore, Gloucestershire, UK) was cut to the same size as the gel and pre-soaked in methanol then transfer buffer (appendix L) and placed on the stack. On top four further, pre-soaked with transfer buffer, filter paper layers were added. This was rolled with a 20 cm plastic tube to disperse air bubbles between the layers, and the blotter lid was added with further weight. Electro-transfer (Hoefer semi-phor) was performed for 60 minutes at 70 milliamps. A duplicate gel was checked with Coomassie blue (Sigma) for a clear indication that the proteins were present and have run correctly on the gel. The membrane was blocked with 5 % w/v powdered milk (Marvel, Premier Brands, UK) in TBS (appendix L) for one hour, rocking at room temperature. The goat anti-human HGF primary antibody (R&D Systems, Oxon, UK) was diluted to 1/100-1000 in 5 % w/v milk/TBS and incubated with the membrane for 16 hours at 4^{o}C. As a control a rabbit anti-human CD44 primary antibody was diluted to 1/200 in 5 % w/v milk/TBS and incubated with the membrane for 16 hours at 4^{o}C, this is because CD44 is implicated in cellular binding and has always shown to be present in these cell lines in the laboratory. The membrane was washed five times for five minutes, rocking at room temperature in 0.1 % v/v tween 20 in TBS. The membrane was incubated in 1/1000 donkey anti-goat secondary antibody (Santa Cruz) in 5% w/v milk/TBS for one hour, rocking at room temperature. The membrane was washed five times for five minutes, rocking at room temperature in 0.1 % v/v tween 20 in TBS. ECL detection mix (Amersham Bioscience, Little Chalfont, UK) was poured onto the membrane and incubated at room temperature for one minute. This was then placed in a film cassette with x-ray photography paper (Sigma) using a red safety light and left for one hour at room temperature. The film was placed in developer for 20 to 60 seconds until the small bands appeared on the film. This was washed in tap water and then placed in fixer for 5 minutes. If the specific protein was present then small bands will appear darker on the opaque film.

7.2.2. RT-PCR to Detect HGF Expression

The primer sequences were taken from a previously published study (Okazaki *et al.*, 2002). These were checked against the cDNA sequence provided by the National Centre for Biotechnology Information Database (NCBI, www.pubmed.com) to be sure they are correct. The primers were produced by Thermo Electron Corporation, Basingstoke, UK.

7.2.2.a. Sample Preparation

T75 flasks (Nunc, Fisher, Loughborough, UK) were seeded with 1ml 50 x 10^4/ml of Het1a and Oe21 cells and grown for 48 hours. The cells were washed three times with PBS. The cells were scraped from the flasks using a cell scraper (Nunc) and a further 10ml PBS was added. This was collected into a 15 ml centrifuge tube and centrifuged at 1200 x g for 10 minutes at 4^{o}C. The cell pellet was collected into a 2ml eppendorf tube and centrifuged at 20,000 x g for 15 minutes at 4^{o}C. The cell pellet was snap frozen in liquid nitrogen and stored at –80°C prior to use. When required, the cells were defrosted on ice and the RNA was extracted using the RNA extraction kit (RNeasy Mini kit, Qiagen, West Sussex, UK). The RNA was quantified. Using the reverse transcriptase kit (Invitrogen Cloned AMV – first strand synthesis kit), the cDNA for each sample was produced, along with a RT-negative sample which incubated without the Reverse transcriptase in the reaction.

7.2.2.b. PCR Reaction

The following PCR reaction components were added together in a thin-walled PCR tube (Okazaki *et al.,* 2002):

- 0.5 µl cDNA sample
- 5 µl 10 x Taq buffer, containing 25mM $MgCl_2$ (Qiagen)
- 1 µl dNTPs (10mM each dNTP) (Qiagen)
- 0.5 µl Each primer (50 pMol)
- 0.25 µl Taq (1.5 units) (Qiagen)
- 37.25 µl Nuclease Free H_2O

A PCR negative control reaction was prepared containing all components except the cDNA, replaced by the equivalent volume of H_2O. A positive control PCR reaction was prepared using the same cDNA as used in the first PCR test reaction, but with β-actin primers. β-actin was chosen as it is a protein implicated with cell motility that is always expressed known as a house keeping gene (Raff *et al*., 1997). The positive control results for are in the appendix J.

Reactions were performed for the following temperature and cycles using a Techne Genius Thermal cycler (Stone, UK).

- 35 cycles of 94°C for 30 seconds, 61°C for 30 seconds and 72°C for 90 seconds
- 1 cycle of 95°C for 30 seconds, 61°C for 30 seconds and 72°C for 5 minutes.

Reaction products (10 µl) were resolved on 2% w/v agarose gels by electrophoresis (Hoefer semi-phor) at 100 volts for 25 minutes and were visualised by soaking the gels in 0.5µg/ml ethidium bromide and stained for 30 minutes. This was then visualised using a UV light box set to a wave length of 254 nm.

7.2.3. Investigating Growth Factor Protein Expression and PGE_2 Treatment

T75 flasks (Nunc) were seeded with 1ml 50 x 10^4/ml cells and incubated in media (cell methods in the appendix E) for 48 hours. The cell specific media (Sigma) was changed for media spiked with 0, 0.0005, 0.05 or 5 µg/ml PGE_2 and labelled A, B, C and D respectively. The cells were grown for different periods of time: 1 hour, 3 hours, 24 hours, 48 hours and 72 hours and labelled 1, 2, 3, 4, and 5 respectively. The cells were collected from the flasks by washing with 10ml PBS and cell scraping in a further 10ml PBS. This was collected and centrifuged at 1200 x g for 10 minutes. The cell pellet was collected into a 2ml eppendorf and centrifuged at 20,000 x g for 15 minutes. The cell pellet was snap frozen in liquid nitrogen and stored at -80^0C. When required the cells were defrosted on ice and the RNA was extracted using RNA extraction kit (Qiagen RNeasy Mini, West Sussex, UK). The RNA was quantified and all the samples were diluted to 50µg/ml with nuclease free water. Using a reverse transcriptase kit (Invitrogen Cloned AMV – first strand synthesis kit), the cDNA of the samples were collected, along with a RT-negative sample, which was incubated without the Reverse transcriptase in the reaction. The cDNA samples where diluted with nuclease free water at the optimised dilutions of 1/5, 1/50 and ½ for EGFR, VEGF and COX 2, respectively. PCR was carried out using the following reaction mixture in each instance:

- 2 µl cDNA Sample
- 5 µl 10 x Taq Buffer (Qiagen)

- 1 µl dNTPs (10mM of each dNTP) (Qiagen)
- 1 µl Each Primer (20 Picomolar) (see below)
- 1 µl Taq (1.5 Units) (Qiagen)
- 39 µl Nuclease free H_2O

A PCR negative reaction was prepared without cDNA and replaced by an equivalent volume of H_2O. A positive PCR reaction was prepared by using the same cDNA as that used in the first PCR reaction (A1), with β-actin primers. β-actin was chosen as it is a protein implicated with cell motility that is always expressed (Raff *et al*., 1997). The primers were manufactured using the cDNA sequence (see appendix K) provided by National Centre for Biotechnology Information Database (NCBI, www.pubmed.com). The primers were produced by Thermo Electron Corporation, Basingstoke, UK.

Primers:

Beta-Actin (End product size: 698 bases)

Sense: 5'-CTA GAA GCA TTT GCG GTG GAC-3'

Antisense: 5'-TGA CGG GGT CAC CCA CAC TGT-3'

EGFR (End Product size: 351 bases)

Sense: 5'-ATG TCC GGG AAC ACA AAG AC-3'

Anti-Sense: 5'-TTC CGT CAT ATG GCT TGG AT-3'

VEGF (End product size: 224 bases)

Sense: 5'-TGC TGT CTT GGG TGC ATT GG-3'

Anti-Sense: 5'-ACA CAG GAT GGC TTG AAG AT-3'

COX 2 (End product size: 305 bases)

Sense: 5'-TTC AAA TGA GAT TGT GGG AAA AT-3'

Anti-Sense: 5'-AGA TCA TCT CTG CCT GAG TAT CTT-3'

The PCR reactions are run at the following thermo-cycles in a Techne Genius Thermal Cycler (Stone, UK).

EGFR and β Actin

- 31 cycles of 95^{o}C for 45 seconds, 54^{o}C for 30 seconds and 72^{o}C for 90 seconds
- 1 cycle of 95^{o}C for 45 seconds, 54^{o}C for 30 seconds and 72^{o}C for 5 minutes

VEGF

- 31 cycles of 95^{o}C for 45 seconds, 60^{o}C for 30 seconds and 72^{o}C for 90 seconds
- 1 cycle of 95^{o}C for 45 seconds, 60^{o}C for 30 seconds and 72^{o}C for 5 minutes.

COX 2

- 35 cycles of 95°C for 45 seconds, 54°C for 30 seconds and 72°C for 90 seconds
- 1 cycle of 95°C for 45 seconds, 54°C for 30 seconds and 72°C for 5 minutes

The RT PCR methods above were optimised to attempt to make the assay semi-quantitative. A range of cDNA concentrations were used in the PCR until the product produced a faded band after electrophoresis. A range of PCR cycles were carried out, collecting the product at different cycle numbers. The intensity of the resulting bands, using band recognition software (Genesnap, Synoptics Ltd, Cambridge), were plotted against cycle number and the centre of the steepest part of the curve selected as the optimum number of cell cycles (exponential part of the reaction), where small changes in the amount of starting material (RNA / cDNA) would give the largest change in band intensity on the resulting electrophoresis gel.

For optimisation of COX 2 the following was tested:

- PCR cycles number (20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40)
- cDNA concentration (1,2, 5, 10. 25, 50 µl cDNA)

For optimisation of VEGF and EGFR the following was tested:

- PCR cycles number (28, 30, 32, 34, 36, 38,)
- cDNA concentration (0.01, 0.1, 0.2, 0.5, 1,2, 5, 10, 25, 50 µl cDNA)

7.3. Results

7.3.1. Western Blotting to Detect HGF Expression

Following optimisation of the protein transfer to membrane, the protein from the oesophageal, bladder and prostate cell lines that had given positive results for CD44 western blots, did not give any positive results with the HGF antibody. Using 30 kilodalton Millipore tubes to filter smaller polypeptides the protein was concentrated and retested, this did not give any improved response. Both the primary and the secondary antibodies were tested at an array of concentrations and this did not give any response. The secondary antibody was developed to bind to the molecular weight ladder developed by Santa Cruz Biotechnology. Therefore this was used to check the binding ability of the secondary antibody, should the secondary antibody not bind then the ladder would not appear. The ladder appeared as normal. To increase antibody binding the secondary binding temperature was increased to 37^{o}C from room temperature, which increased the background but did not show any bands. The binding time and temperature was changed for the primary antibody application, which showed no response. The transfer method was changed from the dry blot to the wet blot, although this did not affect the response.

7.3.2. RT-PCR to Detect HGF Expression

Following the method described by Okazaki *et al.* (2002) that provided the primer sequence, bands failed to appear. Therefore a number of optimisation methods were undertaken. Firstly, the RT-PCR reactions were also carried out on RNA from RT112 (bladder) and OE33 (oesophageal adenocarcinoma cell line) without any positive response. From the original method the annealing temperature was first tested at 50, 52, 54, 58, 61 and 62 without any result. The PCR cycle numbers were tested at 30, 35 and 40 cycles without any result. The primers, $MgCl_2$, dNTP, *Taq* and cDNA were

tested at a range of concentrations without any result. New primers were designed to determine that the primers were working and verify that the gene was present in the DNA (see appendix H for sequence and placement in the gene).

7.3.3. Assessment of EGFR and VEGF Expression Following Treatment using RT-PCR (Het1a and Oe21 cells).

For all of the results the following labelling will be used:

A = No PGE_2 treatment, B = 0.0005μg/ml PGE_2, C = 0.05μg/ml PGE_2 and D=5μg/ml PGE_2.

1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

Electrophoresis of RT-PCR products of EGFR with Het1a cell line

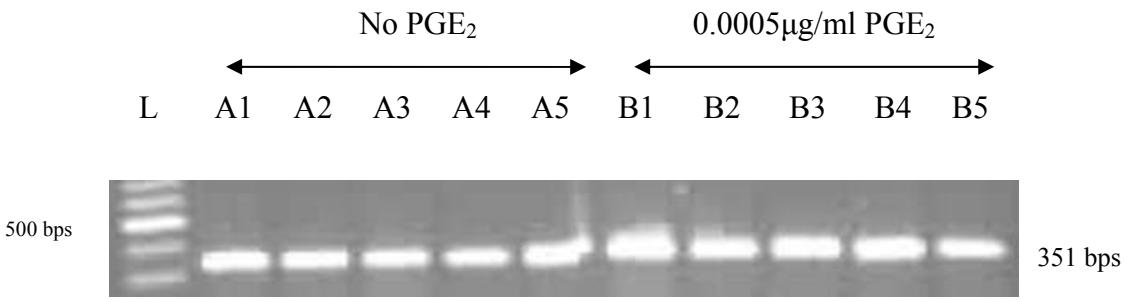


Figure 7.1: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. EGFR RT-PCR products from RNA extracted from 0 (A) and 0.0005 µg/ml PGE_2 (B) treated Het1a cells over different periods of time. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

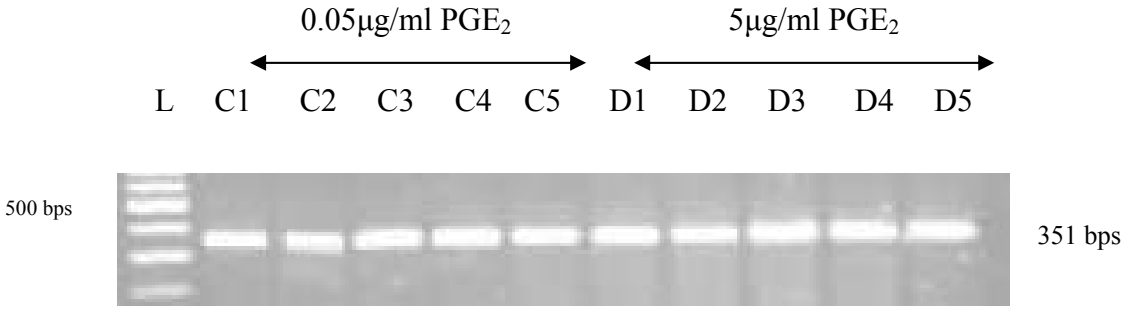


Figure 7.2: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. EGFR RT-PCR products from RNA extracted from 0.05 (C) and 5 µg/ml PGE_2 (D) treated Het1a cells over different periods of time. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

Figures 7.1 and 7.2 show similar band intensities for all Het1a cells over time. The results show there do not appear to be large changes in expression due to PGE_2 concentration.

Electrophoresis of RT-PCR products of EGFR from Oe21 cell line

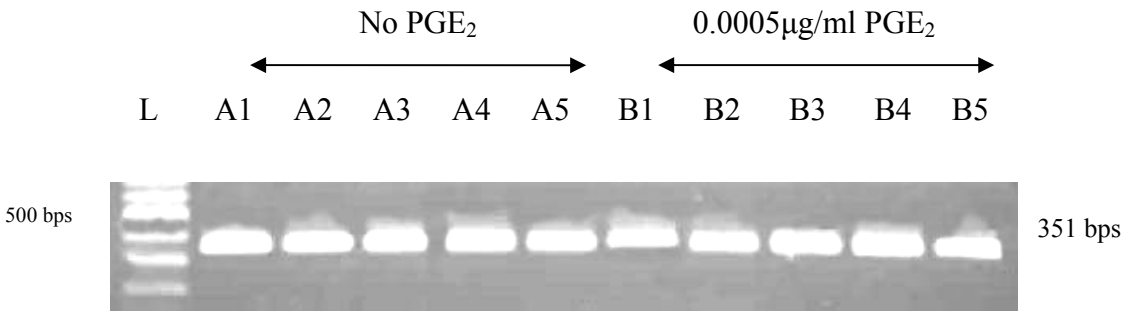


Figure 7.3: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. EGFR RT-PCR products from RNA extracted from 0 (A) and 0.0005 µg/ml PGE_2 (B) treated Oe21 cells over different periods of time. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

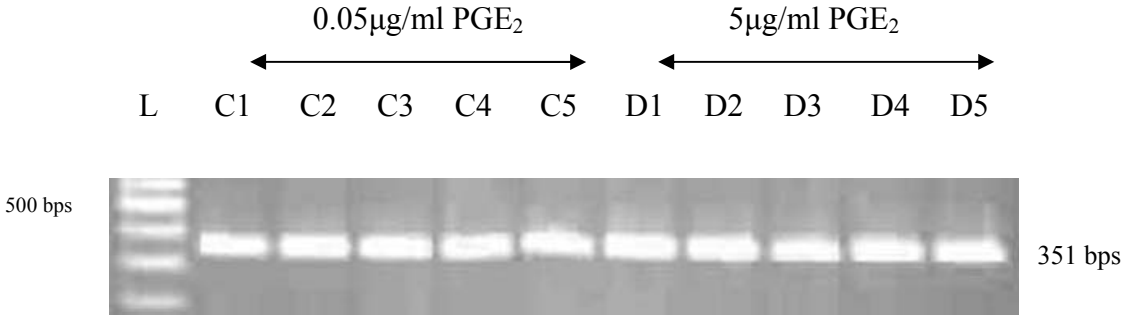


Figure 7.4: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. EGFR RT-PCR products from RNA extracted from 0.05 (C) and 5 µg/ml (D) PGE_2 treated Oe21 cells over different periods of time. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

Figures 7.3 and 7.4 show similar band intensities for PGE_2 treated and untreated Oe21 cells over time. The results show there do not appear to be large changes in expression due to PGE_2 concentration.

Electrophoresis of RT-PCR products of VEGF from Het1a cell line

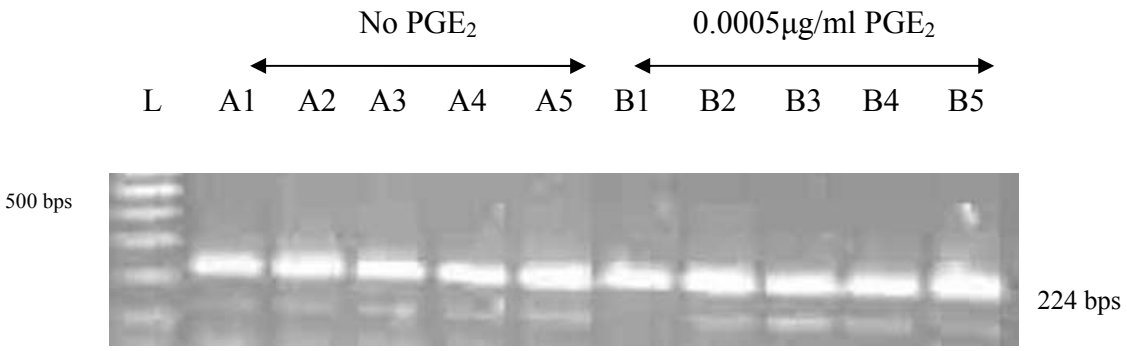


Figure 7.5: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. VEGF RT-PCR products from RNA extracted from 0 (A) and 0.0005 µg/ml (B) PGE_2 treated Het1a cells over different periods of time. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

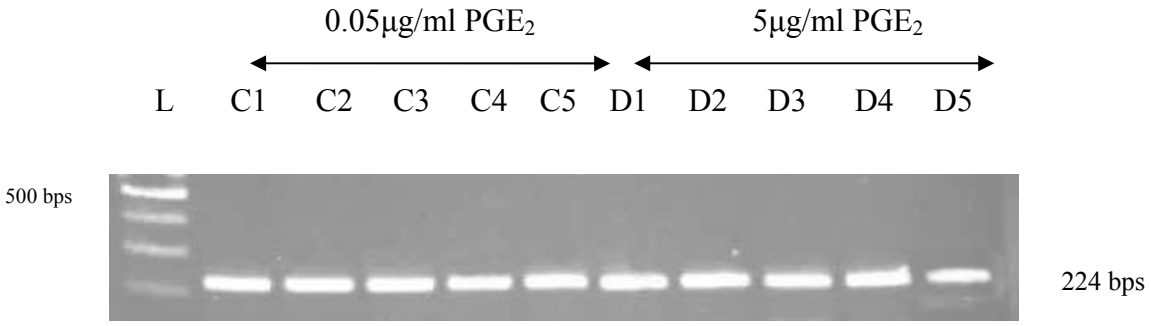


Figure 7.6: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. VEGF RT-PCR products from RNA extracted from 0.05 (C) and 5 µg/ml (D) PGE_2 treated Het1a cells over different periods of time. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

Figures 7.5 and 7.6 show similar band intensities for PGE_2 treated and untreated Het1a cells over time. The results show there do not appear to be large changes in expression due to PGE_2 concentration.

Electrophoresis of RT-PCR products of VEGF from Oe21 cell line

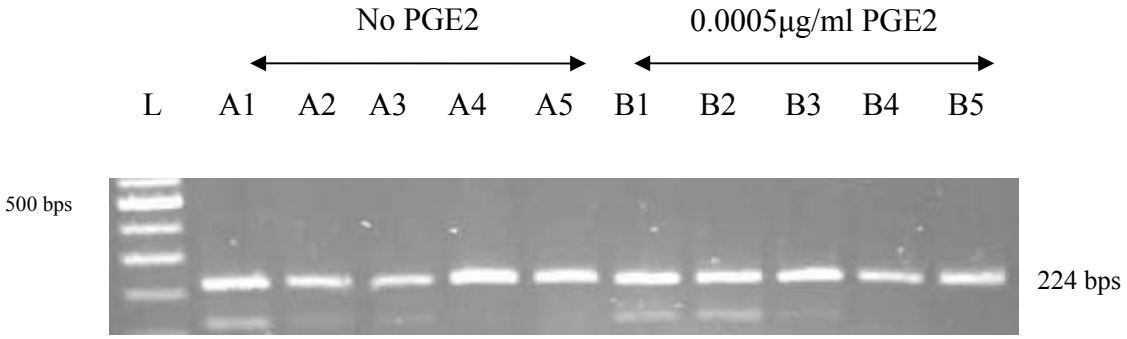


Figure 7.7: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. VEGF RT-PCR products from RNA extracted from 0 (A) and 0.0005 µg/ml (B) PGE_2 treated Oe21 cells over different periods of time. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

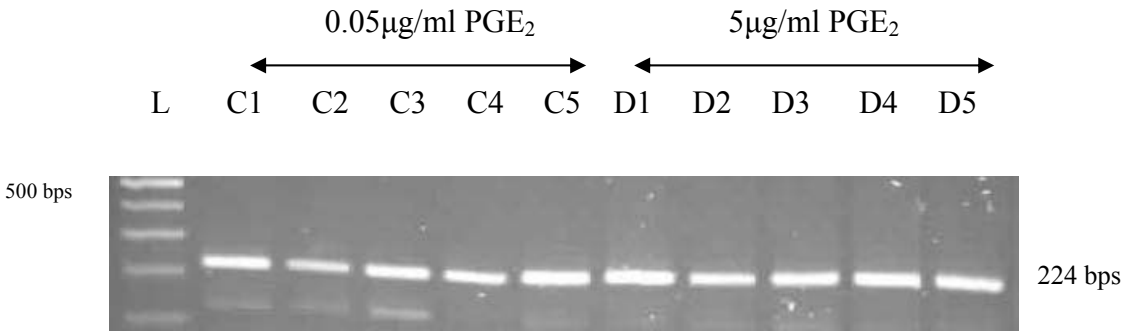


Figure 7.8: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. VEGF RT-PCR products from RNA extracted from 0.05 (C) and 5 µg/ml (D) PGE_2 treated Oe21 cells over different periods of time. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

Figures 7.7 and 7.8 show similar band intensities for PGE_2 treated and untreated Oe21 cells over time. The results show there do not appear to be large changes in expression due to PGE_2 concentration.

7.3.4. Assessment of EGFR and VEGF Expression Following PGE_2 Treatment Using Serum Free Media and RT-PCR (Het1a and Oe21 cells)

It was important to repeat the RT-PCR reactions without the foetal calf serum which is added to the media to allow the cells natural growth stimulation, and may cause increased expression of the proteins that are being investigated, masking the effect of the PGE_2. As found with other studies (Tang *at al.*, 1997), without this foetal calf serum the cells eventually die therefore the cells are serum treated to the point of PGE_2 treatment and then replaced by serum free media.

Electrophoresis of RT-PCR Products of EGFR from Het1a Cell line

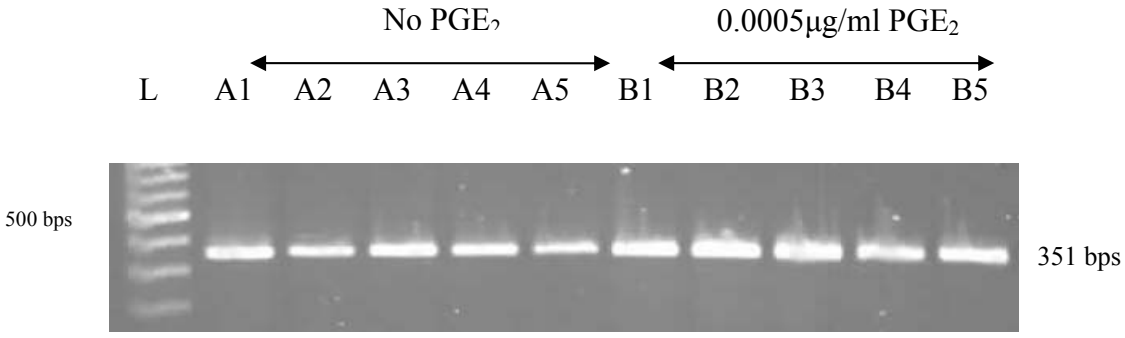


Figure 7.9: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. EGFR RT-PCR products from RNA extracted from 0 (A) and 0.0005 µg/ml PGE_2 (B) treated Het1a cells over different periods of time in serum free media. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

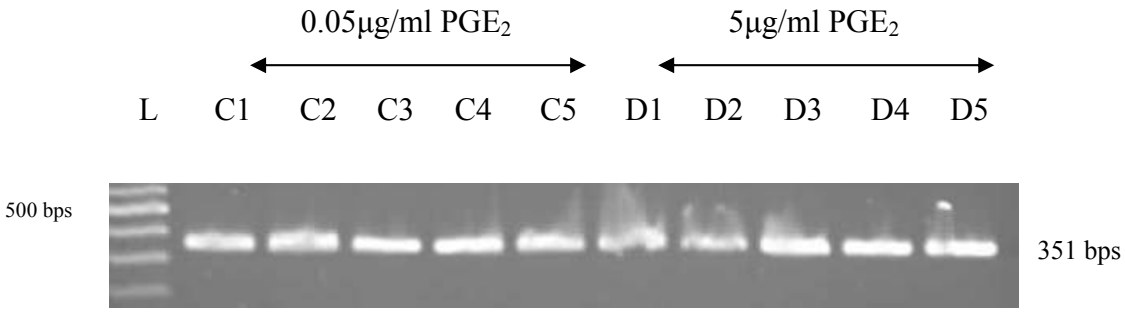


Figure 7.10: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. EGFR RT-PCR products from RNA extracted from 0.05 (C) and 5 µg/ml (D) PGE_2 treated Het1a cells over different periods of time in serum free media. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

Figures 7.9 and 7.10 show similar band intensities for PGE_2 treated and untreated Het1a cells over time. The results show there do not appear to be large changes in expression due to PGE2 concentration.

Electrophoresis of RT-PCR Products of EGFR from Oe21 Cell line

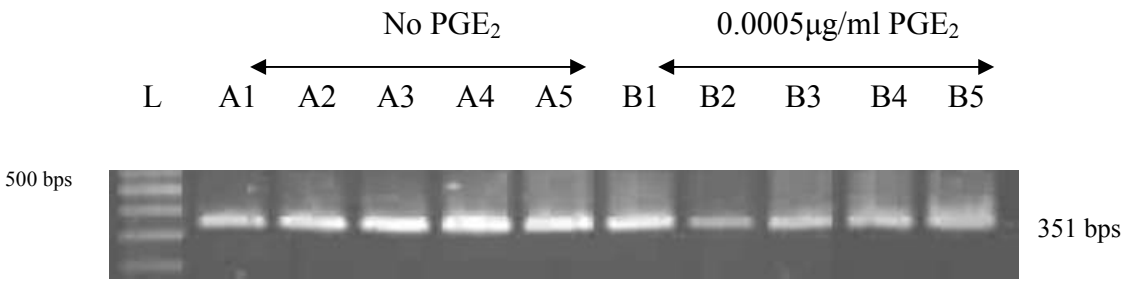


Figure 7.11: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. EGFR RT-PCR products from RNA extracted from 0 (A) and 0.0005 µg/ml (B) PGE_2 treated Oe21 cells over different periods of time in serum free media. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

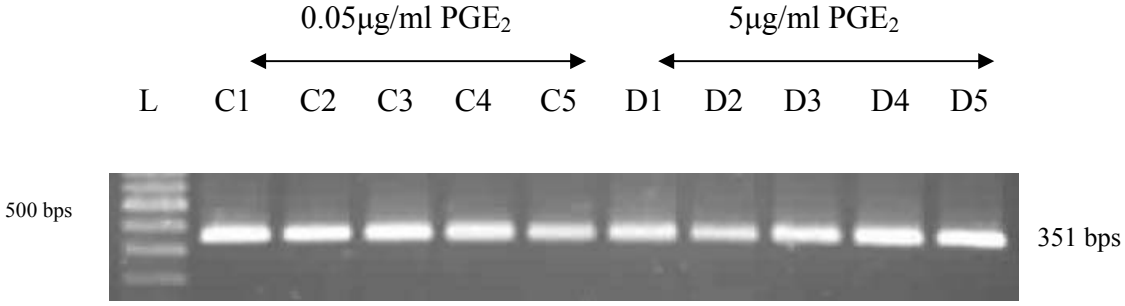


Figure 7.12: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. EGFR RT-PCR products from RNA extracted from 0.05 (C) and 5 µg/ml (D) PGE_2 treated Oe21 cells over different periods of time in serum free media. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

Figures 7.11 and 7.12 show similar band intensities for PGE_2 treated and untreated Oe21 cells over time for the two higher concentrations of 0.5 and 5 μg/ml PGE_2, although the 0.0005 μg/ml had slightly duller bands. As this difference is so small they can be accepted as insignificant. The results show there do not appear to be large changes in expression due to PGE_2 concentration.

Electrophoresis of RT-PCR Products of VEGF from Het1a Cell line

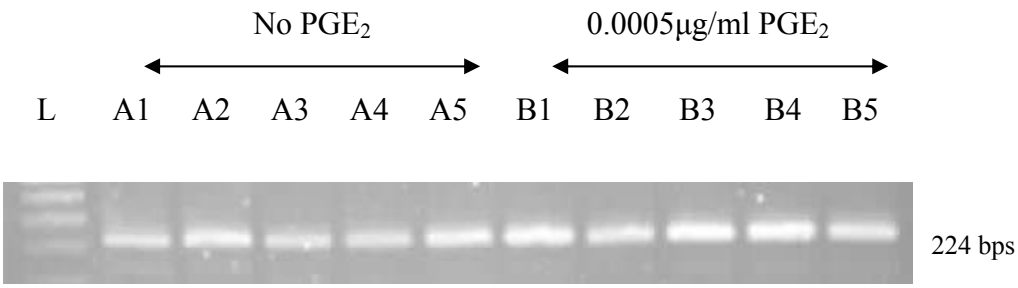


Figure 7.13: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. VEGF RT-PCR products from RNA extracted from 0 (A) and 0.0005 μg/ml (B) PGE_2 treated Het1a cells over different periods of time in serum free media. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

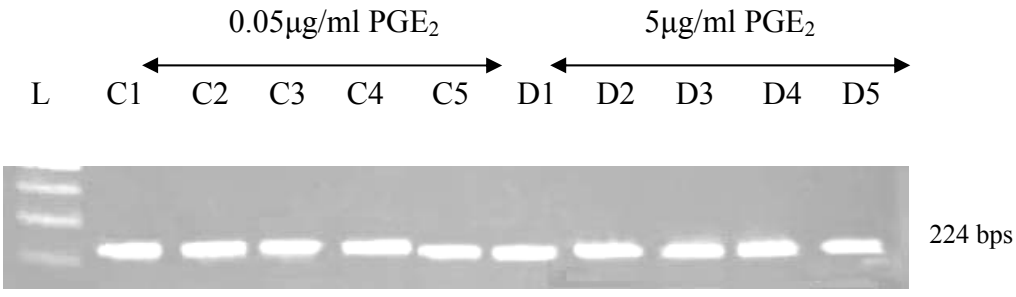


Figure 7.14: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. VEGF RT-PCR products from RNA extracted from 0.05 (C) and 5 μg/ml (D) PGE_2 treated Het1a cells over different periods of time in serum free media. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

Figures 7.13 and 7.14 show similar band intensities for the PGE_2 treated het1a cells over time. The untreated cells appear to be slightly duller and not as intense, although this change do not appear to be enough to be significant. The results show there does not appear to be large changes in expression due to PGE_2 concentration.

Electrophoresis of RT-PCR Products of VEGF from Oe21 Cell line

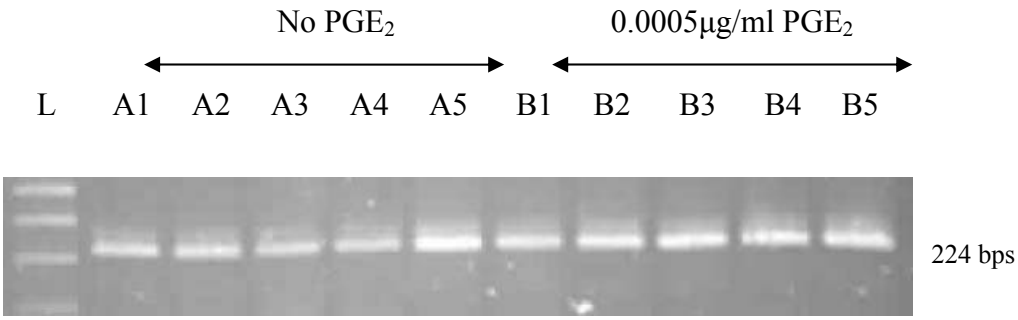


Figure 7.15: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. VEGF RT-PCR products from RNA extracted from 0 (A) and 0.0005 µg/ml (B) PGE_2 treated Oe21 cells over different periods of time in serum free media. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

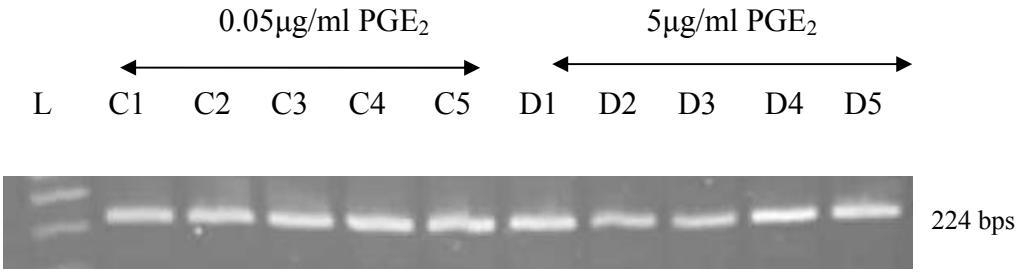


Figure 7.16: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. VEGF RT-PCR products from RNA extracted from 0.05 (C) and 5 µg/ml (D) PGE_2 treated Oe21 cells over different periods of time in serum free

media. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

Figures 7.15 and 7.16 shows similar band intensities for the PGE_2 treated and untreated Oe21 cells over time. The results show there do not appear to be large changes in expression due to PGE_2 concentration.

Assessment of COX 2 expression following PGE_2 treatment using RT-PCR (Het1a and Oe21)

Electrophoresis of RT-PCR products of COX 2 from Het1a cell line

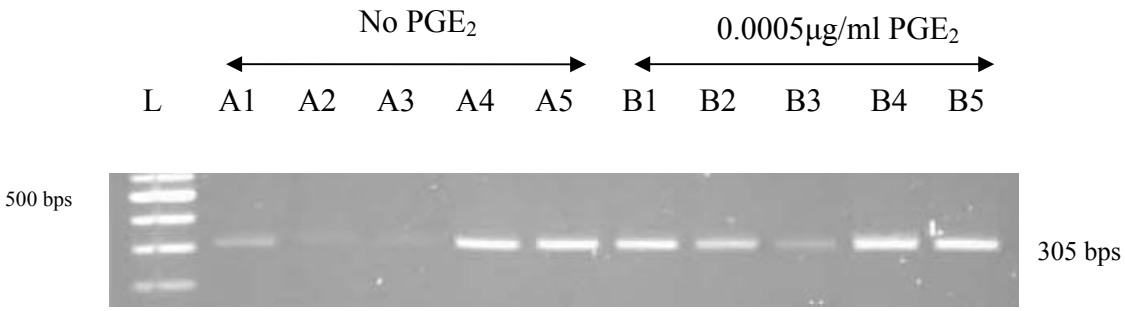


Figure 7.17: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. COX 2 RT-PCR products from RNA extracted from 0 (A) and 0.0005 µg/ml (B) PGE_2 treated Het1a cells over different periods of time. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

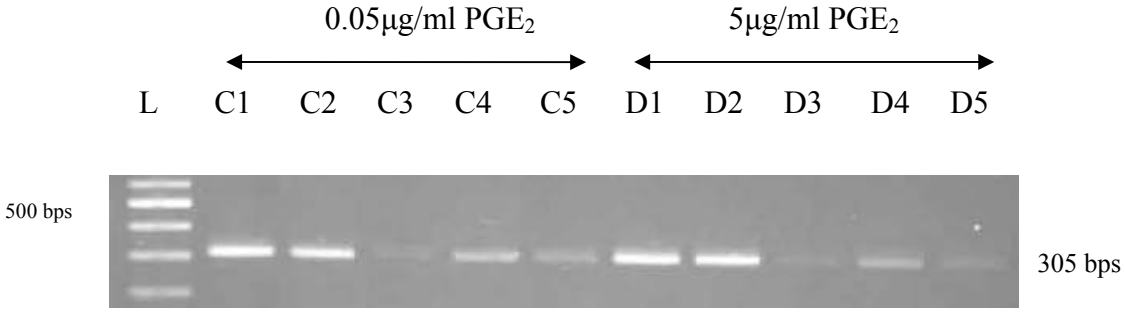


Figure 7.18: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. COX 2 RT-PCR products from RNA extracted from 0.05 (C) and 5 µg/ml (D) PGE_2 treated Het1a cells over different periods of time. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

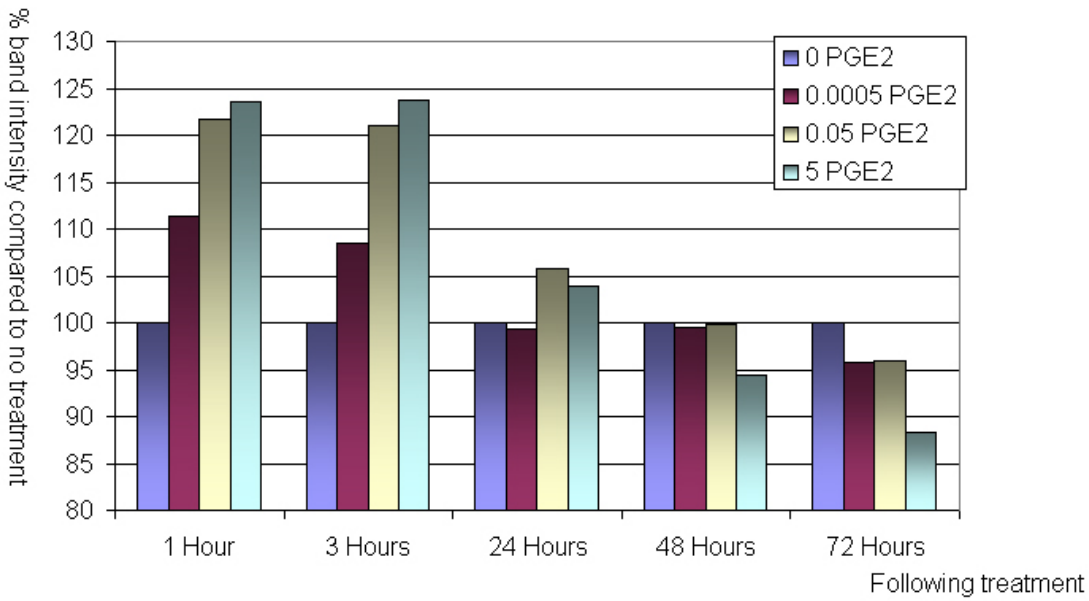


Figure 7.19: The percentage changes of band intensities of COX 2 product taken from the electrophoresis gels of Figures 7.17 and 7.18 compared to the untreated control Het1a cells.

Figure 7.19 show how the expression changes compare to expression of non-treated cells. From this and the Figures 7.17 and 7.18 it can be seen that expression for COX 2 has increased with concentration, although this relationship slowly reduces to 48 and 72 hours where additional COX 2 expression seems to be repressed compared to normal. It is to be stated that this method of measuring expression is just semi-qualitative and a real-time PCR would be required for more accurate results.

Electrophoresis of RT-PCR Products of COX 2 from Oe21 Cell line

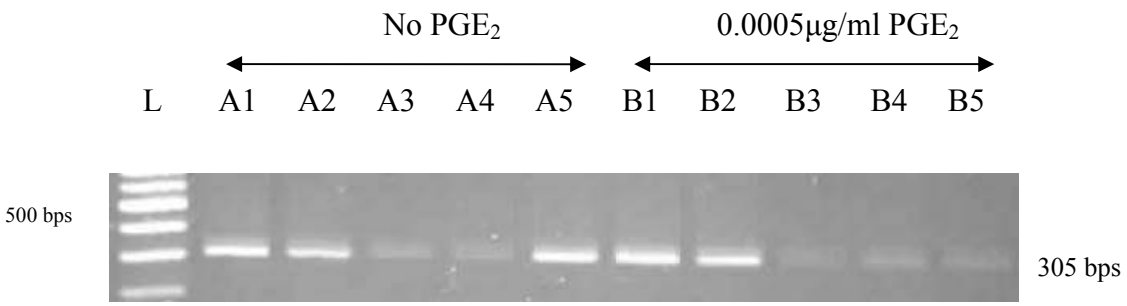


Figure 7.20: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. COX 2 RT-PCR products from RNA extracted from 0 (A) and 0.0005 µg/ml (B). PGE_2 treated Oe21 cells over different periods of time. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

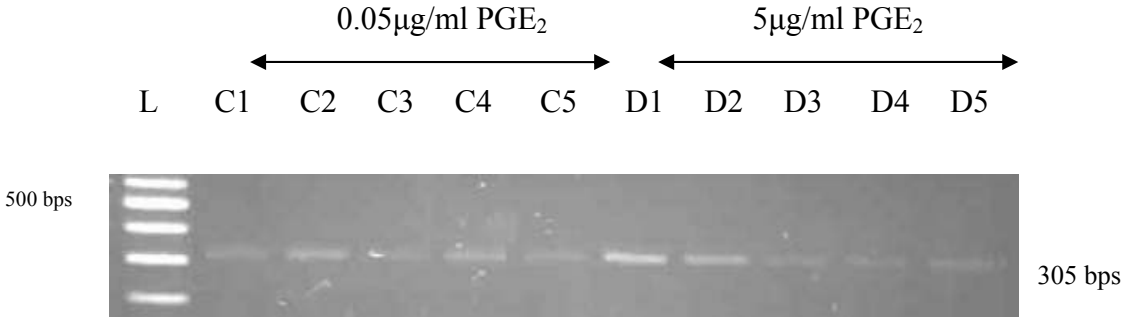


Figure 7.21: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. COX 2 RT-PCR products from RNA extracted from 0.05 (C) and 5 µg/ml (D) PGE_2 treated Oe21 cells over different periods of time. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

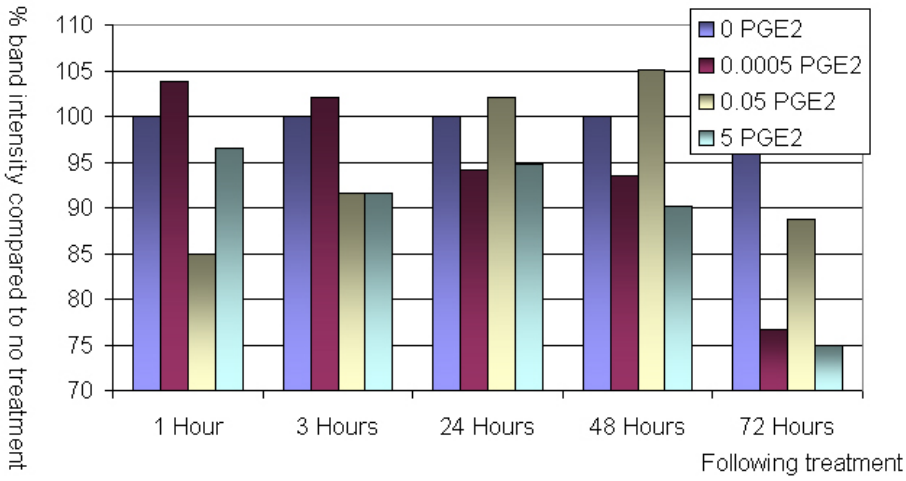


Figure 7.22: The percentage changes of band intensities taken from the electrophoresis gels of Figures 7.20 and 7.21 compared to the untreated control Oe21 cells.

Figure 7.22 shows there is not a clear increase in band intensity compared to the non treated cells. After the initial one and three hours the smallest PGE_2 concentration had an effect on the cells and increased COX 2 expression, although this gradually decreased over the three days. The 0.05 μg/ml PGE_2 has the opposite effect and steadily increases the expression for the first 48 hours. The higher concentration does not increase the expression above that of the untreated cells, Figures 7.20 and 7.21 show that expression is reduced with increased PGE_2 concentration after the shorter time period, although at 24 – 48 hours the two higher concentrations have a longer effect, where the band intensities are corrected by standardising against the 500 bp ladder band. Figure 7.22 show a different pattern of expression of COX 2 in the Oe21 cells from that of the Het1a cells (Figure 7.19). The expression patterns between the two cell lines are similar to the growth patterns shown in Chapter 6 (section 6.3).

7.3.5. Assessment of COX 2 Expression Following PGE_2 Treatment Serum Free Media using RT-PCR (Het1a and Oe21 cells)

Electrophoresis of RT-PCR Products of COX 2 from Het1a Cell line Using Serum Free Media.

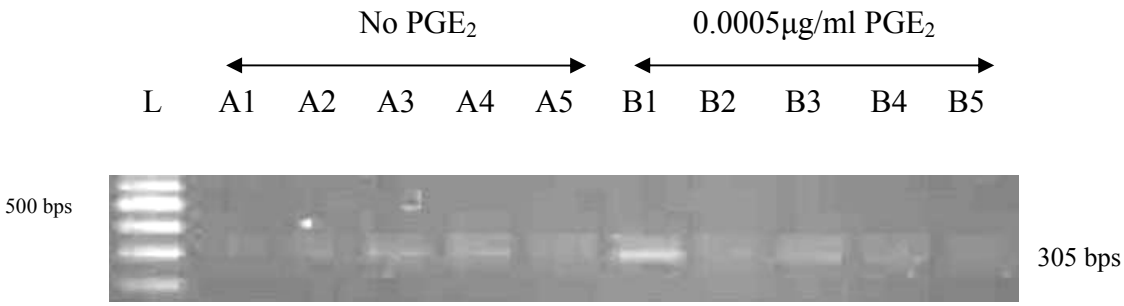


Figure 7.23: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. COX 2 RT-PCR products from RNA extracted from 0 (A) and 0.0005 µg/ml (B) PGE_2 treated Het1a cells over different periods of time in serum free media. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

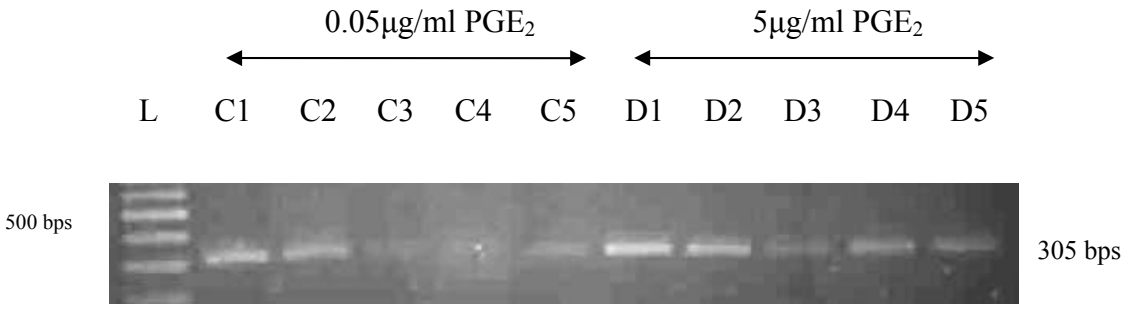


Figure 7.24: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. COX 2 RT-PCR products from RNA extracted from 0.05 (C) and 5 µg/ml (D) PGE_2 treated Het1a cells over different periods of time in serum free media. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

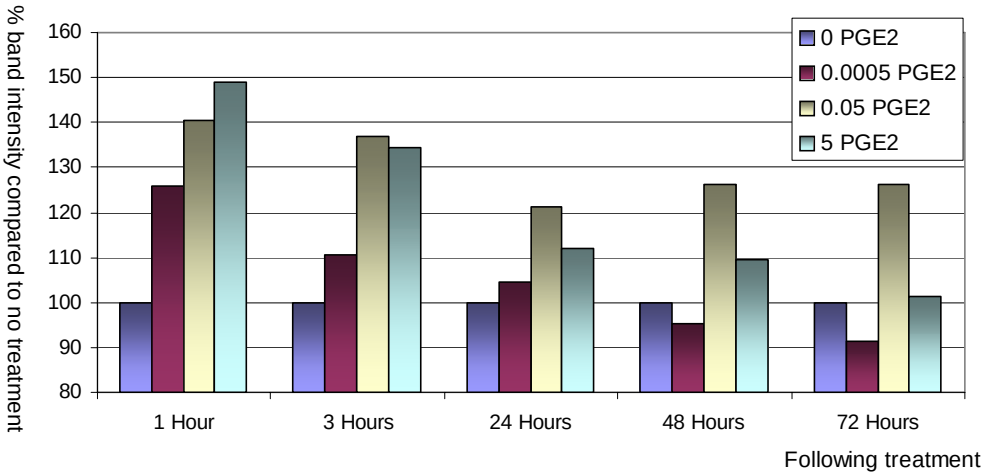


Figure 7.25: The percentage changes of band intensities, measured using Syngene GeneTools (Synoptics, Cambridge, UK) taken from the electrophoresis gels of Figures 7.23 and 7.24 compared to the untreated control Het1a cells.

The Figures 7.23 and 7.24 show an increase in expression with PGE_2 concentration increase. This is poorly represented by the photo due to the software exporting poor images. This is supported by Figure 7.25 when all concentrations have shown to increase expression compared to the normal. As seen with the above Figures the intensity of the PCR bands decline with time from PGE_2 treatment,

Electrophoresis of RT-PCR Products of COX 2 from Oe21 Cell line Using Serum Free Media.

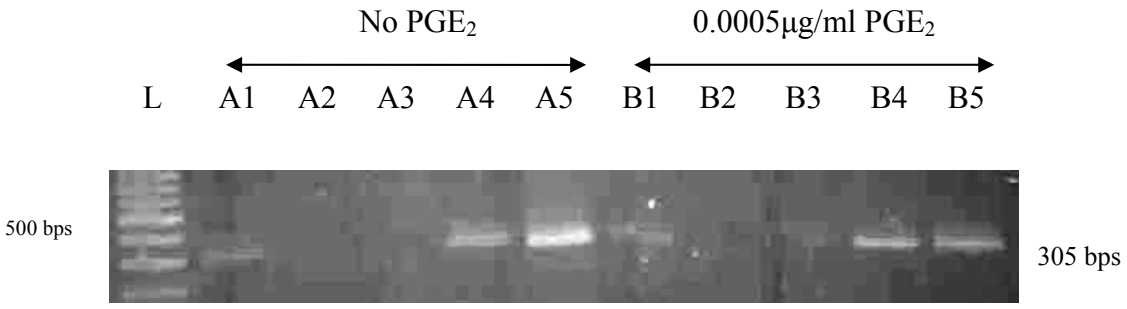


Figure 7.26: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. COX 2 RT-PCR products from RNA extracted from 0 (A) and 0.0005 µg/ml (B) PGE_2 treated Oe21 cells over different periods of time in serum free media. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

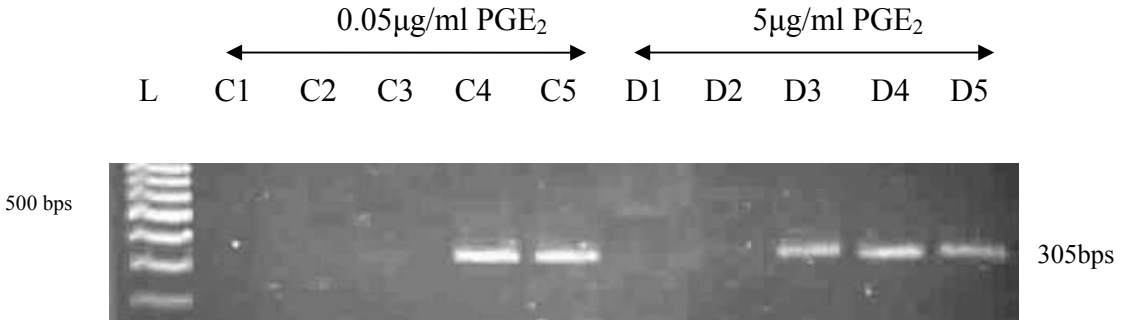


Figure 7.27: RT-PCR products visualised with agarose gel electrophoresis and Ethidium Bromide. COX 2 RT-PCR products from RNA extracted from 0.05 (C) and 5 µg/ml (D) PGE_2 treated Oe21 cells over different periods of time in serum free media. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

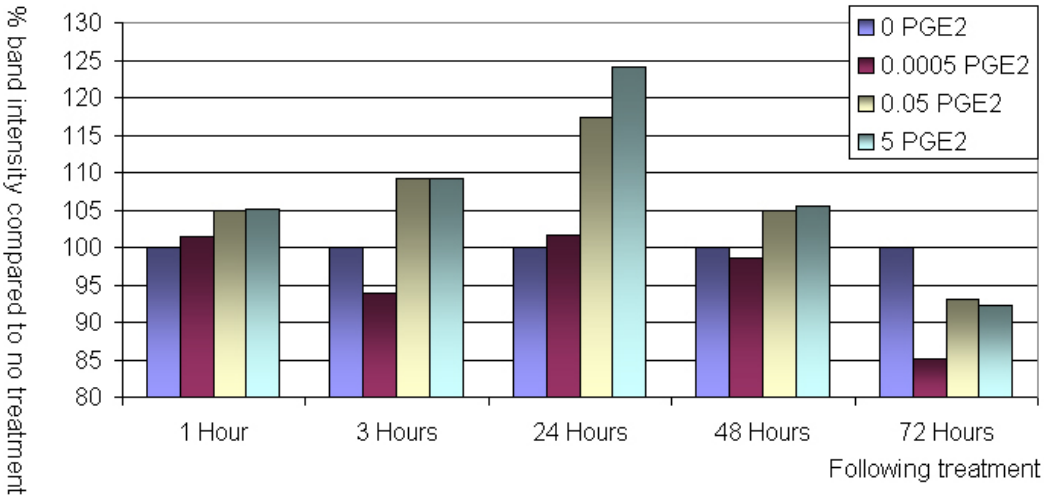


Figure 7.28: The percentage changes of band intensities measured using Syngene GeneTools (Synoptics, Cambridge, UK) taken from the electrophoresis gels of Figures 8.26 and 8.27 compared to the untreated control Oe21 cells.

Figures 7.26 and 7.27 show that after one hour there is a slight increase in COX 2 expression after no and the smallest concentration of PGE_2 treatment, although this disappears when treating the cells with higher concentrations of PGE_2. Both the gels and Figure 7.28 show that there is a significant increase in COX 2 expression following 5 μg/ml PGE_2 treatment after 24 hours showing a doubling in intensity.

7.4. Discussion

The investigations in this chapter have presented some exciting novel results. Due to the importance of the patterns seen in the cell proliferation assays of Chapter 6 (section 6.3) the lack of conclusive results for growth factor expression seen in sections 7.3.2-7.3.4, does not conclusively demonstrate that growth factors are not implicated in the carcinogenesis of oesophageal cancer. It is predicted that the method of how the growth factors respond to the PGE_2 has been at fault. Originally it was postulated that PGE_2 would have a stimulatory effect on the expression, increasing production of VEGF and the EGF receptor, and increasing cellular proliferation. The

results shown here suggest this may not be the case as the expression remains reasonably constant throughout, regardless of stimulation. The double banding seen in Figures 7.5, 7.7 and 7.8 may to be due to different isoforms of VEGF (Sheen *et al.,* 2005). Increases in growth factor expression were expected with increased PGE_2 treatment, but this was not demonstrated in this study. It is postulated that the PGE_2 may have a stimulatory effect on the proteins that orchestrate the cell cycle, which would normally be activated by the growth factors. As discussed below this work has led to the suggestion that it maybe the intricate downstream growth factor secondary proteins that are affected by the PGE_2 to drive cell growth. These results have also led to the conclusion that PGE_2 stimulation may be increasing COX 2 expression forming a positive feedback loop that would stop should the COX 2 substrate, Aracadonic acid (derived from linoleic acid in maize), not be plentiful in the oesophagus.

The optimisation of the western blotting for HGF was thorough but did not yield a clear picture. Due to the lack of results with the western blot, it is not possible to conclude whether the primary antibody was working, although it has been shown that the other components of the method do not appear to be the problem. A great deal of time was spent on this with little result, therefore this method was dropped from the investigation.

The antibody (R&D Systems) did not yield positive results; it has not been tested on oesophageal tissue. It has been shown to work with the western blotting technique. DeFrances *et al.,* (1992) do show positive results for squamous epithelium of the oesophagus using an antibody they developed. This was shown to demonstrate expression using immunohistochemistry, a very different technique with many binding differences. For example the antibodies may be binding to different protein epitopes, due to conformational changes following protein denaturation in western blotting. DeFrances concluded that HGF levels were high in the oesophageal development of rat foetuses, therefore this could be differently structured to human. This would suggest a proliferative drive by HGF providing cellular growth, although without a positive result using human cells the HGF protein can not be discussed as part of the developed theory of this thesis.

The optimisation of the RT-PCR attempted with the HGF primers was thorough. All of the separate components worked, although there was no result from the cDNA of

the oesophageal cell lines. A literature review did not show any studies which have looked at the expression of HGF in oesophageal tissue using a PCR method. Takada *et al*. (1995) investigated the expression of HGF in human oesophageal squamous cell carcinoma using a sandwich ELISA. They discovered HGF expression to be higher in oesophageal tissue compared to normal mucosa, concluding that HGF could be used as a potential biomarker for cancer progression in this tissue. The positive control solution from the HGF primer kit provided positive results for all primers. This should provide the evidence that all the components of the methods work. No presentation of the HGF gene in the cDNA suggests that the HGF protein may not be expressed at the time of extraction. The positive control solution from the kit is made up using manufactured polynucleotides specific for the HGF assay primers.

Further research to look at changes in HGF protein concentrations could be undertaken using an ELISA kit and recombinant HGF. Due to the time and money spent on the HGF investigation and with few solid conclusions, the investigation was moved on to research a different component of the theory stated in Figure 3.2. Other more sensitive methods could have been used for example PCR-ELISA, Surface Plasma Resonance (SPR) or real-time PCR, should the time and equipment be available. The results have not concluded that HGF is important in validating the theory; since, these methods may not be sensitive enough. Further investigations are required to reach a definite conclusion with respect to HGF.

According to RT-PCR band intensity the expression for EGFR and VEGF appeared to remain constant for all PGE_2 concentrations treated for all the periods of time studied. It is to be stated that the results from the reverse transcriptase PCR (RT-PCR) are semi-quantitative therefore to quantitatively measure the expression using RT-PCR would require a Real-Time PCR, such as the TaqMan. It appears that EGFR and VEGF are both being expressed fairly constantly between the one to 72 hours investigated. As these proteins appear to be expressed when there is no PGE_2 or serum treatment this neither supports nor denies a connection between the PGE_2 treatment and the VEGF or the EGFR expression.

There are still large areas to research investigating PGE_2 and VEGF and EGFR activation. Due to the lack of changes with and without treatment over these time periods, it would be interesting to research any changes in the expression of growth

factor related proteins after just a few minutes of treatment; once a growth factor is released and binds its receptors, it only takes 15 minutes for the early response genes to be induced and over an hour for the delayed-response genes to be induced. The main reason behind this time difference is due to the latter requiring protein synthesis (Almendral *et al*., 1988). It is this level of growth factor activation that needs to be investigated next in order to secure a link to PGE_2 growth stimulation and the growth factor activation; this will open up new areas of research and potential therapy. Increased amounts of VEGF and EGFR would increase the cell proliferation drive. However, an increase in the activation of the proteins downstream of these growth factor receptors could have the same stimulatory effect. Rather than investigating the expression of this growth factor and receptor it may provide greater information to research the activation of this growth factor and receptor. By studying specific kinases (e.g. Tyrosine kinase), cyclins or the response genes activation (*fos, jun* or *myc*) then it may be found that PGE_2 activates these growth factors faster or for longer periods of time stimulating greater epithelial growth. Both tyrosine kinase and cyclin D have been shown to be over-expressed in oesophageal cancer (Nemoto *et al*., 1997; King, 2000). They may have been stimulated by natural proteins such as PGE_2.

The Sammon theory postulates that the growth factors are not being broken down by the inhibition of the natural enzymes locally in the oesophagus, although as PGE_2 in this study has been shown to increase cellular proliferation, this means the pre-disposition to cancer could be due to reasons outside the theory; for example, increases in growth factor expression and activation. To fully investigate the channels in which PGE_2 could activate cell growth at a molecular level would take another PhD project due to the complexity of these growth factor pathways, for example, Figure 7.28 shows some of the many proteins that PGE_2 could be stimulating in the EGF pathways.

Stimulated PGE_2 producing receptors are synthesising PGE_2 within one to five minutes, followed by massive degradation (Klein, 1990). Therefore, should the PGE_2 stimulate the growth factor expression through this mechanism then this RNA production may well be initiated between this 15 and 60-minute period and the related cell cycle proteins remain over the three day period. Hence, a study over this short period may show differences in the time of expression for the proteins providing a

clearer understanding of what is happening on a molecular basis rather than a snapshot of the natural cell cycle after effects of stimulated cell growth. A majority of the results for COX 2 (Figures 7.25 and 7.28) show a gradual decrease in expression following 24 hours therefore this could be due to a reduction in localised PGE_2, through degradation, decreasing local response. These extraction times were chosen due to them producing effective results in a previous study (Tjandrawinata *et al.*, 1997).

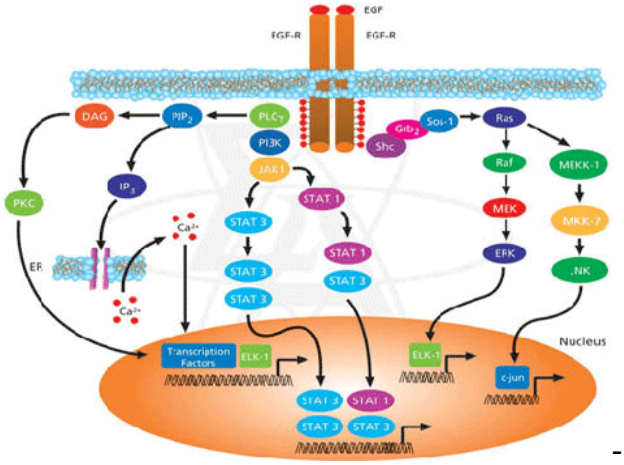


Figure 7.28: Key molecular pathways of Epidermal growth factor (EGF) regulation. Growth factor signalling from EGF, results in the expression of target genes, including transcription factor genes essential for cell cycle progression. (Sigma-Aldrich study aids, 2005)

Increased EGFR has already been shown to enhance tumour growth and progression in the oesophageal tissue (Mestre *et al.*, 1997), thus should EGFR be up regulated or activated by the PGE_2, this could explain the hyperplasia present. EGFR expression has been shown to increase in oesophageal disease, although an explanation of how is still to be determined (Jankowski *et al.*, 1992; Janskowski *et al.*, 1993). Forming a connection to this paper, PGE_2 is implicated with the inflammation of disease, which could explain the proliferative findings of this study (section 1.6.7). Whether EGF, the ligand for EGFR, is produced in the human oesophagus is a matter of debate (Mukaida *et al.*, 1990; Jankowski *et al.*, 1992). The EGF concentration in the oesophagus could be the result of the EGF produced in saliva, as reports show that if

saliva glands are removed from a rat there is a decrease in oesophageal mucosa protection, which is reversed by the addition of recombinant form EGF (Poulsen *et al.*, 1986).

Regurgitation of gastric fluid in the target population could also increase the EGF oesophageal level, and if the Sammon theory is correct in that, an alkali environment suppresses the breakdown of these growth factors, there should be an increase in proliferation from this external influence. However, growth factors are fragile proteins therefore they would not be expected to last very long in the oesophagus. There are other suggestions of how this high PGE_2 presence would have a cell growth stimulus. Work carried out by Pai *et al.* (2002) and Buchanan *et al.* (2003) over the period of this investigation makes such a suggestion. They treated human colon cancer cells with PGE_2 and found increased EGFR phosphorylation levels, a trans-activation which had the effect of increasing the proliferation of the cells. Using western blotting and real time PCR techniques they measured phosphorylated states of intracellular growth secondary proteins, such as PI 3 Kinase and Akt. This suggests the need for the study of this PGE_2 trans-activation of growth factor receptors in oesophageal tissue, as this could explain the whole basis of the increased proliferation seen in chapter 6. Ozama *et al.* (1989) suggested that the EGFR levels have a prognostic significance in oesophageal squamous cell carcinoma, as survival rates decrease as EGFR increases. Negishi *et al.* (1995) have explained that PGE_2 exerts its effects by working on the PGE_2 receptors coupled with G proteins, also implicated with natural growth factor mechanism. These activate or inhibit secondary messenger systems that could cause an influx of Ca^{2+}. Li and Villalobo (2002) later discovered that when calmodulin is complexed with this Ca^{2+} then the activity of EGFR is inhibited via tyrosine kinase activity, which suggests a relationship between PGE_2 and EGFR. It is important that changes in expression are not to be confused with the activation of the growth factors themselves.

This association between PGE_2 and growth factor activation is of huge importance in the carcinogenesis of oesophageal cancer. The method used in this study has not provided a great deal of information on this relationship but has opened up new avenues of the growth factor pathways that can be analysed. At the start of this thesis the general trend of cancer research was to look at the expression and maybe

activation that one protein has on another. New technology has expanded the direction of cancer research to investigate whole cellular pathways working together over one time period. The knowledge of common oncogenes has produced a body of research to show how the cell cycle pathways flow and interact together, although this should be taken a step further. It is often mutated proteins within these cycles that led to chain reactions of carcinogenesis by affecting many pathways involved in apoptosis, proliferation and angiogenesis. The future of cancer research is embracing new technology to look at cell cycle pathways as a whole, and not just individual proteins. This approach needs to take place in this research. PGE_2 treatment of the cells, pushing proliferation through growth pathways, needs to be investigated as a whole. This may be possible now with the right facilities as equipment such as micro-array technology is becoming more reliable and computer capabilities to analyse the results are now available, for example, the GeneChip by Affymetrix, Santa Cruz, USA.

Due to cell death with sustained serum deprivation, during the first 96 hours of growth the cells in this study were fed with media supplemented with serum to allow them to establish growth before comparing expression after different treatments. Tang *et al.* (1997) and Iihara *et al*. (1993) stated that they found it difficult to look at growth factor modulation following serum deprivation as after 48 hours the cells tend towards apoptosis. The cells in this investigation appeared to last around two days longer than this allowing testing to be extended to 72 hours without cell death. The flasks were washed with serum free media but the stimulus for the VEGF and EGFR expression may have come from the internal cellular reactions caused by the previous serum media, maybe promoting autocrine growth factor production and release. If the cells are deprived of serum for around an hour they should enter an altered state, known at G_0 in the cell cycle, or resting phase. It is then predicted that it takes eight hours to emerge from this state and all protein synthesis is depressed (Pardee, 1989), although in this study the cells still seem to be expressing these proteins after one and three hours in control non- PGE_2 treatments. As the cells are being serum starved over a period of days then the expression of such proteins may change as a response for survival, promoting growth in the case before cell death. There were no peer reviewed papers to support cellular serum deprived stress and PGE_2 links. The issue is that there is not another significant reliable model to investigate growth factors without using an animal, which itself will present masses of external influence issues. The

body is a very complex organism therefore a good model will also need to have similar molecular influences. In the results of this study the different PGE_2 concentrations had increased growth responses compared to no treatment, therefore the effects of PGE_2, on the cell line can be discussed with confidence that PGE_2 has the largest effect on the results.

In the cellular model, these cells taken from human tissues were immortalised by various methods, so they may have acquired a mutated growth stimulatory system, a factor which contributes to their survival *in-vitro*. Without studying the cells at a point of before they have VEGF and EGFR expression, it is difficult to determine whether it is the mutation of the cells that has caused this automated expression. The negative control here was to treat the cells with serum free media in a period that the cells do not show this expression, for example when serum starved and the cells enter senescence (explained above). This did not occur in the short term, as the cells in this investigation are starved for 3 days without any change in expression. As this chapter shows, the Het1a cell line can grow in serum free conditions in the short term; this could suggest that the cells have not entered a state of senescence maybe due to autocrine regulation of these required growth factors. Stern *et al.* (1987) have shown in an autocrine model that fibroblasts transfected with localised EGF release, exhibited uncontrolled proliferation. These same autonomous expression results are provided for the Oe21 cell line.

The action of angiogenesis in the process of carcinogenesis should not be underestimated. Without this introduction of nutrients to the tumour, it cannot grow any larger than 1mm in diameter, hence, the huge amount of drug company attention on VEGF and cancer therapy. As discussed in section 1.6.5, it is commonly presented that VEGF has connections with the development of angiogenesis in tumours and some investigators have linked this to COX 2 interaction. A paper printed after the research for this thesis was undertaken, showed how polyunsaturated fatty acids like those found in fish oils, reduce the VEGF expression in human colon cancer cells. This was shown to interact with the growth factor activation pathway and to suppress the actions of PGE_2, through COX 2 inhibition (Calviello *et al*., 2004). The process of the polyunsaturated acids suppressing VEGF could be due to this being a competitive substrate for the same enzymes as PGE_2, hence, the decrease in the PGE_2 action could

reduce the stimulation of VEGF activation. Investigations into this could provide information as to why people in the target population have a smaller chance of developing oesophageal cancer if they have a mix of fatty acids in their diet. Cianchi *et al.* (2004) suggest that the COX 2 activation of VEGF in colorectal cancer could be due to COX 2 activation mediated through the effect of nitric oxide.

Overall it cannot be proven that there are any connections between the expression of VEGF and EGFR, and PGE_2 treatment in the oesophageal cell lines from the results of this study without further models and methods in place. Should results suggest that VEGF is being up regulated then this could be via a positive feedback mechanism common in cell cycle pathways, as VEGF has been shown to increase the expression of COX 2 (Murphy and Fitzgerald, 2001).

The research into the changes that PGE_2 would have on the expression of the enzyme that produces it, COX 2, produced some very interesting results (section 7.3.5) that were not expected but seem to make sense, as described below. The COX 2 expression, as suggested by the presence of the RT-PCR product bands from the treatment of different concentrations of PGE_2 over various periods of time, is clearly different to that with no PGE_2 treatment. A majority of the above results for both serum and serum free treatment of both cell lines has shown an increase in COX 2 expression following PGE_2 treatment compared to no treatment (Figures 7.19, 7.22, 7.25 and 7.28). Figure 7.19 shows that after the 0.0005, 0.05 and 5 μg/ml PGE_2 treatment there were significant expression increases after one and three hours of the treatment in the "normal" Het1a cell line. After 48 hours and 72 hours the 0 and 0.0005 μg/ml PGE_2 treated COX 2 bands were only faintly seen, but the higher concentrations of 0.05 and 5 μg/ml had no bands, suggesting a loss of most of this expression. This suggests that the expression of the COX 2 has been brought on earlier by the PGE_2 treatment in the Het1a cell line (Figure 7.17 and 7.18). To keep the cells healthy they have to be fed every 48/72 hours, therefore as the serum is used up by the cells over the first day or two then COX 2 in the cells may be expressed when the cells are starved, stressed or reach 'crisis' point, shown after 48 and 72 hours. Increased PGE_2 concentration may well cause this same stressed state more quickly therefore bringing the expression forward. A connection can be made as PGE_2 is released when tissues are damaged and stressed leading to inflammation in tissue,

stimulating the release of growth promoters to maintain local tissue damage (section 1.6.7). It may also suggest that PGE_2 is being broken down and COX 2 is being produced to replace this. Figure 7.17 shows that after the Het1a cells were treated with 0.0005 μg/ml of PGE_2, the COX 2 was expressed after one and three hours as well as after 48 and 72 hours. This is for a much longer period of time than the control of no PGE_2 treatment.

As shown with the proliferation assays in chapter 6, the growth curves gave a bell sigmoidal growth curve (section 6.3), hence an optimal concentration of PGE_2 has a maximum response, lesser or higher concentrations of PGE_2 have a reduced response. If more COX 2 is produced over this period of time and the enzyme's substrate is locally abundant then this may suggest that a higher concentration of PGE_2 would be produced. It was shown in the previous chapter (section 6.3) that PGE_2 has a stimulatory effect on the proliferation of cells. By combining the results in this chapter and the last it would suggest a positive feedback system where additional PGE_2 can stimulate the expression of the COX 2 enzyme which itself could produce more PGE_2. Providing the PGE_2 does not reach too high a concentration to cause an inhibitory effect (seen in this study, figures 6.1 and 6.2) this repeated process could lead to increased cellular proliferation. In the normal oesophagus locally released PGE_2 may promote cellular restitution following sloughing of cells by food. This localised damage and PGE_2 may stimulate COX 2 to provide more PGE_2 to orchestrate this restitution. As the substrate for the COX 2 is used up then this positive feedback mechanism breaks down. However, as a maize diet may damage the oesophagus, as stated previously by sharp ground maize (Rose, 1979) and provide unlimited arachidonic acid (derivative of linoleic acid in maize) for PGE_2 production, then this positive feedback mechanism could last for much longer periods. Also, the regurgitation of stomach fluids provides increased cellular damage, PGE_2 and arachidonic acid content, further pushing this local proliferative drive.

Chapter 6 shows that the Oe21 cell line growth response is not as high following stimulation as that found with the Het1a Cell line. This could be for reasons previously discussed (section 6.4). This includes the concept that mutated proteins with in the cell cycle could amplify the receptor stimulus signal causing a slowing of cell growth through regulatory processes, producing normally distributed

concentration of growth curves. These results show the PGE_2 concentrations for the Oe21 cell line seem to be too high for increased expression, as the increasing concentrations of PGE_2 treatment seems to inhibit the expression of COX 2 (Figure 7.22). The cells that did not receive any PGE_2 treatment seem to express COX 2 after one, three and 72 hours. The cells that were treated with PGE_2 gradually have decreased expression as concentration increases. After one and three hours following 0.0005 μg/ml PGE_2 treatment the expression response was smaller, than one and three hours after 0.05 and 5 μg/ml PGE_2 treatment.

The RT-PCR band Figures 7.23, 7.24, 7.26 and 7.27 suggest that there does seem to be a small amount of COX 2 expressed following the addition of serum free media between the one and 72 hours. This suggests that the serum has an effect on the expression of COX 2 when these results are compared to the previous serum treated cells. The 0.0005 μg/ml PGE_2 treatment showed a positive band after one hour of treatment, but not after any other time period. The 0.5 μg/ml PGE_2 showed expression over one and three hours and the 5 μg/ml PGE_2 showed strong expression after one and three hours, but also faint expression after 24, 48 and 72 hours. It appears that the cell proliferation assays (Chapter 6) and the serum treated cells showed a normal distribution in relation to PGE_2 concentration and growth response, the expression studies have also shown this positive PGE_2 concentration and response relationship. This provides strong evidence for the argument stated previously that these cells respond via a positive feedback mechanism, where increased PGE_2 concentration increases COX 2 expression, which with the right local substrate concentrations would lead to further PGE_2 concentration. Therefore, the increasing oesophageal cellular proliferation caused by the PGE_2 treatment stated in Chapter 6, supports a case of pre-disposition to cancer. The Oe21 cell line shows similar results without such a positive relationship, which may be due to amplified stimulus response. This amplified response may cause the cell to shut down with large concentrations of stimulus. This may suggest that only very small amounts of stimulus provide a larger response, as previously discussed (section 6.4). The 0, 0.0005 and 0.05 μg/ml PGE_2 treated cells are expressing COX 2 after 48 and 72 hours and the 5 μg/ml treated cells are expressing the COX 2 gene after just 24 hours as well as the 48 and 72 hours - a longer period of time, but this does not indicate whether more of the COX 2 enzyme

is being produced. Using COX 2 protein measurements and further COX 2 expressions methods would validate whether the PGE_2 has a positive feedback mechanism increasing COX 2 expression.

Overall, there is no strong evidence to suggest that PGE_2 has an effect on the expression levels of EGFR and VEGF, but in light of recent research (Pai *et al.*, 2002; Buchanan *et al.*, 2003) it would be more productive to look at the activation of these growth factors . In the Het1a cell line it has been shown that PGE_2 increases the expression intensity and time of COX 2, which could suggest a positive feedback mechanism, which, with increased local substrate, could be a cause of hyper-proliferation in the oesophagus.

Summary of results supporting suggested theory:

- Further investigation for HGF would be required, although the results would suggest that HGF is not present in the cell lines; therefore this does not provide a good model for this area of research.
- The expression of VEGF and EGFR did not appear to change following PGE_2 treatment and remained constant. This does not support or disprove the theory. Further work needs to be carried out on the activation rather than the increased expression of, growth factor, as highlighted in recently released papers.
- PGE_2 treatment in the "normal cell line" increases COX 2 expression over a longer period of time compared to the control.
- In a "normal" population the oesophagus may release PGE_2 due to sloughing of cells by food, to promote localised growth. This damage and PGE_2 may stimulate COX 2 faster to provide more PGE_2 to orchestrate restitution. As the substrate for the COX 2 is used up, then this positive feedback system breaks down. In the target population the maize diet reflux may provide a much greater amount of PGE_2 and arachidonic acid, further pushing local cellular proliferative drive. This could be exacerbated by the sharp ground maize ingested, as previously documented (Rose, 1969).

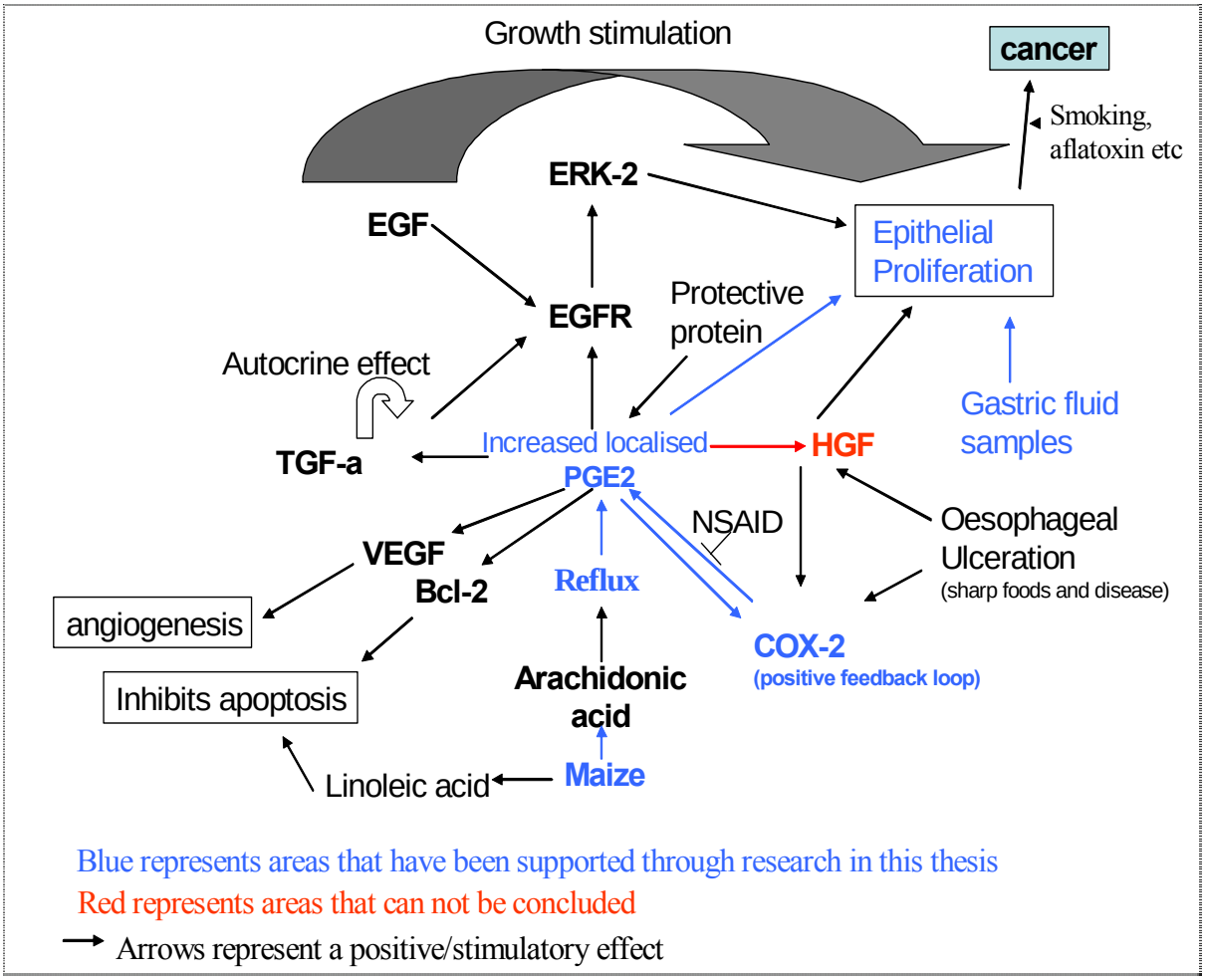


Figure 7.29:The Sammon Pink theory and how the research to this point supports it.

8. Validation of the COX 2 Positive Feed Back Loop

8.1. Introduction

It was established in the previous chapter that PGE_2 could have a stimulatory effect on the expression of COX 2 in the oesophagus. Increases in COX 2 levels in the presence of the right substrate could further produce PGE_2. This would form a positive feedback loop. It has been shown in Chapter 6 that PGE_2 promotes oesophageal cellular proliferation at certain concentrations. Arachidonic acid, the precursor of PGE_2, has been shown to be high in the maize diet ingested by the target population. It is suggested that PGE_2 relaxes the oesophageal-gastric sphincter promoting regurgitation (known as 'cleansing' in Transkei), as witnessed in the target population (Chapter 2). This causes the stomach contents to flow back up into the oesophagus. This thesis therefore presents the novel theory that natural oesophageal restitution caused by food ingestion and gastric reflux would lead to localised PGE_2 and arachidonic acid (derivative of linoleic acid of maize), respectively, in the oesophagus. Should PGE_2 stimulate COX 2 production, then this along with arachidonic acid, would produce more PGE2, hence, providing a positive feedback loop. PGE_2 has a short half life *in-situ* which may work as a sensitive control mechanism, forming a positive feedback loop only when the required substrate is available. This could be due to PGE_2 having huge energy and resource expenditures for the body, such as cellular proliferation in restitution. The high concentrations of arachidonic acid in the reflux of the gastric content could provide this substrate driving this feedback loop.

As much of this research has been investigated on a model in this thesis, it is important to be sure that PGE_2 is stimulating COX 2 expression. In the previous chapter strong evidence for this was provided by using the reverse transcriptase polymerise chain reaction (RT-PCR). This is a fundamental method for analysing gene expression, although testing with another method would validate this work. Therefore a Northern blotting technique and quantitative nucleotide ELISA assay was

also used. As RNA is only stable for short periods of time, to achieve quantitative results new cells were PGE_2 treated. The RT-PCR was repeated to check that it matched previous results and the new methods were carried out. To show that this expression related to COX 2 production, the COX 2 protein levels were recorded for the various PGE_2 concentration treatments on the cells using an ELISA assay.

8.2. Methods

8.2.1. Cellular Model Used for COX 2 Expression Studies

As described in section 7.2.3, Het1a and Oe21 cells were grown in T75 flasks with media containing different concentrations of PGE_2 (A = 0, B = 0.0005μg/ml, C = 0.05 μg/ml and D = 5 μg/ml) over different periods of time (1 = 1 hour, 2 = 3 hours, 3 = 24 hours, 4 = 48 hours and 5 = 72 hours). The RNA and protein was extracted for COX 2 investigation.

8.2.2. COX 2 Expression Studies using RT-PCR

The methods stated in section 7.2.3 were repeated by extracting RNA (RNeasy, Qiagen) from PGE_2 treated cells. The cDNA was created by a reverse transcriptase reaction (Invitrogen Cloned AMV – first strand synthesis kit) from the RNA and the COX 2 expression levels were then investigated using the RT-PCR method.

8.2.3. COX 2 Expression Studies Using an ELISA Assay

The Quantikine colourimetric mRNA quantitation kit (R&D Systems, Abingdon, UK) was used for the detection of COX 2 expression in samples from Het1a and Oe21 cells following different PGE_2 concentration treatments over different periods of time (same method as section 8.2.1), to validate the results from the RT-PCR method. Unless stated the component composition was not disclosed by the manufacturer. Total RNA was extracted using the Qiagen RNeasy method, quantified and diluted so that all of the samples each have 50μg/ml total RNA. From these samples, 20μl was diluted with 280μl sample dilutent (composition unknown). A RNA calibrator

containing concentrations of cloned COX 2 cDNA was prepared using 800, 400, 200, 100, 50, 25, 12.5, 6.2 and 0 aMol/mL, to produce a concentration curve to compare the samples against.

The polystyrene 96 well microplate was washed with wash buffer (composition unknown). In to each well, 50µl of the COX 2 specific biotin-labelled capture oligonucleotide probe and 150µl of prepared sample/calibrator sample were hybridised at 65^{o}C for one hour. A streptavidin coated plate was washed with wash buffer (composition unknown) and 150µl of hybridization mixture was added and incubated at room temperature for one hour on a shaker (500 rpm), binding to the plate. This was washed four times and 200µl of polyclonal antibody against digoxigenin conjugated to alkaline phosphatase was added to each well and placed on a shaker at room temperature for one hour. This plate was emptied and washed six times and 50µl of lyophilized nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) substrate was added and incubated on a shaker for one hour at room temperature. To this 50µl of amplifier solution was added and incubated for 30 minutes followed by 50µl of stop solution. This was read spectrophotometrically on a microplate reader (MRX, Dynex technologies, Worthing UK) at (490nm). Figure 8.1 shows a schematic diagram of how the ELISA is built up on the plate.

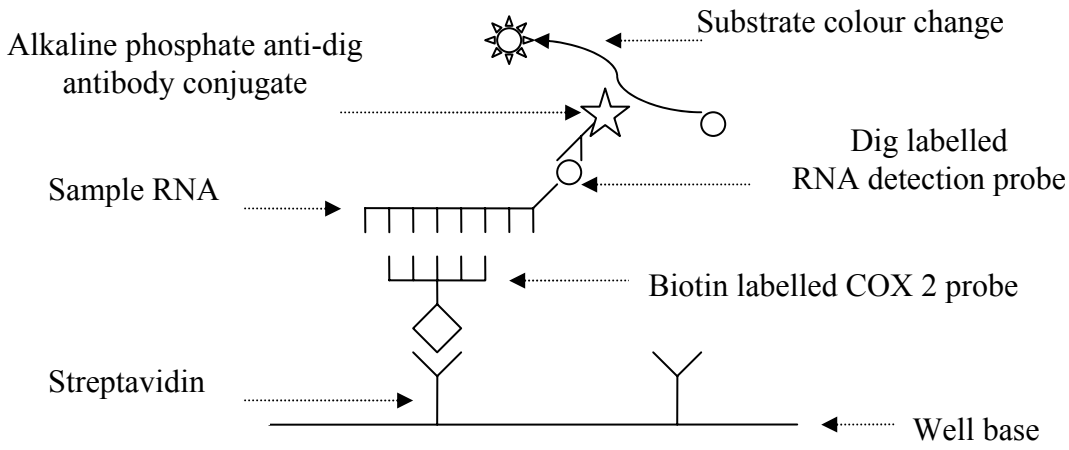


Figure 8.1: A schematic diagram of how the ELISA was constructed to produce a colourimetric change proportional to the RNA concentration.

The results obtained from this assay were compared against the results gained by the COX 2 RT-PCR electrophoresis results.

8.2.4. COX 2 Expression Studies Using the Northern Dot Blot Technique

To validate that COX 2 expression has changed by increases in PGE_2 concentration in oesophageal cell lines a Northern dot blot technique (also known as RNA dot blot) was carried out on the same samples as stated above. Due to the lack of positive results when using the full Northern blot technique of formaldehyde electrophoresis a dot blot technique was adapted from Maniatis (1982). This method gave a positive result for beta-actin, but failed to give a result with COX 2. The optimisation methods used include different probes (PCR primers and RT-PCR products), different sample cell types previously shown to express COX 2 (SKMES, RT112 and OE33), different nucleotide cross linking techniques (heat and UV) and wash stringency tests. For optimum results with the dot blot assay the mRNA was substituted for cDNA due to the increased hybridisation strength of Sample DNA-DNA probe, previously stated as binding 25% more than RNA-DNA (Sagesser *et al.,* 1997).

Before any experimentation took place all the equipment was treated with RNase-ZAP (Ambion) and all solutions were made up using DEPC treated H_20. The cDNA samples were prepared as stated in section 8.2.2. Of each sample, 5.4µl was mixed with 5.4µl 6M glyoxal (all chemicals from Sigma), 16µl DMSO and 3µl 0.1M sodium phosphate (pH 7). These were incubated at 50^{o}C for one hour and then chilled on ice. To a Hybond H+ membrane (Amersham), 2µl of prepared sample was applied without touching the surface, and allowed to dry. The sample nucleotides were cross linked to the membrane by baking the membrane at 80^{o}C for two hours. To remove the glyoxal the membrane was soaked in 20mM Tris-HCl for five minutes. Hybridisation buffer was prepared using 6 x SSC, 2 x Denhardt's reagent, 0.1% SDS w/v, 0.5M NaCl and 5% w/v blocking reagent (Amersham) and heated to 42^{o}C. The membrane and 4ml of hybridisation buffer were incubated for one hour in a revolving hybridisation chamber at 42^{o}C. The COX 2 probe was prepared by using the PCR product from a RT-PCR reaction using DIG labelled dUTP (Roche). Added to the hybridisation buffer was 20µl DIG labelled COX 2 probe at 10ng/ml and denatured

for 5 minutes at 95°C. This was hybridised for 16 hours at 42°C. The membrane was washed with primary wash buffer (appendix L) for 20 minutes at 42°C. The membrane was then washed with antibody wash (containing 0.1M maleic acid, 0.15M NaCl, NaOH to pH 7.5 and 0.3% v/v tween), for five minutes at room temperature. The membrane was blocked with the above wash solution containing 0.5% w/v hybridisation block for one hour at room temperature. The membrane was incubated with 1:200 horseradish peroxidase labelled anti-DIG antibody (Roche) in the above block solution for one hour at 37°C. The unbound components were washed away with antibody wash, twice, for 15 minutes each. The membrane was incubated with Enzymic ChemoLuminescence (ECL) detection solution for 1 minute, placed in cling-film. This was placed in a film cassette with x-ray photography paper (Sigma) using a red safety light and left for one hour at room temperature. The film was placed in developer for 20 to 60 seconds until the small bands appeared on the film. This was washed in tap water and then placed in fixer for 5 minutes. If the specific protein was present then small bands will appear darker on the opaque film. A schematic diagram of this process is represented in Figure 8.2.

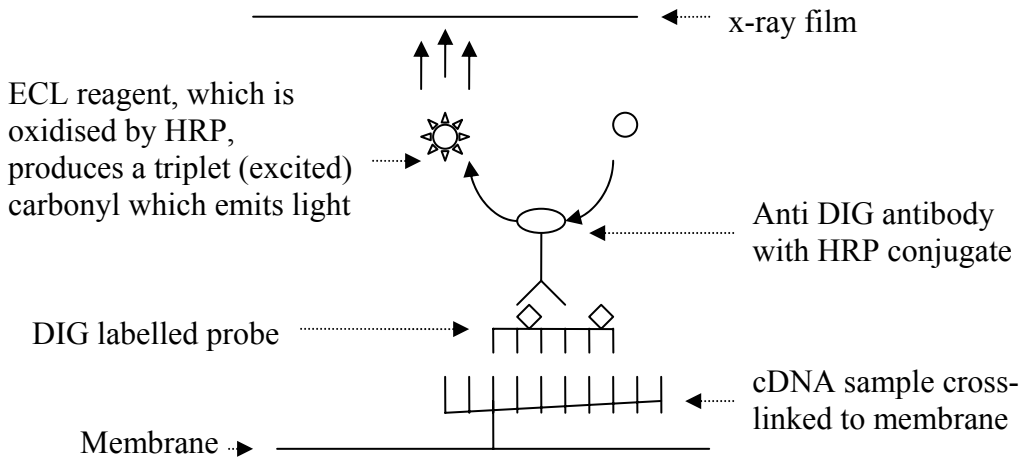
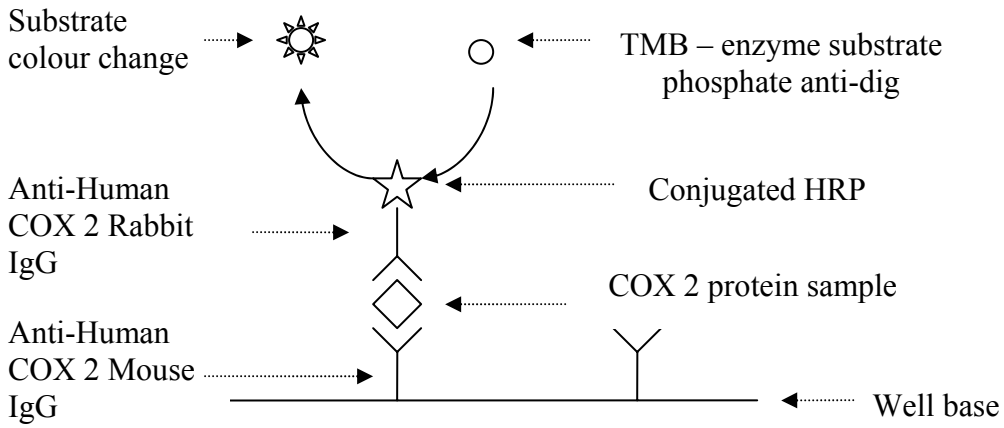


Figure 8.2: A schematic diagram of how the Northern dot blot assay was constructed to produce a visual change proportional to the RNA concentration.

8.2.5. COX 2 Protein Quantification Studies Using an ELISA Assay.

The additional expression assays described above were used to validate previously carried out work (section 7.3.5) that PGE_2 increases COX 2 expression, this was extended by investigating COX 2 protein concentrations. Section 7.2.1.a explains how the protein samples were extracted from cells treated with PGE_2 over different periods of time. As this is a commercial assay from IBL (Gunma, Japan) some of the concentrations or components are not stated.

A polystyrene 96 well microplate, pre-treated with Anti-Human COX 2 Mouse monoclonal antibody, was filled with 100µl EIA buffer (1% BSA, 0.05% tween 20 in PBS). The plate was incubated for one hour at 37^{o}C following the addition of 100µl of sample or standard (68.75, 34.38, 17.19, 8.59, 4.3, 2.15 and 0 ng/ml). Each well was washed seven times with 0.05% tween 20 in phosphate buffer. To each well 100µl of HRP conjugated anti-Human COX 2 rabbit polyclonal antibody was added and incubated for 30 minutes at 4^{o}C. The wells were washed nine times with 0.05% tween 20 in phosphate buffer. To each well enzyme substrate tetra methyl benzidine was added and this was incubated at room temperature for 30 minutes. This gave a colour change which was proportional to COX 2 protein concentration. A stop solution (1M H_2SO_4) was added. This was read spectrophotometrically on a microplate reader (450nm). A schematic diagram of this assay is represented in Figure 8.3.



8.3: A schematic diagram of how the COX 2 protein ELISA assay was constructed to produce a colourimetric change proportional to the COX 2 protein concentration.

8.3. Results

8.3.1. COX 2 Expression Results Using RT-PCR and RNA ELISA Assay

The results for the quantitative RNA expression ELISA and RT-PCR results, both taken using the same samples, have been integrated below to make it easier to compare them both.

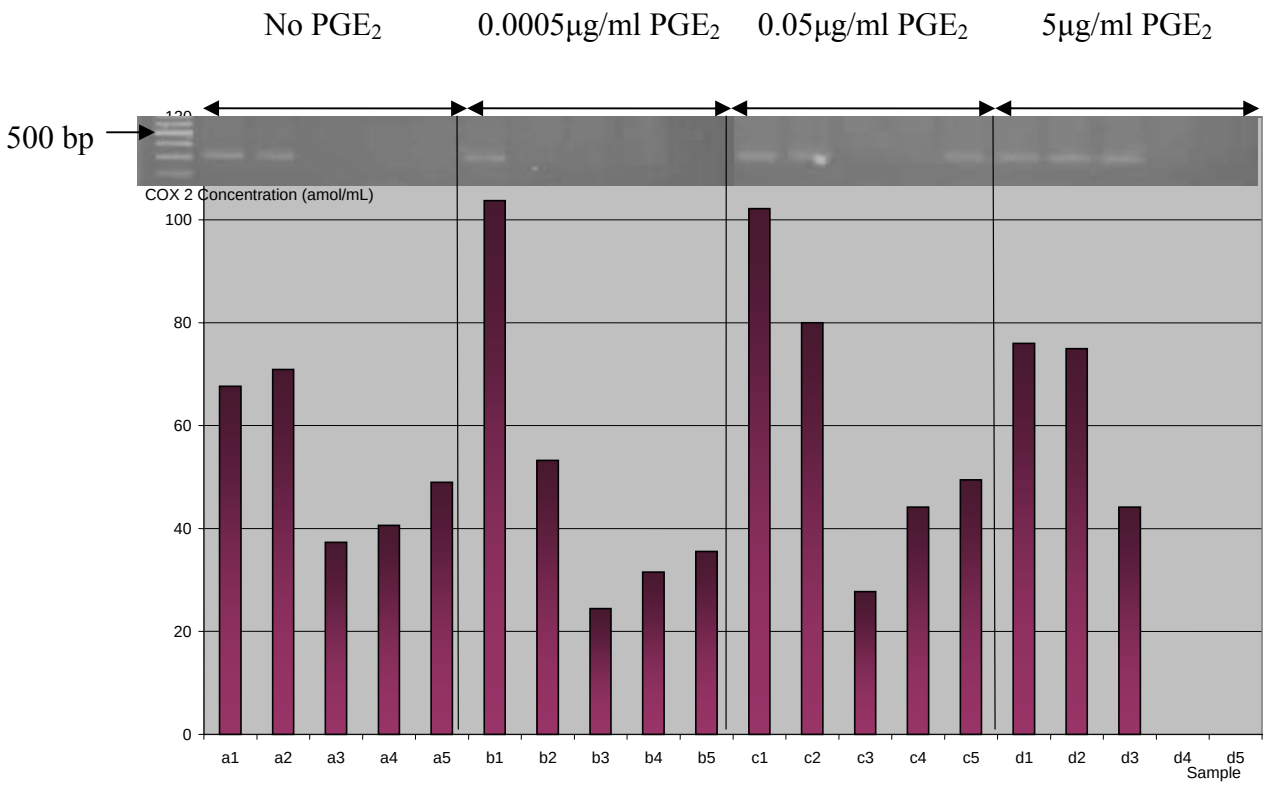


Figure 8.4: The graph shows a mean of two sets of ELISA COX 2 expression concentration readings from RNA samples extracted from Het1a cells treated with different concentrations of PGE_2 over different time periods. These are given by using a standard concentration curve. Above this graph the matching PGE_2 treatment RT-PCR result is shown using the same RNA samples. The sample labels letter refers to PGE_2 treatment concentration (A = 0, B = 0.0005µg/ml, C = 0.05 µg/ml and D = 5 µg/ml) and the number; different periods of treatment time (1 = 1 hour, 2 = 3 hours, 3 = 24 hours, 4 = 48 hours and 5 = 72 hours).

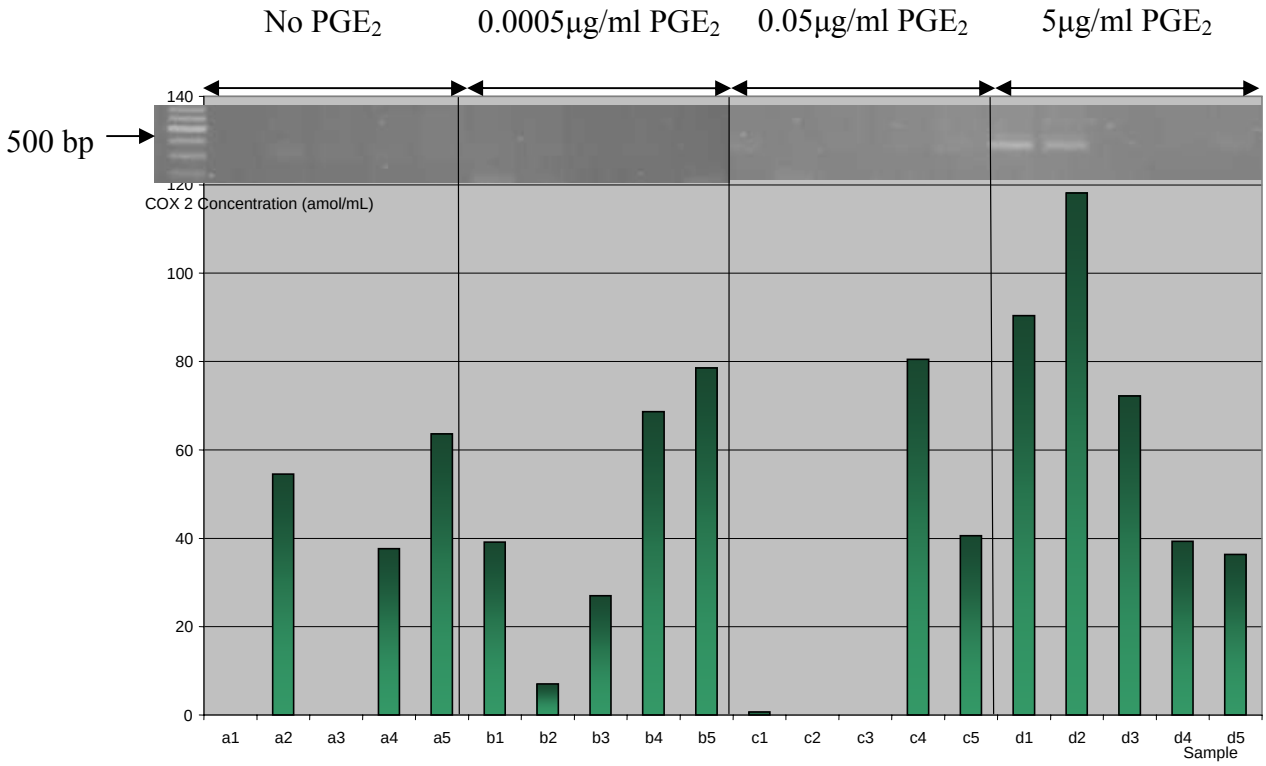


Figure 8.5: The graph shows a mean of two sets of ELISA COX 2 expression concentration readings from RNA samples extracted from Oe21 cells treated with different concentrations of PGE_2 over different time periods. These are given by using a standard concentration curve. Above this graph the matching PGE_2 treatment RT-PCR result is shown using the same RNA samples. The sample labels letter refers to PGE_2 treatment concentration (A = 0, B = 0.0005µg/ml, C = 0.05 µg/ml and D = 5 µg/ml) and the number; different periods of treatment time (1 = 1 hour, 2 = 3 hours, 3 = 24 hours, 4 = 48 hours and 5 = 72 hours).

It is clear from Figures 8.4 and 8.5 that the RT-PCR results show similar results to the RNA ELISA for both cell lines. These can be used to validate the results from the previous chapter showing a PGE_2 increase compared to the time period of expression. Shown in Figure 8.4 the lower PGE_2 concentrations for 0.005 and 0.05µg/ml gave a much higher expression result from the ELISA assay compared to no PGE_2 treatment. Shown in Figure 8.5 for the Oe21 cell line, the expression has increased at 5µg/ml PGE_2 treatment compared to no treatment.

8.3.2. COX 2 Expression Results Using the Northern Dot Blot Technique

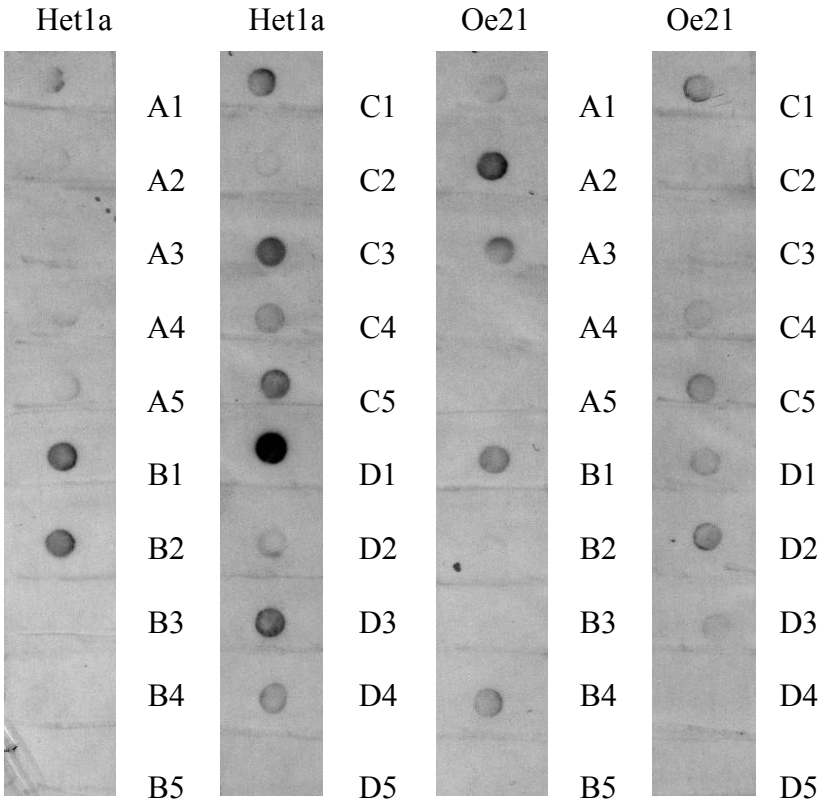


Figure 8.6: Typical Northern dot blot results gained from cDNA of RNA samples extracted from Het1a and Oe21 cells treated with different concentrations of PGE_2 over different time periods. A = No PGE_2 treatment, B = 0.0005µg/ml PGE_2, C = 0.05µg/ml PGE_2 and D=5µg/ml PGE_2. 1 = 1 hour treatment, 2 = 3 hours treatment, 3 = 24 hours treatment, 4 = 48 hours treatment and 5 = 72 hours treatment.

The Northern dot blot results showed similar results to that of the two other techniques, therefore validating the previous theory that increases in PGE_2 causes COX 2 expression over longer periods of time. Figure 8.6 show similar intensities to that of the peaks on Figures 8.4 and 8.5. This also shows the same high values on the graph generally correspond to more intense spots on the dot blot, therefore, increases

of COX 2 expression over longer periods of time for increased PGE_2. The results for Oe21 in Figure 8.6 also show this increase in COX 2 expression over longer periods of time compared to lower concentrations of PGE_2, as seen in Figure 8.5.

Therefore these results validate the theory that increased PGE_2 concentration in oesophageal tissue causes increases of COX 2 expression for longer periods of time.

8.3.3. COX 2 Protein Quantification Studies Using an ELISA Assay.

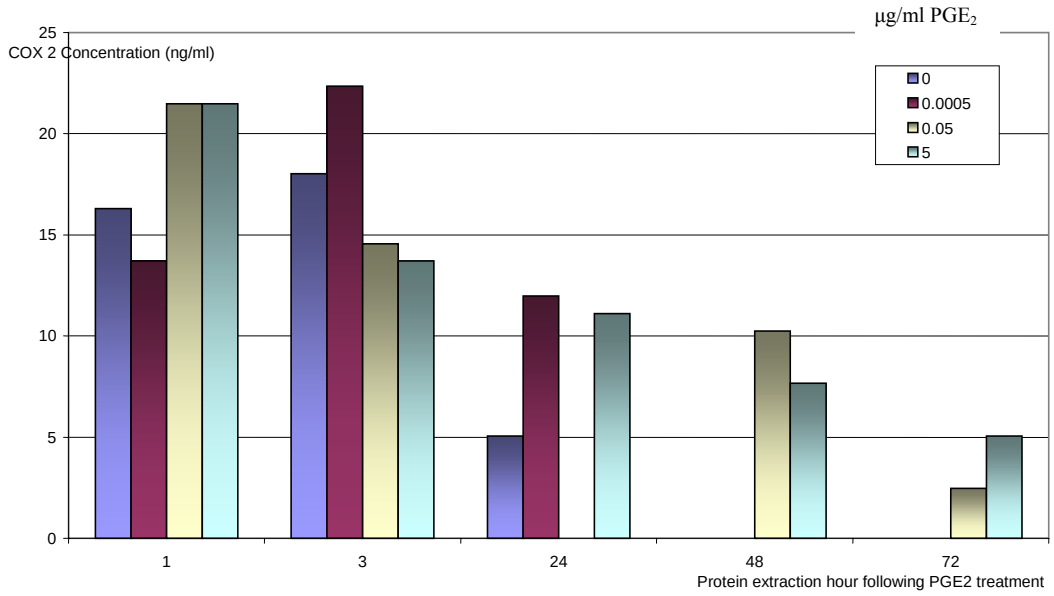


Figure 8.7: The mean of two sets of ELISA COX 2 protein concentration readings from protein samples extracted from Het1a cells treated with different concentrations of PGE_2 over different time periods.

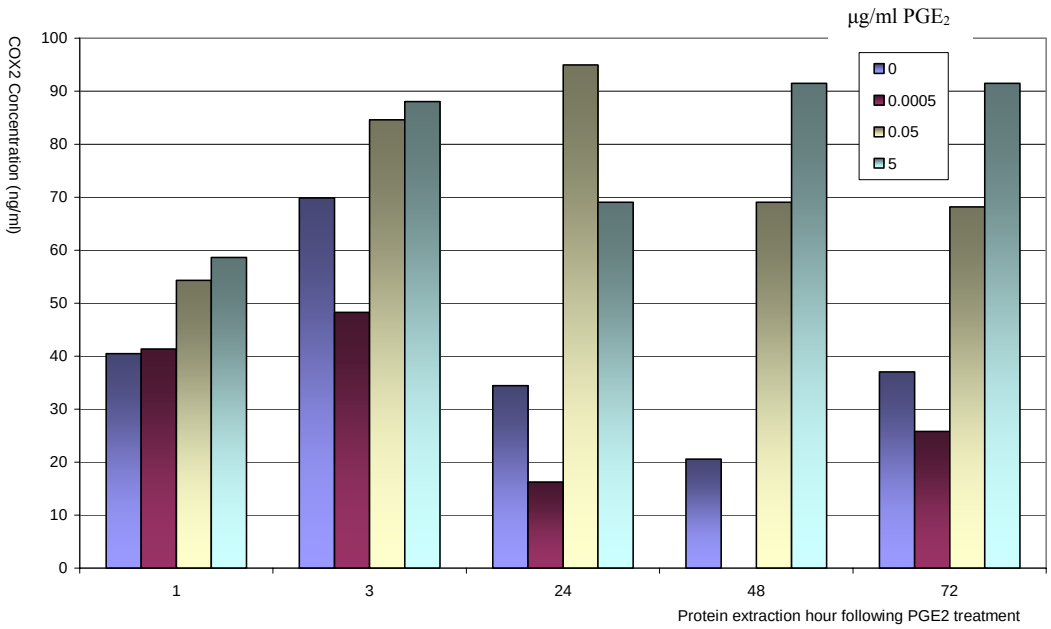


Figure 8.8: The mean of two sets of ELISA COX 2 protein concentration readings from protein samples extracted from Oe21 cells treated with different concentrations of PGE_2 over different time periods.

Shown in Figures 8.7 and 8.8 the two higher PGE_2 concentration treatments of 0.5 and 5 µg/ml, in a majority of cases, show COX 2 protein to be higher compared to no PGE_2 treatment. In Het1a, the COX 2 concentration reduced over time for all results, although the COX 2 remained at similar levels over the time period for the Oe21 cell line treated with the two highest PGE_2 concentrations.

8.4. Discussion

The different methods in this chapter were all carried out with the same aim: To test the theory that increased PGE_2 stimulates the expression of COX 2 in oesophageal cell lines. This is to provide evidence that PGE_2 increases in oesophageal cancer resulting from a high maize diet caused by gastric reflux and cell restitution, this would promote COX 2 expression. This along with the high PGE_2 substrate (arachidonic acid, high in maize) would provide a positive feedback loop causing

further PGE_2 concentration increases. It has been shown in Chapter 6 that increases in PGE_2 cause increases in cellular proliferation, thus predisposing the tissues to cancer. The results above would suggest that this is the case. The different methods used to measure COX 2 expression all support the suggestion that cellular COX 2 levels would also increase in the presence of increased PGE_2 over various periods of time. This is also supported by the increased COX 2 concentration following PGE_2 treatment shown by using the COX 2 protein ELISA assay.

The RNA quantitative ELISA results from Figures 8.4 and 8.5 appear to have broadly similar results to the RT-PCR electrophoresis results. When the ELISA results reach a required COX 2 concentration, around 55 amol/ml for Het1a and 90 amol/ml for Oe21 cell line, a band is seen from RT-PCR. This sensitivity difference could suggest two fundamental differences between the two methods. The RT-PCR method only provides a semi-quantitative method, were the ELISA is a direct quantitative method. This is because the RT-PCR requires a reverse transcriptase method to produce cDNA from RNA, adding error to the sample. The RT-PCR requires an optimisation process (determining the PCR cycle number that produces the greatest differential in the brightness of the RT-PCR electrophoresis result), therefore several steps where it can be limited and error occur. This would be overcome by use of a quantitative technique such as Taq Man which was unavailable for this study. The ELISA uses a direct binding mechanism (Figure 8.1). Therefore the validation shown in this chapter was required to be confident of the PGE_2 affect conclusion summarised below.

When treated with PGE_2, the Het1a cells (Figure 8.4) show an increase in the amount of expression over the first three hours. After one hour of 0.0005 and 0.05 μg/ml PGE_2 treatment there was a large increase in expression compared to one hour of untreated cells. This increase in COX 2 expression is also shown with the Oe21 cell line. After the maximum PGE_2 treatment in the Oe21 cells the COX 2 expression is around double that of the cells not treated with PGE_2 over 24 hours. These ELISA results show that this quantitative increase is taking place over the first few hours of PGE_2 stimulation compared to no PGE_2 treatment. As this expression is increased with PGE_2 concentration then any secondary promoters within the long chain of numerous cell communication proteins may also be stimulated and activated proportionally to PGE_2 concentration. The orchestrated effect of cell surface receptors

may have a fundamental effect on many common pathways. As this increase in PGE_2 has both shown to increase cellular stimulation (section 6.4) and promote the expression of the COX 2 protein then many secondary proteins may have been stimulated to reach this expression promotion in the nucleolus (for an example see Figure 7.28). It can be suggested that the proliferative effect of PGE_2 could be caused by an interaction with these secondary proteins, as suggested in the discussion of chapter 7 referring to growth factor path stimulation. Hence, mutations in these proteins could have an exacerbated effect on this intracellular stimulation. Environmental factors found in the sample population, such as smoking, fungus on maize and disease, that also have an effect on these same pathways may work in synergy with the PGE_2 growth pathway promotion making the people of Transkei at much higher risk of uncontrolled cell growth, hence cancer. This exacerbated effect on the molecular growth pathways would explain why this target population has a much higher oesophageal rate than other countries that have similar environmental factors i.e. Central Africa. These results support the view that there would be a huge benefit of carrying out future work on the affect PGE_2 has on the secondary proteins of the growth factor pathways.

The Northern dot blot results show COX 2 expression to increase over longer periods following PGE_2 treatment compared to no PGE_2 treatment (Figure 8.6). The results appear to be different from the RT-PCR and ELISA methods, although this uses a very different method. This assay uses cDNA as the sample, similar to the RT-PCR but a direct probe for the detection of the COX 2 gene, similar to the ELISA.

Further validation using a real time PCR was not available. However, these three methods have been judged to provide enough evidence to strongly suggest that PGE_2 has a stimulatory effect on the expression of COX 2 in the oesophageal cell lines.

The ELISA assay for detection of COX 2 protein has supported the theory that PGE_2 stimulates the expression of COX 2. The analysis of protein shows the gene has been transcribed. The protein being analysed in this case is COX 2, therefore recording the COX 2 protein levels are essential to validate this work. As explained in the results the higher PGE_2 concentration treatments promote greater COX 2 production over longer periods of time compared to no PGE_2 treatment (Figure 8.7 and 8.8). This is

the same for both cell lines. The Het1a results, shown in Figure 8.7, decrease with time, where the Oe21 results, shown in Figure 8.8, remain comparatively high with time. This provides an indication that the 'normal' Het1a cells may have an intact protective pathway inhibiting the production of COX 2, should the production not normally be necessary. For the 'carcinoma' Oe21 cells this feedback system may not be in place allowing the cells to manufacture COX 2 under a tightly controlled external receptor stimulatory system. There is a difference in the internal growth pathways caused by the carcinogenesis. It was questioned in a previous chapter that these cell lines could not be compared against each other as changes in their growth characteristics were unknown. These results may support the concept that these cell lines present two different characteristics fundamental to a normal versus carcinoma model; the normal cells control protein growth and the carcinoma cells do not in this case. Although this could not be stated without further validation using tissue and animal studies.

Therefore the different assays used in this model have provided evidence that strongly supports the conclusion that PGE_2 has a stimulatory effect on the expression of COX 2 and its production in oesophageal tissue. Further work would be required to confirm this and analyse how PGE_2 achieves this. It is predicted that PGE_2 binds to an extra cellular receptor on the membrane, which in turn leads to a complex orchestrated cellular growth pathway producing by products such as COX 2. Careful intervention with this pathway could provide treatment for this population, although they could not afford treatment therefore a change of diet would provide a cheaper alternative. The study of this mechanism could provide information on the unknown drug action of treatments such as paracetamol and aspirin.

9. Conclusion

Cox 2 has become a huge area of research; since this work began, the papers documented on Pubmed linking Cyclooxygenase to cancer have more than doubled, increasing by around 25% every year. This is likely to be driven by the commercial interest in COX 2 inhibitors as cancer suppressors. This thesis supports this research at a time when the importance of COX 2 in cancer is being realised and can add valuable insights into cancer research overall. Further developments of this field are likely in the future. Following the presentation of this research at the American Association of Cancer Research (AACR), a paper is being submitted at the request of the editor of the 'Nutrition and Cancer' journal (Lawrence Erlbaum Associates Publishing).

Much of the research in the literature review (Chapter 1) has stated the importance of COX 2 in the development of oesophageal cancer, mainly due to the increased use of knock out/down methods. From this exciting work it can be suggested that PGE_2 has a particular effect due to the knowledge that COX 2 helps to produce PGE_2. Therefore this current study postulates that PGE_2 is the important factor increasing oesophageal cell proliferation, represented here by the use of an *in-vitro* cell line model. This investigation is novel in the context of showing PGE_2 to have a mitogenic effect on oesophageal cell lines, not previously shown, and that PGE_2 itself may have an effect on its own production via a positive feedback system (chapters 7 and 8). Growth patterns observed in these results have supported recent studies in that the PGE_2, shown in this thesis to be high in gastric fluid of the sample population caused by a high maize diet (chapter 6), may be activating growth factor pathways downstream of the normally stimulated receptors, having a pre-disposing carcinogenic effect. The PGE_2 may be lasting in the oesophageal lumen for much longer periods of time protected by transporter proteins. This, constitutes molecular evidence with which to expand the present Sammon theory (section 1.9).

9.1. The Mitogenic Effect of a High Maize Diet

The measurement of the PGE_2 concentrations in the gastric fluid samples of the people from the target population, was predicted to be higher than for people that ingest a "western diet", using related pieces of research (Figure 5.3). Results in Chapters 5 and 6 support the theory that the high maize diet consumed by the target population increases PGE_2 content in the stomach. This can suppress the smooth muscle of the gastric sphincter allowing gastric reflux into the oesophagus (section 1.9.3). Gastric reflux has been documented in about 60% of the target population oesophagus (Sammon, 1994).

The novel results of Chapter 6 show that in squamous cells, PGE_2 has a proliferative effect, pre-disposing the tissue to oesophageal cancer. From this model it can be deduced that the regurgitation of gastric fluid, exacerbated by localised PGE_2 released for natural restitution caused by sharp maize damage, would increase PGE_2 to high concentrations in the oesophageal lumen. These studies show that, at a high enough concentration, this would drive a localised proliferative effect. This was supported by increased cellular proliferation of oesophageal cells following gastric fluid treatment, suggesting that the gastric fluid from the sample population promotes oesophageal cell hyperplasia due to its high concentration of PGE_2.

This concurs with present research and the Sammon theory. Sammon suggests that changes in oesophageal pH levels, due to this alkali regurgitation of gastric fluid suppresses the natural breakdown of growth factors by inhibiting the enzymes that cleave them, hence allowing growth factor stimulation to remain. Due to the short half life of growth factors this may not likely to be the case, but increases in local PGE_2 could have a direct proliferative effect on the squamous tissue. Therefore, the increased PGE_2 levels have a direct influence on local squamous growth stimulation, perhaps through the intracellular manipulation of these growth factors.

9.2. The Mitogenic Effect of Maize at a Molecular Level

Chapter 5 disscussed a study investigating the possible detrimental effects of the three day transportation of the samples. This showed the expected results of a drop in PGE_2 levels in the samples after transportation. Additionally a very interesting pattern was revealed. As explained in section 5.4 the PGE_2 in the gastric fluid after the journey, and the PGE_2 levels after the simulated journey showed a similar drop to a specific concentration in all the samples. It was suggested that these remaining PGE_2 proteins, not degraded by the journey, appear more stable, perhaps bound to their transporter proteins (MRP4 and ABCC4), similar to that found with the related Prostaglandin I_2 stabilised by Apolipoprotein (Aoyama *et al.* 1990). This may mean that not only could the increased localised PGE_2 have a stimulatory effect on the cells, but due to increased stability the PGE_2 would remain in the oesophagus for longer periods of time. Investigating this molecular interaction further would provide an insight into how a protected epithelium, like the mucus lined oesophagus, may be stimulated to an extent that could lead to cancer by short half-life unstable proteins.

The mitogenic effect PGE_2 on the oesophageal cell lines was optimal at a certain concentration, showing similar patterns to that found when treating cells with a growth factor, suggested as the PGE_2 proliferative action. Therefore, it could be postulated that the PGE_2 treatment may have a stimulatory effect on the growth factors directly, as suggested in recent papers (Pai *et al,* 2002; Buchanan *et al.,* 2003). After discussing the results in Chapter 7, it became evident that it would be more significant to investigate the activation of growth factors and their receptors, for example, by looking at decreases in Rb phosphorylation (King, 2000), early response genes (Almendral *et al.,* 1988) or changes in cyclins (Takeno *et al*., 2002). Since the start of this project it has been discovered that breast cancer progression can be inhibited through blocking COX 2, itself blocking vascularisation caused by VEGF (Saji, 2004). The VEGF and EGFR investigated in chapter 7 are two very important proteins that are implicated in oesophageal cancer. There has been growing interest in these proteins from the larger drug companies such as Astra Zeneca and Roche, In February 2004 the FDA approved the use of Avastin (Genentech Inc) for colon cancer patients which it thought to work by reducing tumour stimulated angiogenesis by inhibiting VEGF. This is marketed in the UK by Roche and is called Bevacizumab

and was given NICE appraisal in January 2007. Astra Zeneca have recently invested around 500 million dollars in buying Cambridge Antibody Technologies developing growth factor binding human specific antibodies for cancer therapy. This was achieved through phage library technology that would be ideal for mapping growth factor downstream protein interactions in this study, but is hugely expensive.

PGE_2 may naturally up-regulate EGFR and VEGF in normal tissues, as PGE_2 is implicated in foetal development, and angiogenesis and epithelial proliferation are both required for tissue growth. Therefore, this shows the importance of pursuing research in this field.

Throughout the development of the aims, the thesis concentrated on the PGE_2 effect on growth factor expression rather than intracellular activation of these growth factor pathways. The concept of PGE_2 having an effect on the activation of the growth factor downstream components was only realised following a talk on PTEN tumour suppressor gene and its unrelated interactions with other proteins, as well a recent discussion of this work following its presentation. This led to the late discovery of Pai *et al's.* (2002) and Buchanan *et al.*'s (2003) work on PGE_2 activation of growth factors in other tissues. The study of molecular and cellular biology has dramatically changed over the years the last few years. The groups at the forefront of cellular biology are studying how a protein affects many other cellular pathways at the same time; this is through software such as cPath, pathBLAST, Pathway Tools and microarray technology. Although the technology is expensive, this would greatly progress this research.

9.3. Is this the Right Model?

There are fundamental questions to be asked about the model used in this thesis. It is not that this was the wrong model, but issues have to be considered. This model is fundamental in cancer research. It is relatively cheap and represents the molecular pathology only second to carrying out animal studies.

This model only allows the study of a single cell population, although the suggested methods of carcinogenesis caused by increased maize ingestion would involve many cells interacting with each other. Therefore this model is limited in representing the proliferative interactions seen with in the human body. This model has its limitations but also allows single cell understanding without further variables affecting the stimulation of cells by paracrine factors. It is fundamental to take one step at a time with these complicated orchestrated cellular interactions. Once these are established then extra cell lines and animal models can be pursued as the next stage in model development.

The study of growth factors outlined in Chapter 7 showed difficulties with using this model. Only after the observation of constant VEGF and EGFR expression following serum treatment in media, did it become apparent that the foetal calf serum may have a stimulatory effect on the growth factor expression rates. This presents a problem for designing a significant model that examines the measurement of expression just caused by PGE_2 itself. As the cells require serum to stay viable, by removing the serum moments before RNA extraction for an expression measurement, there would still be no certainty that the serum was not having a stimulatory effect on the growth factor expression. Conversely, by serum starving the cells, it cannot be positively shown that the response is not due to serum withdrawal causing cellular stress, leading to various survival proteins to be expressed. Tang *et al.* (1997) and Iihara *et al*. (1993) stated that using a cellular model it is difficult to investigate growth factor modulation following serum deprivation, as after 48 hours the cells tend undergo apoptosis. This is not the case for the PGE_2 treatment increasing COX 2 expression as discussed (section 7.4).

Therefore, for the analysis of growth factors, it would be of more value to investigate the activation of growth factors quantitatively. For example, the activation of growth factors and receptors caused by PGE2, though the activation of ras or tyrosine kinase could be monitored using protein ELISA assays. This may present a protein that could be studied that is not hugely affected by the serum, but more by the PGE_2 itself.

9.4. The COX 2 Positive Feedback Theory

Shown in Chapters 7 and 8, the PGE_2 treatment on oesophageal cell lines gave significant differences of COX 2 expression over a period of one to 72 hours. The results demonstrated a very novel concept, that at an optimum concentration of PGE_2 treatment, the COX 2 is expressed for longer periods of time (Chapter 7), both at the RNA and protein levels (Chapter 8).

If these findings revealed by this model have direct implications on the sample population, then increased PGE_2 from the high maize diet is refluxed into the oesophagus for reasons previously explained. This PGE_2 could have a proliferative effect on the oesophageal cells and should this localised PGE_2 also stimulate COX 2 expression, increases in its substrate (arachidonic acid in abundance in the gastric fluid following a high maize meal), then further PGE_2 would be produced, having further stimulatory effects. This positive feedback mechanism may work as a natural process of oesophageal cellular restitution following damaged caused by swallowing food. Hence, the arachidonic acid content may be low inhibiting many cycles of this positive feedback loop. This increase in cellular proliferation could amplify the mutational effect of environmental carcinogens. Due to the COX 2 enzyme having both oxygenase and peroxidase functions, one carcinogenic action could be to catalyse the conversion of pro-carcinogens to carcinogens. COX 2 activity is thought to be responsible for the conversion of the pro-carcinogen benzo(a)pyrene, present in tobacco smoke, into the carcinogen of mutagen benzo(a)pyrene diolepoxide (Kelley *et al.*, 1997).

9.5. Updating the Sammon Theory

Much of this research supports the Sammon theory (section 1.9) by validating the theoretical postulates with evidence from these cellular models. As well as providing evidence on the mitogenic actions of the maize diet, it also provides understanding as to what may be happening on a molecular level and how this may affect the general

pathology seen in the target population. The general pathology is further supported by physiological factors witnessed in the target population, such as gastric regurgitation. Therefore, this suggests new areas for study in different genres of life sciences to further this work.

There are many factors that could affect the homeostasis of the oesophageal cells, therefore it is hard to pin point which factors could be driving the cancer progression, especially with an unfamiliar target population studied for such a short time. As explained above, the results suggest that increased PGE_2 in the diet, hence in gastric fluid, as seen in the Transkei population, may occupy the oesophagus following regurgitation. The Sammon theory states that this increase in pH would suppress the localised pepsin that would normally breakdown the growth factors present (Sammon & Alderson, 1998). This may allow the growth factors to stimulate cellular growth for increased periods of time. The results from this thesis suggest that the increase in PGE_2 has a direct mitogenic effect on the oesophageal cells, bypassing growth factor release, although this cellular growth may still be through intracellular growth factor activation. The proposed method suggested by Sammon may be questionable due to the growth factor's short half-life. It can be predicted that in order to have constant cellular stimulation from the growth factors, they will have to be reproduced or activated at a constant rate. This could be due to changes in the habits of the cells, mutation, growth factor stimulating mechanisms such as tyrosine kinase or mutations in the production of *ras*. Direct stimulation by the PGE_2 may produce an exaggerated proliferative effect explaining the large luminal oesophageal tissue growths seen in the target population.

In order for oesophageal cell growth to take place many proteins will be expressed and activated. Through this constant increase in cellular proliferation there is increased production of cellular proteins, therefore, a greater chance for mutations in DNA to take place. A proto-oncogene could be mutated, hence, cancer initiated by tobacco smoke or fungal infection from the maize. It is not postulated here that the increased PGE_2 from the high maize diet is the single carcinogenic factor, but it increases the pre-disposition of oesophageal cells to cancer by external carcinogens. This is not helped by the increased prevalence of local disease and high PGE_2

amounts suppressing the immune system (Harris *et al.,* 2002), therefore preventing carcinogenic reactions and carcinogens from being disposed of naturally.

9.6. How Can this Help the Target Population?

As the Sammon theory is updated with the work that has been presented in this thesis, it has become clearer how this research could translate into aiding the target population with its oesophageal cancer problem. This thesis has shown how fundamental PGE_2 through diet is in the carcinogenesis of the oesophageal cancer in the Transkei population.

To help the target population it is important to find a preventative rather than a treatment, as treatments are often expensive, which developing areas cannot afford. This is exacerbated by the fact that many of the Transkei people only visit their health centres when the symptoms have become greatly developed, past the stage of treatment. Should this developed "Sammon-Pink theory" be right, it would be important to investigate other dietary fats that would be cheap to produce, that naturally compete (Kinsella *et al.,* 1990) with arachidonic acids for the COX 2 enzyme, decreasing the gastric PGE_2. Ideally a more varied diet would be undertaken that included other vitamins and vegetables, as all of these have been shown to have inhibitory effects on oesophageal cancer (Barone *et al.,* 1992; De Stefani *et al.,* 1999; Franceschi *et al.,* 2000). Various South African plants have shown COX 2 inhibiting properties that could be investigated. The bark from the *Erythrina* species of plant, used by traditional healers, has been shown to have anti-bacterial and prostaglandin synthesis inhibiting properties (Pillay *et al.,* 2001). This was also discovered in the extracts of other local plants: *siphonochilus*, *aethiopicus* and *solanum* (Lindsey *et al.,* 1999; Jäger et al., 1996). It is fundamental that the South African population are educated about how diet affects their health. Prevention through education is the cheapest and the most efficient way of eradicating this disease by allowing the population to take steps to helping themselves.

COX 2 inhibitors and NSAIDs, are of great interest to the large drug companies such as Pfizer and Merck. This is not only due to their suppression of PGE_2 relieving arthritis sufferers, but also through their promise in the suppression of tumour growth. Aspirin is known to inhibit PGE_2 production through COX1 and COX 2 suppression. Therefore it could be suggested that Aspirin could inhibit this excess PGE_2 production in the target population caused by COX 2. A clinical trial could be set up where patients take aspirin or a placebo and the amount of regurgitation/cleansing is measured. Should the aspirin reduce this then histological studies investigating pathological changes could be carried out to see if these patients are at lower risk of oesophageal cancer than the patients not taking the aspirin. Even though aspirin has a detrimental effect on the stomach lining, the reduction in oesophageal cancer at the cheap price of aspirin may be considered worthwhile. As aspirin is no longer under patent there are little profits in its use, therefore clinical trials would have to be paid by the charitable grants. Even though the treatments are promising it is fundamental that this population are educated on how to help themselves through a more varied diet.

9.7. Research Summary

The key findings of this project are summarised as follows:

- Gastric fluid from a population that regularly ingests high amounts of maize, contains high amounts of PGE_2
- PGE_2 at certain concentrations increases oesophageal cellular proliferation, thought to be linked to growth factor increase due to common characteristics.
- Proteins found in the gastric fluid from the sample population increases oesophageal cellular proliferation. It is postulated that the PGE_2 in the sample may cause this hyper-proliferation.
- HGF is not expressed at detectable levels in the cell lines used.

- It is suggested that the VEGF and EGFR mechanisms are activated by increased PGE_2, rather than pH changes in the oesophagus not breaking down growth factors as originally suggested in the Sammon theory.
- PGE_2 increases the time period of COX 2 expression causing a positive feedback loop that, in the presence of COX 2 substrate and gastric reflux, leads to continuous local mitogenicity, predisposing the squamous epithelium to cancer.
- Other local factors such as fungal infection of the maize, smoking and disease may then progress this proliferative state to *in-situ* carcinoma.

Collectively, these findings contribute to the development of the Sammon theory, although much remains to be done to validate this, and translate the theory into actions which can help save lives of the people in the Transkei through inhibition of PGE2. An updated schematic representation of the Sammon theory is presented in Figure 9.1.

9.8. Suggested Pathways Contributing to Carcinogenesis of Oesophageal Cancer in Transkei, South Africa:

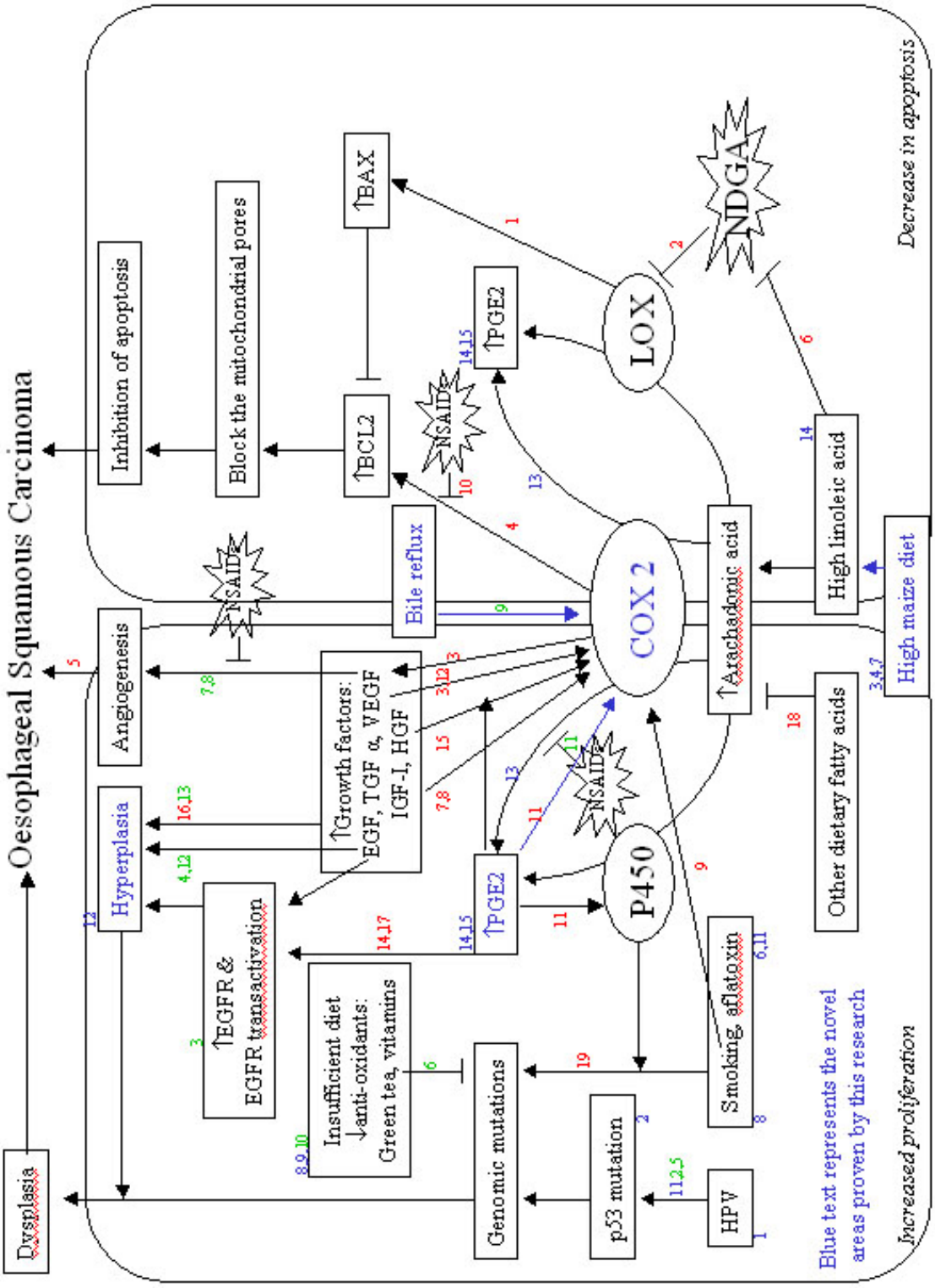


Figure 9.1: Suggested epidemiological Carcinogenesis of oesophageal cancer in Transkei, South Africa

The References for Figure 9.1 on the epidemiological carcinogenesis of oesophageal cancer in Transkei, South Africa

References

Shown in relation to Oesophageal cancer, not necessarily in South Africa:

14. Takada *et al.*, 1995
15. Sur and cooper, 1998
16. Ozawa *et al*, 1989
17. Juhl *et al*., 1995
18. Ono *et al.*, 1994
19. Muller *et al*, 1997
20. Inoue *et al*, 1997
21. Koide *et al*., 1999
22. Zhang *et al.*, 1998
23. Gao *et al.*, 1994
24. Farrow *et al.*, 1998
25. Jankowski *et al.*, 1992
26. Jimenez *et al.*, 1998

Shown in other tissues:

20. Tang *et al*., 1996
21. Goodman *et al.*, 1994
22. Murphy and Fitzgerald 2001 (colon)
23. Liu *et al*., 2001 (mammary)
24. Folkman, 1990
25. Rouzer *et al.*, 1990 (leukocytes)
26. Perkins and Kniss, 1997 (WISH cells)
27. Diaz *et al.*, 1998 (fibroblasts)
28. Kelley *et al*., 1997 (Oral)
29. Liu *et al.*, 1998 (prostate carcinoma)
30. Brueggemeier *et al.*, 1999 (breast)
31. Gallo *et al*., 2002 (Head and Neck Squamous Cell Carcinoma)
32. Fosslien, 2000
33. Pai *et al.*, 2002 (colon)
34. Jones *et al*., 1999 (colon)
35. Schmassmann *et al.*, 1997 (gastric)
36. Buchanan *et al*., 2003 (colorectal carcinoma)
37. Raedersdorf and Moser, 1992
38. Mace et al., 1997

High incidence in oesophageal cancer in South Africa:

16. Williamson *et al.*, 2002
17. Vos *et al.*, 2002
18. Van Rensburg *et al*., 1985
19. Van Rensburg *et al*., 1981
20. cooper *et al*., 1995
21. Rheeder *et al.*, 1992
22. Rose, 1982
23. Van Rensburg, 1987
24. Dayne and Munoz, 1982
25. Matsha *et al.*, 2002
26. Matsha *et al.*, 2002
27. Hamilton and Isaacson, 1985
28. Grant *et al.*, 1988
29. Sammon, 1994
30. Sammon and Morgan, 2002

10. Further Work

Although the research in this thesis has presented some very interesting findings, up dating the Sammon theory, this has just touched the surface of what can be investigated. From this work and peer reviewed studies, a diagram of the suggested molecular carcinogenesis of oesophageal cancer in the sample population of Transkei, South Africa was created (Figures 9.1). This shows huge areas of research required to further develop an understanding of the Sammon theory, both, the mitogenic action of PGE_2 is required to understand why people in Transkei suffer from oesophageal cancer up to ten times more than other areas of the world, and what can be done to help them. In the following chapter the fundamental research areas are outlined.

10.1. Further Investigation into the Discussion Points in this Thesis

10.1.1. Activation of Growth Factors by PGE_2

As discussed in the conclusion, further investigations are required to study the relationship between the PGE_2 proliferative effect on the oesophageal cells and the intracellular activation of growth factors. This information would be of greater value than the expression studies carried out in this thesis (Chapter 7). By studying specific kinases (e.g. Tyrosine kinase), secondary proteins (ras, p53), cyclins or the early response genes activation (*fos, jun* or *myc*), it may appear that PGE_2 activates these growth factors faster or for longer periods of time, stimulating greater epithelial growth. Both tyrosine kinase and cyclin D have been shown to be over-expressed in oesophageal cancer (Nemoto *et al*., 1997; King, 2000). This increased stimulation could have been the cause of natural proteins such as PGE_2. Mutational studies of these intracellular growth factor pathway proteins in the target population could raise progression and promotion factors in carcinogenesis, exacerbated by the proliferative effect of PGE_2. New innovative techniques are on the market to investigate whole molecular pathways in short periods of time. Microarrays such as the Genechip, sold by Allymetrix, claims to be able to study tens of thousands of intracellular genes at one time, modelling pathways such as growth factors. This would be ideal for this

study, but the equipment is very expensive at £250,000 for the chip reader and between £50- £500 for the chips. The price is expected to fall as the chips are produced in a plastic format rather than metal and demand increases as more laboratories realise that, in the words of Vogelstein "the future of cancer research is based on the interaction of whole pathways" (AACR 2006). The study of how protein A affects protein B, tells us only about the effect, which may bypass many of the true cancerous proteins.

10.1.2. PGE2 and Transporter Protein Protection

In Chapter 5 the concept of PGE_2 transporter protein protection was discussed following a pattern of intriguing results (section 5.4). The PGE_2 levels seem to remain at higher concentrations for longer periods of time than previously documented (Klein, 1990). Aoyama *et al.* (1990) discovered that the half-life of prostaglandin I_2 (similar to PGE_2) was stabilised by apolipoprotein A-I (Apo A-I), a major apolipoprotein of high density lipoprotein. Apolipoprotein synthesis in the intestine is regulated principally by the different fat contents of the diet, similar to this study (Eichner *et al,* 2002). Apo A-I bound proteins were stabilised for up to 90 minutes compared to one to six minutes without. The PGE_2 and prostaglandin I_2 have very similar structures, produced from the same protein, Prostaglandin H_2, both produced by COX 2 (Figure 1.10); both could have stabilising proteins. By investigating the PGE2 transporter proteins like MRP4 and ABCC4 (Ruis *et al.,* 2005), this could provide information on the protection of PGE_2 from natural degradation, stimulating the oesophageal tissue for longer periods of time.

10.1.3. Experimental Models

Using oesophageal cell lines as a model has its limitations, due to many reasons, including this not representing localised different cell to cell interactions and the cell lines being developed to stay alive in synthetic conditions. Therefore, changes in the cell lines predominant growth characteristics compared to the body is inevitable. The

addition of foetal calf serum to cell media is required for cell growth; these contain large amounts of growth factors, therefore, when growth factor levels are being investigated the use of a more significant model should take place. Others have also discovered this; Tang *et al.* (1997) and Iihara *et al*. (1993) stated that they found it difficult to look at growth factor modulation following serum deprivation as after 48 hours the cells tended to lead to apoptosis. They did not suggest a preferred method. The closest model to that of a human would be to use animals, such as mice. Mice could be fed with various concentrations of maize, linoleic acid and PGE_2, and the protein and RNA levels in the oesophageal and gastric tissue could be investigated. Should PGE_2 have a carcinogenic effect, this could be investigated and staged with an animal model.

10.1.4. Laboratory Based *in-vivo* Studies

Using a sensitive immuno-cyto-fluorescent method for tissue analysis, such as that shown in Figure 10.1 from a preliminary study, could take place investigating the areas *in-vivo* where the PGE_2 and COX 2 may be produced. Slices of human or animal tissue from different stages of oesophageal squamous cell carcinoma could provide an insight into the PGE_2 and COX 2 effect on the general stages of carcinogenesis, as this thesis can only suggest that PGE_2 has a pre-carcinogenic effect increasing the chance of initiation, rather than promotion and progression.

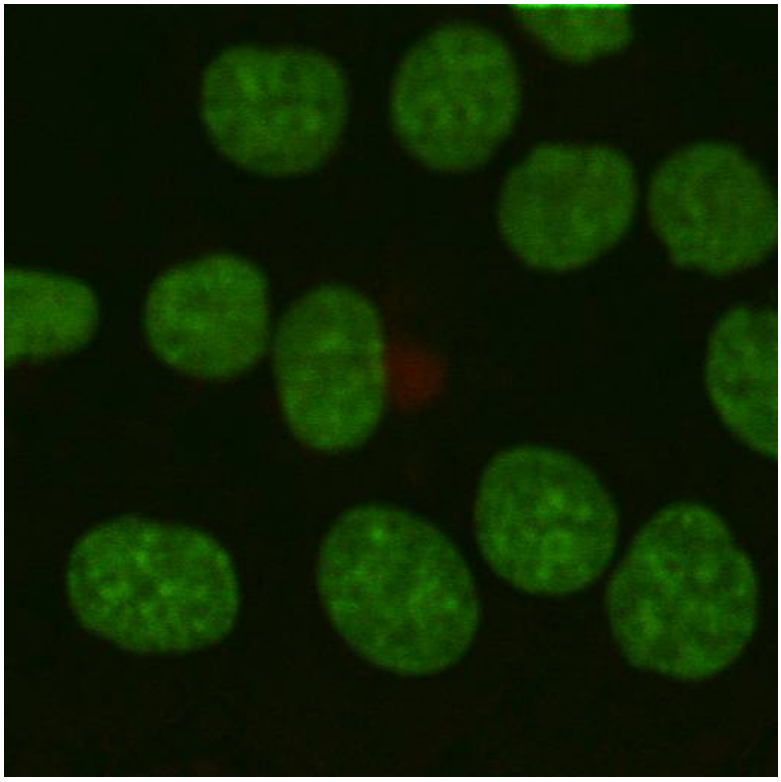


Figure 10.1: A sensitive immuno-cyto-fluorescent method here used for staining the nucleus (green) and COX 2 protein (red). This was a preliminary study carried out on RT112 bladder cells visualised with the confocal microscope.

10.2. Further Research into the Components of the Sammon Theory

10.2.1. Gastric Reflux

The reflux of gastric fluid into the oesophagus is fundamental to the Sammon theory. Due to the many components of gastric fluid, greater analysis of the effect the sample population's gastric fluid, and the gastric fluid of a population eating a regular diet, has on squamous tissue should take place. This could include the analysis of other maize related constitutes. It was discovered by Sammon (2002) that the gastric fluid of the sample population, which refluxes into the oesophagus, is more alkali than the expected low pH of the stomach. This change in pH could have an effect on many of the chemical structures, hence, molecular reactions in the oesophagus. Therefore, investigating high pH changes on gastric or dietary components in the oesophagus

could present differences to suggest why Barretts oesophagus pre-disposes the oesophagus to adenocarcinoma. This may show an insight into any connections of the carcinogenesis between the squamous cancer and, any already discovered links from the heavily researched, adenocarcinoma.

10.3. Epidemiological Investigations

10.3.1. Environmental Factors and Mutagenicity

The research in this thesis suggests that PGE_2 has a proliferative effect on the oesophageal cells, therefore this may be a pre-disposing factor aiding initiation and promotion. Hence, studies need to be made to investigate any mutagenic reaction from the PGE_2. These studies should also report on the synergistic action PGE_2 may have with known environmental carcinogens. Testing the gastric samples and PGE_2 for mutagenicity with methods like the Ames test and its variations could support this. There are many potential carcinogenic environmental factors seen in the target population, which themselves present large areas of research. Examples of such environmental factors include aflatoxins found in the fungus of maize, and the consumption of tobacco products. Aflatoxins and tobacco consumption may be connected to specific transversions or transitions in the genome, therefore, the genetic analysis of the target population is oesophageal samples may present more information as to whether they have an effect. A full epidemiological study may help in the discovery of carcinogenic factors.

10.3.2. Disease and Carcinogenesis

A majority of the Transkei can be classed as economically and sociably poor compared to the western world. Diseases that are eradicated, or efficiently treated in the UK, are prevalent in many areas of South Africa, including the areas with high number of oesophageal cancer cases. There is a large chance that diseases such as *H pylori* gastric infections, tuberculosis, HPV and AIDS have an effect on the

development of oesophageal carcinomas. If this is not through direct molecular interactions then it could be through suppression of the immune system. There is little peer reviewed evidence to support this connection, although the chances are high and the research has not yet been carried out. For example, *Helicobacter pylori* is associated with gastric cancer (Jiang & Wong, 2003) and it was shown that *H. Pylori* infection may induce atrophic gastritis, which results in a less acidic gastric reflux, by neutralising gastric acid through the production of ammonia (Richter, Falk & Vaezi, 1998). This may explain the increases in the pH of gastric fluid in the target population. It can be argued that many epithelial tissues react similarly to extra cellular stimulus, therefore if *H. pylori* has a carcinogenic effect on gastric tissue the conditions may apply for oesophageal tissue. Hence, studies in this area would be of value.

Bachelor *et al*. (2004) has linked skin cancer to COX 2 increases, through mediated skin tumour promotion. Populations that ingest a high maize diet may be more at risk to skin cancer than people ingesting a varied diet. This theory could be tested in the target population who ingest a high maize diet, should the theory be correct that PGE_2 stimulates the expression of COX 2.

10.4. Expanding the Sammon Theory

10.4.1. Apoptosis (BCL and BAX)

As shown in Figure 9.1 it is suggested that the same enzymes that produce prostaglandins, from the linoleic acid found in a high maize diet, could inhibit apoptosis upsetting the fine balance in normal cellular homeostasis. Research could be carried out on this half of the cellular growth control balance, affecting the rate of proliferation, hence, pre-disposition to cancer. Changes in linoleic acid treatment and apoptotic proteins such as BCL2 and BAX could be researched. Activational studies of mitochondrial enzymes could show the state of apoptosis as this is thought to be the area that initiates apoptotic cell death. By investigating the inhibition of any

adverse reactions caused by this high maize diet, for example LOX inhibitors, apoptosis may be stimulated slowing the development of cancer in the target population.

10.4.2. Prostaglandin E Synthase

This thesis shows that PGE_2 has a proliferative effect on the oesophageal cell lines (section 6.4). In the production of PGE_2, an enzyme called prostaglandin E synthase, which functions further downstream to COX 2, converts prostaglandin H2 to prostaglandin E2 (Figure 1.10). This enzyme has been implicated with tumourigenesis in colon cancer cell lines (Kamei *et al.,* 2003) and non-small cell lung cancer, thought to be connected to ras (a growth factor secondary protein) activation increase (Yoshimatsu et al., 2001). Little has been investigated into the carcinogenic actions of this enzyme, although like COX 2, it is expected to be due to the production of PGE_2. This enzyme could be researched by its suppression caused by anti-inflammatory glucocorticoids (Murakami *et al.,* 2002). Inhibition of these enzymes could be as potent as COX 2 inhibition in the suppression of cancer.

10.4.3. PGE_2 Markers

Should increased PGE_2 prove to be the potent protein in the development of carcinogenesis, the measurement of PGE_2 effects in the body could be used as a marker for the potential of oesophageal cancer. PGE_2 attenuates vasopressin-induced NaCl reabsorbtion in the kidneys (Negishi *et al.,* 1995), increases internal calcium levels (Toborek *et al.,* 1997), is linked to periodontal disease (Harris *et al.,* 2002) and induces bone formation (Kobayashi *et al.,* 2002) PGE_2 has been postulated as a histopathologic predictor for proliferation and invasion in urothrelial carcinomas of the bladder due to its relationship with cancer aggressiveness and concentration (Eschwège *et al.,* 2003). These physiological states offer markers to predict high PGE_2 levels in the body. Using these are markers to screen oesophageal cancer in the

target population could be too expensive, but by adapting this knowledge for cancer screening or treatment in the western world may provide a economic benefit for research in Transkei.

10.4.4. Food Related Studies

There are a number of foods that can be implicated by this study that are postulated to suppress the harmful effects of a high maize diet, providing ideal research opportunities. High levels of oleic acids, found in olive oil, have been shown to reduce the synthesis of arachidonic acid, by competing for the desaturase enzymes (Holman, 1964). De Schrijver and Privett (1982) showed how dietary fish oils high in polyunsaturated fatty acids depress the desaturase enzymes decreasing the arachidonic acid levels. Calviello *et al* (2004) has recently shown that these fish oil polyunsaturated fatty acids reduce VEGF expression in human colon cancer cells, modulating the COX 2 / PGE_2 pathway. This reduction in VEGF blocking angiogenesis has also been shown with flavonoids and curcumin (also known as turmeric) (Fotsis *et al.,* 1997). Aggarwal *et al.* (2003) later found curcumin to suppress the proliferation of tumour cells through the down-regulation of the expression of COX 2, LOX and EGFR. Another natural flavonoid, quercetin, has also been shown to inhibit COX 2 in the adenocarcinoma Oe33 cell line (Cheong *et al.,* 2004). Genistein, rich in soybean and some forage plants, has been shown to decrease the incidence of breast and prostate cancer (Nakagawa *et al*., 2000). Ye *et al*. (2004) showed that genistein inhibited COX 2 action in head and neck cancer cells similar to that of commercial COX 2 inhibitors. All of these could be tested in trial conditions offering a cheap preventative treatment, should variations of these foods be able to grow in Transkei.

10.4.5. Immunology

It is established that PGE_2 suppresses the immune system. Research into this area is growing due to the implications of COX 2 inhibitors being used in the treatment of arthritis sufferers. The detailed pharmacological effect on the epithelial tissue is unknown. The immune system has fundamental actions in suppressing the development of cancer, therefore its suppression is detrimental. T-helper lymphocytes have been shown to be suppressed by PGE_2 (Choudhry *et al.,* 1999;Cosme *et al.,* 2000). Studies into the relationship of immuno-suppression caused by a high maize diet are essential, not only in the study of cancer.

10.4.6. Non-Steroidal Anti-Inflammatory Drugs

Throughout the journals showing investigations of PGE_2 and COX 2, it has been be shown that non-steroidal anti-inflammatory drugs (NSAID) suppress the action of COX 1 and fundamentally COX 2, which decreases the production of PGE_2 (section 1.7.1). This therapeutic action is widely used in the treatment of arthritis. It is suggested that aspirin and paracetamol relieve pain by suppressing the inflammatory action of PGE_2. Therefore, clinical studies could be set up in the target population at high risk of oesophageal cancer. It can be tested if a cheap NSAID, such as aspirin, has a suppressive effect on the regurgitation of gastric fluid, high in PGE_2.

Appendix A: Copyright Permission

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Appendix B – Ethic Information

The following sheets.

Appendix C: PGE_2 concentration of the samples

1) Raw results for PGE_2 ELISA of the Gastric fluid Samples

Label	Reading 1	Reading 2	Average	% B/Bo	Minus NSB
TA	0.229	0.24	0.2345		
sb	0.13	0.131	0.1305		
Bo	0.343	0.342	0.3425	100	0.2125
NSB	0.13	0.13	0.13		
st 5000	0.135	0.141	0.138	3.764706	0.008
st 2500	0.141	0.154	0.1475	8.235294	0.0175
st 1250	0.155	0.162	0.1585	13.411765	0.0285
st 625	0.175	0.179	0.177	22.117647	0.047
st 312	0.204	0.224	0.214	39.529412	0.084
st 156	0.243	0.254	0.2485	55.764706	0.1185
st 78	0.284	0.288	0.286	73.411765	0.156
st 39	0.329	0.318	0.3235	91.058824	0.1935
1	0.258	0.25	0.254	74.16058	0.124
2	0.252	0.236	0.244	71.24088	0.114
3	0.237	0.236	0.2365	69.05109	0.1065
4	0.275	0.293	0.284	82.91971	0.154
5	0.287	0.27	0.2785	81.31387	0.1485
6	0.276	0.274	0.275	80.29197	0.145
7	0.306	0.294	0.3	87.59124	0.17
8	0.309	0.311	0.31	90.51095	0.18
9	0.305	0.287	0.296	86.42336	0.166
10	0.282	0.281	0.2815	82.18978	0.1515
11	0.198	0.188	0.193	56.35036	0.063
12	0.296	0.304	0.3	87.59124	0.17
13	0.288	0.289	0.2885	84.23358	0.1585
14	0.28	0.269	0.2745	80.14599	0.1445
15	0.216	0.205	0.2105	61.45985	0.0805
16	0.279	0.287	0.283	82.62774	0.153
17	0.323	0.3	0.3115	90.94891	0.1815
18	0.276	0.259	0.2675	78.10219	0.1375
19	0.261	0.274	0.2675	78.10219	0.1375
20	0.324	0.299	0.3115	90.94891	0.1815

2) The PGE_2 Concentrations of the Gastric Fluid Samples

The PGE_2 concentrations from the above PGE_2 ELISA on the samples now and the concentrations of the PGE_2 from the same samples taken when fresh before a long journey from South Africa to England, not at optimum temperatures.

Sample no.	Old PGE2 (pg/ml)	New PGE2 (pg/ml)
1	3920.00	600
2	3950.00	850
3	2590.00	910
4	8150.000	540
5	2600.00	590
6	1530.00	600
7	1720.00	430
8	2350.00	400
9	4870.00	480
10	3500.00	550
11	628000.00	1070
12	2910.00	450
13	29300.00	510
14	3830.00	610
15	79700.00	1030
16	442000.00	540
17	3060.00	400
17	7000	400
18		650
19	2860.00	650
20	96800.00	400
21	3880.00	400
22	64600.00	820
23	55600.0	550
24	119000	740

Appendix D: Sample transportation information from airplane specialists

1) Professor John Fielding, Head of Aerospace Engineering group in the School of Engineering, Cranfield University.

Q: *What would the temperature of the baggage cabin be throughout the flight?*

A: About 20^{o}C (same pressure and temp as cabin – poss slightly cooler)

Q: *How different would the pressure be in the baggage cabin compared to the passenger cabin?*

A: About 20^{o}C (same pressure and temp as cabin – possibly slightly cooler)

Q: *Would take off or landing have any effects on the temperature of the baggage cabin?*

A: No - not significantly. depends on how long it was on the plane before take off - it could be up to 2 hours turn around.

Q: *Can I quote you on this for the thesis?*

A: Yes

2) Dr Craig Lawson, Airframe Systems Design, Aerospace Engineering Group, Power, Propulsion and Aerospace Engineering Dept., School of Engineering, Cranfield University.

Q: *What would the temperature of the baggage cabin be throughout the flight?*

A: During cruise: usually slightly cooler than the passenger cabin - typically maintained around 15 Celsius, say +/- 5 Celsius.

Before take-off and after landing (i.e. before/after the engines are shut down): depends on ambient temperature - the cabin temperature is still conditioned during boarding/disembarkation, but may not be regulated as tightly. For the baggage hold, the most extreme case is likely to be reaching ambient temperature, if the aircraft was to sit at the gate for a long time. It's worse for the passenger cabin; it may reach 5-10 Celsius above ambient temperature, because of the windows and passengers.

Q: *How different would the pressure be in the baggage cabin compared to the passenger cabin*?

A: It is the same. So, typically varying from 1 bar to 0.7 bar.

Q: *Would take off or landing have any effects on the temperature of the baggage cabin?*

A: No. At all times while the engines are running, the cabin is subject to

the same temperature regulation.

Q: *Is there anything else I should be thinking about that is effected by the flight and loading/unloading?*

A: The ambient temperature is the significant factor during loading and unloading. Typically, baggage handlers are not gentle.

Q: *Can I quote you on this for the thesis?*

A: Yes, I hope this helps. Let me know if you need any clarification or want more details.

Appendix E: Cell culture methods

1) Conditions

Cell lines were maintained at 37^{o}C and 5% CO_2 in an incubator. All manipulations were carried out in a class II laminar flow cabinet using aseptic technique.

2) Reagents

All reagents were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated.

3) Tissue Cultureware

All tissue culture plasticware was obtained from NUNC (Ruskilde, Denmark) unless otherwise stated.

4) Media Preparation

In the class II laminar flow cabinet 50ml fetal calf serum (FCS), 5ml Penicillin/Streptomycin, 1ml Amphotericin was added to a 500ml bottle of RPMI 1640 with bicarbonate media. Ten ml of this was aliquoted into a small vial and placed in the incubator to check for infection. A separate bottle was provided for each cell line, to minimize risk of cross-infection.

5) Thawing the mammalian cell lines

The cell line vials were removed from the liquid nitrogen bank and the details on the vial was recorded. Silver foil was placed over the lid of the vial and it was held in a beaker of 37oC until the contents had thawed. The vial contents were placed into a 15ml centrifuge tube. Media was pipetted into the tube one drop at a time and gently swirled until after 5 minutes the tube contents reached 5ml. Over the next minute a further 5 ml was added with gentle swirling. This was centrifuged at $4^{o}C$ at 100 x g (ALC MRC, model PK131R) for 5 minutes. The suspension was pipetted off and the pellet was resuspended in 1ml of media. This was mixed by pipetting and placed into a T25 cell culture flask with a breathable lid. A further 6 ml of media was added to the T25 and a gentle swirl of the flask ensured an even distribution of the cells. This

was then left to grow in a infection free 37^{o}C incubator supplemented with 5% CO_2 and >95% humidity to prevent evaporation.

6) Feeding the cell lines

The old media was pipetted from the flask into equivalent amounts of Virkon disinfectant and new media at 37^{o}C was pipetted into the flasks. Six ml of media was placed in a T25 and 9ml of media was placed in a T75.

7) Splitting the cell cultures

Following 70-80 % confluence of cells in the flasks they were split into 1:3. The media was removed from the flask and rinsed with sterile PBS. Five ml of Trypsin x 1 was pipetted into the flask and incubated at 37^{o}C for 5 minutes. The flask was tapped and placed under the microscope to check the cells had come away flask base, if not this was repeated until all the cells were in the suspension. In a 15ml centrifuge tube 5ml of media was added along with the suspension from the flask. This was centrifuged at 4^{o}C at 66 x g for 5 minutes. The pellet was resuspended in 3ml of media and pipetted equally into three T75 flasks. To these flasks 9ml of media was added and gently swirled to ensure dispersion of the cells. The flasks were then placed into the incubator.

8) Creating Frozen Cell Stocks

The cells were stored in liquid nitrogen (-146^{o}C) banks. Seventy to eighty percent confluent T75 flasks were washed with sterile PBS, 5ml trypsin was added and incubated at 37^{o}C for 5 minutes. The contents was added to a 15ml centrifuge tube

along with 5ml of media. For 5 minutes the tube was centrifuged at 66 x g at 4^{o}C. The suspension was added to Virkon and discarded. Three ml of FCS was used to resuspend the cells. Three ml Freezing media, made from 10% DMSO in FCS, with gentle swirling was slowly added to the cells. One ml aliquots were labeled with the cell line, passage number, dated and named. In each of the 6 aliquots 1ml of the cells, FCS and mixed freezing media was added. These were wrapped in three layers of cloth, placed in a freezing plastic box and were put in the –80^{o}C for 24 hours. These were then transformed to the liquid nitrogen banks.

9) Slide flasks for cell cultures

For immunocytochemistry both cell lines were grown on slide flasks. At 70 - 80 % confluence the T75 flask was split into 10 slide flasks, by trypsinising the flask as above and the contents was centrifuged to obtain a cell pellet. The pellet was then suspended in 5 ml of media and 0.5ml of this suspension was placed into each of the slide flasks along with 2.5ml of media. Feeding with 2.5ml of media after two days. These were placed in the incubator. Once the slides were 100% confluent then they were fixed and stored.

10) Fixing the slide flasks

The 70% confluent slide flasks were washed with sterile PBS, repeated three times gently rolling the slide flasks over as not to wash off the cells. The slide and cells were submersed in 4% paraformadyhyde (Sigma) for 10 minutes, then cold methanol for 10 minutes at 4^{o}C, and left to dry at 4^{o}C. These were then labeled and wrapped in foil and stored at –20 ^{o}C until used.

Appendix F: Statistical Analysis of the Power of the Gastric Fluid Samples

The basic statistical analysis of the PGE2 concentrations of the gastric fluid samples:

Variable	Valid N	Mean	Confidence -95.000%	Confidence +95.000%	Sum	Minimum	Maximum	Variance	Std.Dev.	Standard Error
Var3	25	606.8000	525.8706	687.7294	15170.00	400.0000	1070.000	38439.33	196.0595	39.21190

T-test analysis results of the power calculation from the 24 samples:

Null Hypothesized Mean (Mu0)	312.0000
True Population Mean (Mu)	606.8000
Population S.D. (Sigma)	196.0600
Standardized Effect (Es)	1.5036
Group Sample Size (N)	25.0000
Type I Error Rate (Alpha)	0.0500
Critical Value of t	2.0639
Power	1.0000

The estimated sample size required to have at least a 90% power goal:

Null Hypothesized Mean (Mu0)	312.0000
True Population Mean (Mu)	606.8000
Population S.D. (Sigma)	196.0600
Standardized Effect (Es)	1.5036
Type I Error Rate (Alpha)	0.0500
Power Goal	0.9000
Actual Power for Required N	0.9089
Required Sample Size (N)	7.0000

Appendix G: Cell Counts, MTT and BrdU Assay Raw Data

1) PGE2 treated cell counts raw data

Cell counts taken from two sets of T25 of Het1a cells treated with different concentrations of PGE2 over different periods of time:

PGE2 (µl/ml)	1 Day	Average	2 Days	Average	3 Days	Average	4 Days	Average
0	12 / 20	16	20 / 28	24	92 / 112	102	184 / 206	195
0.0005	16 / 20	18	36 / 36	36	81 / 111	96	186 / 174	180
0.005	13 / 17	16	42 / 33	37.5	96 / 138	117	396 / 342	369
0.05	12 / 8	10	23 / 24	23.5	73 / 64	68.5	208 / 250	229
0.5	15 / 19	17	27 23	25	74 / 65	69.5	218 / 220	219
5	8 / 12	10	5 / 9	7	6 / 6	6	10 / 4	7

Cell counts taken from two sets of T25 of Oe21 cells treated with different concentrations of PGE2 over different periods of time:

PGE2 (µl/ml)	1 Day	Average	2 Days	Average	3 Days	Average	4 Days	Average
0	6 / 7	6.5	14 / 34	24	90 / 77	83.5	240 / 258	249
0.0005	11 / 11	11	36 /20	28	141 / 154	147.5	262 / 342	302
0.005	4 / 3	3.5	54 / 48	51	112 / 147	129.5	392 / 302	347
0.05	10 / 6	8	52 / 48	50	83 / 79	81	312 / 246	279
0.5	18 / 12	15	28 / 26	27	97 / 63	77	154 / 214	184
5	0	0	0	0	165 / 175	170	192 / 192	192

2) PGE_2 treated MTT Assay Raw Data

Optical density of four sets of results from the MTT assay on the two cell lines treated with different concentrations of PGE_2:

Optical Density For Het1a Cell Line (550nm)

PGE_2 (µl/ml)	1	2	3	4	Average
0	0.106	0.126	0.113		0.115
0.0005	0.191	0.168	0.15	0.162	0.16775
0.005	0.18	0.192	0.179	0.194	0.18625
0.05	0.135	0.195	0.212	0.139	0.17025
0.5	0.153	0.204	0.213	0.179	0.18725
5	0.171	0.19	0.194	0.175	0.1825

Optical Density For Oe21 Cell Line (550nm)

PGE_2 (µl/ml)	1	2	3	4	Average
0	0.485	0.542	0.472		0.499667
0.0005	0.618	0.554	0.51	0.658	0.585
0.005	0.605	0.515	0.558	0.468	0.5365
0.05	0.569	0.609	0.534	0.652	0.591
0.5	0.492	0.665	0.545	0.671	0.59325
5	0.661	0.659	0.545	0.671	0.634

3) PGE_2 Treated BrdU Assay Results

Optical density of two sets by four repeats of results from the BrdU assay from the Het1a cell line treated with different concentrations of PGE_2 (μg/ml):

Het1a set 1

	1	2	3	4	average	minus unspec	% of A
blank	0.086	0.083	0.087	0.085	0.08525		
unspecific binding	0.106	0.085	0.087	0.092	0.0925		
a	0.344	0.342	0.352	0.318	0.339	0.2465	100
b	0.296	0.326	0.34	0.333	0.32375	0.23125	93.81338742
c	0.307	0.34	0.355	0.339	0.33525	0.24275	98.47870183
d	0.342	0.351	0.367	0.337	0.34925	0.25675	104.158215
e	0.365	0.39	0.397	0.334	0.3715	0.279	113.1845842
f	0.361	0.335	0.314	0.348	0.3395	0.247	100.2028398

het1a set 2

	1	2	3	4	average	minus unspec	% of A
blank	0.088	0.085	0.094	0.079	0.0865		
unspecific binding	0.081	0.082	0.099	0.113	0.09375		
Control	0.283	0.317	0.31	0.35	0.315	0.22125	100
0.0005	0.313	0.326	0.311	0.285	0.30875	0.215	97.17514124
0.005	0.323	0.314	0.246	0.199	0.2705	0.17675	79.88700565
0.05	0.315	0.368	0.36	0.319	0.3405	0.24675	111.5254237
0.5	0.349	0.315	0.317	0.329	0.3275	0.23375	105.6497175
5	0.307	0.306	0.297	0.292	0.3005	0.20675	93.44632768

Optical density of two sets by four repeats of results from the BrdU assay from the Oe21 cell line treated with different concentrations of PGE_2 (μg/ml):

oe21 set 1

	1	2	3	4	average	minus unspec	% of A
blank	0.088	0.081	0.086	0.082	0.08425		
unspecific binding	0.093	0.1	0.099	0.097	0.09725		
a	0.404	0.108	0.092	0.104	0.177	0.07975	100
b	0.106	0.404	0.446	0.348	0.326	0.22875	286.8338558
c	0.407	0.353	0.374	0.415	0.38725	0.29	363.6363636
d	0.408	0.383	0.377	0.396	0.391	0.29375	368.338558
e	0.417	0.394	0.475	0.326	0.403	0.30575	383.3855799
f	0.367	0.353	0.313	0.374	0.35175	0.2545	319.1222571

oe21 set 2	1	2	3	4	average	Minus Unspec	% of A
blank	0.084	0.086	0.101	0.086	0.08925		
unspecific binding	0.087	0.104	0.105	0.103	0.09975		
Control	0.121	0.1	0.109	0.112	0.1105	0.01075	100
0.0005	0.341	0.423	0.426	0.42	0.4025	0.30275	2816.27907
0.005	0.378	0.417	0.351	0.376	0.3805	0.28075	2611.627907
0.05	0.367	0.38	0.422	0.382	0.38775	0.288	2679.069767
0.5	0.412	0.411	0.376	0.382	0.39525	0.2955	2748.837209
5	0.329	0.352	0.33	0.36	0.34275	0.243	2260.465116

4) MTT Assay Treated with the Gastric Sample Raw Data

Optical density of two sets of results from the MTT assay from the Het1a cell line treated with gastric fluid samples:

Sample No.	1 (OD-550nm)	2 (OD-550nm)	Average
N	0.163	0.085	0.100777778
1	0.454	0.44	0.447
2	0.611	0.65	0.6305
3	0.527	0.497	0.512
4	0.48	0.624	0.552
5	0.419	0.38	0.3995
6	0.607	0.549	0.578
7	0.424	0.533	0.4785
8	0.38	0.461	0.4205
9	0.506	0.444	0.475
10	0.518	0.581	0.5495
11	0.425	0.394	0.4095
12	0.492	0.533	0.5125
13	0.462	0.492	0.477
14	0.54	0.524	0.532
15	0.394	0.394	0.394
16	0.419	0.434	0.4265
17	0.42	0.41	0.415
18	0.527	0.357	0.442
19	0.296	0.225	0.2605
20	0.569	0.457	0.513
21	0.38	0.301	0.3405
22	0.422	0.351	0.3865
23	0.517	0.384	0.4505
24	0.433	0.427	0.43

Optical density of two sets of results from the MTT assay on the Oe21 cell line treated with gastric fluid samples:

Sample No.	1 (OD-550nm)	2 (OD-550nm)	Average
N	0.117	0.085	0.106666667
1	0.231	0.218	0.2245
2	0.141	0.15	0.1455
3	0.2	0.142	0.171
4	0.34	0.247	0.2935
5	0.224	0.248	0.236
6	0.257	0.294	0.2755
7	0.271	0.308	0.2895
8	0.244	0.283	0.2635
9	0.243	0.242	0.2425
10	0.262	0.282	0.272
11	0.262	0.25	0.256
12	0.277	0.263	0.27
13	0.326	0.328	0.327
14	0.305	0.323	0.314
15	0.239	0.307	0.273
16	0.224	0.253	0.2385
17	0.28	0.233	0.2565
18	0.298	0.22	0.259
19	0.332	0.241	0.2865
20	0.214	0.19	0.202
21	0.296	0.255	0.2755
22	0.247	0.28	0.2635
23	0.262	0.362	0.312
24	0.211	0.331	0.271

5) MTT Optical Density Raw Data from Heat Treated Gastric Fluid Samples

Optical density of two sets of results from the MTT assay from the Het1a cell line treated with gastric fluid samples that have been heat treated:

Sample No.	1	2	Average
N	0.071	0.088	0.0795
4	0.17	0.187	0.1785
7	0.192	0.119	0.1555
8	0.283	0.199	0.241
13	0.224	0.111	0.1675
18	0.192	0.154	0.173

Optical density of two sets of results from the MTT assay from the Oe21 cell line treated with gastric fluid samples that have been heat treated:

Sample No.	1	2	Average
N	0.123	0.161	0.142
4	0.197	0.242	0.2195
7	0.261	0.259	0.26
8	0.201	0.262	0.2315
13	0.231	0.252	0.2415
18	0.191	0.282	0.2365

6) Raw Results for the Gastric Sample Treated Cells from the BrdU Assay

Optical density of two sets of results from the BrdU assay from the Het1a and Oe21 cell lines treated with gastric fluid samples:

het1a

	1	2	3	4	average	minus unspec	% of A
blank	0.123	0.129	0.088	0.088	0.107		
unspecific binding	0.121	0.119	0.141	0.101	0.1205		
control	0.503	0.459	0.4	0.372	0.4335	0.313	100
1	0.459	0.458			0.4585	0.338	107.9872204
2	0.201	0.229			0.215	0.0945	30.19169329
3	0.707	0.487			0.597	0.4765	152.2364217
4	0.528	0.379			0.4535	0.333	106.3897764
5	0.516	0.464			0.49	0.3695	118.0511182
6	0.572	0.437			0.5045	0.384	122.6837061
7	0.566	0.457			0.5115	0.391	124.9201278
8	0.583	0.528			0.5555	0.435	138.9776358
9	0.409	0.458			0.4335	0.313	100
10	0.401	0.366			0.3835	0.263	84.02555911
11	0.45	0.449			0.4495	0.329	105.1118211
12	0.395	0.535			0.465	0.3445	110.0638978
13	0.604	0.493			0.5485	0.428	136.7412141
14	0.43	0.608			0.519	0.3985	127.3162939
15	0.114	0.577			0.3455	0.225	71.88498403
16	0.507	0.456			0.4815	0.361	115.3354633
17	0.417	0.436			0.4265	0.306	97.76357827
18	0.345	0.406			0.3755	0.255	81.46964856
19	0.479	0.603			0.541	0.4205	134.3450479
20	0.409	0.484			0.4465	0.326	104.1533546
21	0.495	0.493			0.494	0.3735	119.3290735

22	0.535	0.502			0.5185	0.398	127.1565495
23	0.502	0.527			0.5145	0.394	125.8785942
24	0.531	0.577			0.554	0.4335	138.4984026

oe21

	1	2	3	4	average	minus unspec	% of A
blank	0.092	0.113	0.098	0.099	0.1005		
unspecific binding	0.127	0.145	0.143	0.136	0.13775		
control	0.61	0.637	0.578	0.517	0.5855	0.44775	100
1	0.558	0.644			0.601	0.46325	103.4617532
2	0.692	0.601			0.6465	0.50875	113.6236739
3	0.595	0.622			0.6085	0.47075	105.1367951
4	0.601	0.521			0.561	0.42325	94.52819654
5	0.613	0.705			0.659	0.52125	116.4154104
6	0.684	0.545			0.6145	0.47675	106.4768286
7	0.647	0.678			0.6625	0.52475	117.1970966
8	0.679	0.601			0.64	0.50225	112.171971
9	0.652	0.658			0.655	0.51725	115.5220547
10	0.477	0.527			0.502	0.36425	81.35120045
11	0.599	0.612			0.6055	0.46775	104.4667783
12	0.661	0.59			0.6255	0.48775	108.9335567
13	0.644	0.596			0.62	0.48225	107.7051926
14	0.67	0.642			0.656	0.51825	115.7453936
15	0.716	0.666			0.691	0.55325	123.5622557
16	0.524	0.68			0.602	0.46425	103.6850921
17	0.627	0.662			0.6445	0.50675	113.1769961
18	0.553	0.59			0.5715	0.43375	96.87325516
19	0.571	0.54			0.5555	0.41775	93.2998325
20	0.675	0.579			0.627	0.48925	109.268565
21	0.69	0.618			0.654	0.51625	115.2987158

22	0.749	0.599	0.674	0.53625	119.7654941
23	0.656	0.698	0.677	0.53925	120.4355109
24	0.638	0.595	0.6165	0.47875	106.9235064

Appendix H: Optimisation Methods for the Detection of HGF

1) Optimisation of HGF Western Blotting:

Following failure to obtain results the following optimisation methods were undertaken:

Coomassie blue staining to check that there was protein present, that the protein was running on the gel and the membrane transfer was working

Use of protein from different cell lines that were known to give results with different antibodies (RT112 (bladder) cells and PC-3 (prostate) cells).

Increased protein concentration using size specific Millipore microconcentrators (Millipore, Gloucestershire, UK).

Changed the primary antibody concentration (1/25, 1/40, 1/100, 1/500, 1/1000, 1/2000).

Changed the secondary antibody concentration (1/100, 1/500, 1/1000 and 1/2000).

Tested the secondary antibody on the ladder

Increased secondary binding temperature

Changed primary antibody binding time and temperature (one hour at room temperature and 48 hours at $4^{o}C$.

Compared wet and semi-dry blotting methods

2) Optimisation methods of HGF RT-PCR

Following failure to obtain results using the previously published method the following optimisation methods were undertaken:

Used RT112 (Bladder) and OE33 (Oesophageal adenocarcinoma) cell lines as an alternate source of RNA

Changed annealing temperature (50, 52, 54, 58, 61, 62)

Changed PCR cycles number (30, 35, 40)

Changed primer concentration (20 and 50 picomolar)

Changed $MgCl_2$ concentration (0, 2, 4, and 6 μl of 25mM)

Changed dNTP concentration (0.5 and 1 μl of 10mM each dNTPs)

Changed Taq concentration (0.25, 0.5 and 1 μl of 5 Units/μl)

Changed cDNA concentration (2, 5, and 10 μl from RT reaction, exact concentration unknown)

Made DNA primers to check that the RT primers separately work (see appendix 7C)

Used a commercial HGF primer kit (R&D Systems)

Checked the original and HGF primer kit with HGF control

3: HGF Gene and Primers

Homo sapiens HGF gene for Hepatocyte growth factor:

CGCTTTCTTCTTATGCTGCTTCCCCTTCCTCTTTTCCCAAATAGATATATAAACACATGTATTTTCCTGTTTAAATTG
AGCGAATTGGTCCCCTGCCTGTGCCTTGATTTAGCCATTGGGCTCAGCCTTGCTCCTCCCTTCCTTACTCGGATAGG
AGCCACTGGGATCTGGAGCTCCAGCTTCCAAATTGAAGCTGGCCTCAGGCCAGGTGACTTTTCTTTGTAAGTTTCTT
TCCTAAGCGTGGGGTTGGGGGGAGGCGGGGAATGGGGGGGGTTGCAGGGATCTGTTTGGTGCTGTTGAAGGGGGG
GCGAGTGAGGAAAGGAGGGGGCTGGAAGAGAGTAAAGGGCTGTTGTTAAACAGTTTCTTACCGTAAGAGGGAGT
TCAGACCTAGATCTTTCCAGTTAATCACACAACAAACTTAGCTCATCGCAATAAAAAGCAGCTCAGAGCCGACTG
GCTCTTTTAGGCACTGACTCCGAACAGGATTCTTTCACCCAGGCATCTCCTCCAGAGGGATCCGCCAGCCCGTCCA
GCAGCACCATGTGGGTGACCAAACTCCTGCCAGCCCTGCTGCTGCAGCATGTCCTCCTGCATCTCCTCCTGCTCCCC
ATCGCCATCCCCTATGCAG(exon1)GTTAGTTCCCTTCTTCTTCTTCATTATTAGTATTAGTATTTAACTCTCCTGCTA
ACCTTCCCTATTCCTTTTAACACCCTCTTTTTACCCTATTCCCAGCAATCACTTTCTTGTAGATGATCCTTAGGATTT
TTTTTATATATGAGCCTTAAAAACATTTCTCCAATGTTTATTTTTTTAAAACATTTTGTATTTAATAGAGGGACAAA
GGAAAAGAAGAAATACAATTCATGAATTCAAAAAATCAGCAAAGACTACCCTAATCAAAATAGATCCAGCACTG
AAGATAAAAACCAAAAAAGTGAATACTGCAGACCAATGTGCTAATAGATGTACTAGGAATAAAGGACTTCCATTC
ACTTGCAA(exon2)GTAAGTTACTTCATTTTCTTCTTAGAATAATTTTCCAAATATAGCATGCATGTATTATAATCATG
TGTCTTTTGTGTTGTAGTCAATGTGATTTGATCATATATTGTCCATATGCCCATGGACAAGATGAACCATATGCGCA
TTCTTTAAATACTGTATAATAGTAGTATTTCTTCTTTTCATTCTGATATTTTTTTCAGGGCTTTTGTTTTTGATAAAGC
AAGAAAACAATGCCTCTGGTTCCCCTTCAATAGCATGTCAAGTGGAGTAAAAAAAGAATTTGGCCATGAATTTGA
CCTCTATGAAAACAAAG(exon3)GTAACTGACTTCTCCCTAAATATTGCATAATGAAATAAAGTATAATGAAATGTA
TGTTTGTAATTTCCTGCCAGAGTGGTCTTTCTCACTGATTTTTCTATTACAAGAGTTAATTACTTACAGGTTTGAAC
GGAATTTTTTTCTTAAATACTGCATATGTTTTGCATAGTTGCAATAATTTTATCTACTTTCTTCAATAGACTACATTA
GAAACTGCATCATTGGTAAAGGACGCAGCTACAAGGGAACAGTATCTATCACTAAGAGTGGCATCAAATGTCAGC
CCTGGAGTTCCATGATACCACACGAACACAG(exon4)GTAAGAACAGTATGAAGAAAAGAGATGAAGCCTCTGTCT
TTTTTACATGTTAACAGTCTCATATTAGTCCTTCAGAATAATTCTACAATCCTAAAATAACTTTCTACCTTCAGCAA
AAACATACCCACTAATTAGTAAAATTAATAGGCAAAAAAAAGTTGCATGCTCTTATACTGTAATGATTATCATTTT
AAAACTAGCTTTTTGCCTTCGAGCTATCGGGGTAAAGACCTACAGGAAAACTACTGTCGAAATCCTCGAGGGGAA
GAAGGGGGACCCTGGTGTTTCACAAGCAATCCAGAGGTACGCTACGAAGTCTGTGACATTCCTCAGTGTTCAGAA
G(exon5)GTAAATAAACCTGAATGCCATGTGGGCCATTCTATTCCCCCTATGTGTAGAACTGTAACTCACATTAAAG
GTTAACAGCAACGAATCAATCATAACAAATAAATCTGTTTACTAAACAGGTGCTGTTCTTTAAATGGAACAGTGGT
GTTTTTGATTTTGCTGTCAGAAACAATTAACATGCCATACTTTAATTTTATTAGTTGAATGCATGACCTGCAATGGG
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CGGCACAAATTCTTGCCTGAAAG(exon6)GTAAAATATTAATGAATCATGCTTTCAGTGATTCTTTACAAACTATTAT
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Appendix I: RNA methods

1) RNA quantification

Absorbances readings are taken of the RNA samples at 260nm and 280nm. The ratio was used to give the purity of the sample: Pure RNA: A260/A280 > 2.0 (Pure). The yield was gained by the following:

Yield = 260nm x dilution x 40 (50 for DNA) = xµg/ml

2) Reverse Transcriptase method for cDNA manufacture:

Mix 1µl Random Primer (all Invitrogen), 5µl RNA, 2µl 10mM dNTP mix and add DEPC-treated water Up to 12µl. Denature the RNA and primer by incubating at 65C for 5 min and then place on ice. Vortex the 5x cDNA synthesis buffer for 5 sec immediately before the next step. Prepare following master reaction mix on ice and vortex (per reaction):

4µl 5 x cDNA synthesis buffer

1µl 0.1m DTT

1µl Rnase out (40U/µl)

1µl DEPC-treated water

1µl Cloned AMV RT (15U/µl)*

* - if RNA less than 1ng reduce to 0.5µl increase DEPC-H2O to 1.5µl

Pipette 8µl of master mix into each reaction tube on ice and place in the thermocycler for the following:

cDNA synthesis:

25^{o}C for 10minutes

50^{o}C for 30 minutes

Terminate the reaction by incubating at 85^{o}C for 5 minutes

Store at –20C.

Appendix J: Controls for RT-PCR

RT-PCR controls for Het1a and Oe21 cell lines for EGFR. This includes a β-actin positive control (+), β-actin negative control (-) and a RT reaction without reverse transcriptase

Het+ Oe+ Het- Oe- RT-

RT-PCR controls for Het1a and Oe21 cell lines for VEGF. This includes a β-actin positive control (+), β-actin negative control (-) and a RT reaction without reverse transcriptase:

Het+ Oe+ Het- Oe- RT-

RT-PCR controls for Het1a and Oe21 cell lines for EGFR without the cells being treated with fetal calf serum in the media. This includes a β-actin positive control (+), β-actin negative control (-) and a RT reaction without reverse transcriptase:

Het+ Het- Oe+ Oe- RT-

RT-PCR controls for Het1a and Oe21 cell lines for VEGF without the cells being treated with fetal calf serum in the media. This includes a β-actin positive control (+), β-actin negative control (-) and a RT reaction without reverse transcriptase:

Het+ Het- Oe+ Oe- RT-

RT-PCR controls for Het1a and Oe21 cell lines for COX 2. This includes a β-actin positive control (+), β-actin negative control (-) and a RT reaction without reverse transcriptase:

Het+ Oe+ Het- Oe- RT-

Appendix K: Gene Sequences and Primers Designed

1) The Complete Human Sequence for the COX 2 Gene, Showing the Primers and the Amplification Area

GAGCTCACATTAACTATTTACAGGGTAACTGCTTAGGACCAGTATTATGAGGAGAATTTACCTTTCCCGCCTCTCTT
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AGTAAAGATGTTTGAATACTTAAACACTATCACAAGATGGCAAAATGCTGAAAGTTTTTACACTGTCGATGTTTCC
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AATATTTTGTTGAGATATTCCAGAATTTGTTTATATGGCTGGTAACATGTAAAATCTATATCAGCAAAAGGGTCTA
CCTTTAAAATAAGCAATAACAAAGAAGAAAACCAAATTATTGTTCAAATTTAGGTTTAAACTTTTGAAGCAAACTT
TTTTTTATCCTTGTGCACTGCAGGCCTGGTACTCAGATTTTGCTATGAGGTTAATGAAGTACCAAGCTGTGCTTGAA
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CCTCATAAAATACCTCTTCAAAATGCTTAAATTCATTTCACACATTAATTTTATCTCAGTCTTGAAGCCAATTCAGT
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ACAGTCTTCTCATCACTTCGTTTCTCCTATTTTGTTTTACTAGTTTTAAGATCAGAGTTCACTTTCTTTGGACTCTGC
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GTTTCAGTGCCTCAGACAAATGTGTATTTAACTTATGTAAAAGATAAGTCTGGAAATAAATGTCTGTTTATTTTTGT
ACTATTTAAAAAAAAAAAAAAAAATCGATGTCGACTCGAGTC

2) The Human cDNA Sequence for the COX 2 Gene, Showing the Primers and Amplification Area

CTTGCAGTGAGCGTCAGGAGCACGTCCAGGAACTCCTCAGCAGCGCCTCCTTCAGCTCCACAGCCAGACGCCCTCA
GACAGCAAAGCCTACCCCCGCGCCGCGCCCTGCCCGCCGCTGCGATGCTCGCCCGCGCCCTGCTGCTGTGCGCGGT
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TCCTCCTGTGCCTGATGATTGCCCGACTCCCTTGGGTGTCAAAGGTAAAAAGCAGCTTCCTGATTCAAATGAGATT
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AATATCAGATAATTGATGGAGAGATGTATCCTCCCACAGTCAAAGATACTCAGGCAGAGATGATCT (305 bases)
ACCCTCCTCAAGTCCCTGAGCATCTACGGTTTGCTGTGGGGCAGGAGGTCTTTGGTCTGGTGCCTGGTCTGATGAT
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TCCATATCACATTGCAAAAGTAGCAATGACCTCATAAAATACCTCTTCAAAATGCTTAAATTCATTTCACACATTA
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TTTCTTCTTTTAGCCATTTTGCTAAGAGACACAGTCTTCTCATCACTTCGTTTCTCCTATTTTGTTTTACTAGTCTTAA
GATCAGAGTTCACTTTCTTTGGACTCTGCCTATATTTTCTTACCTGAACTTTTGCAAGTTTTCAGGTAAACCTCAGCT
CAGGACTGCTATTTAGCTCCTCTTAAGAAGATTAAAAAAAAAAAAAAAA

3) The Human cDNA Sequence for the EGFR Gene, Showing the Primers and Amplification Area

CGCCGCCCAGACCGGACGACAGGCCACCTCGTCGGCGTCCGCCCGAGTCCCCGCCTCGCCGCCAACGCCACAACC
ACCGCGCACGGCCCCCTGACTCCGTCCAGTATTGATCGGGAGAGCCGGAGCGAGCTCTTCGGGGAGCAGCGATGC
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CCCTGGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCTGTGCCATCCAAACTGCACCTACGGATGCACTGG
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CAAATGGCATCTTTAAGGGCTCCACAGCTGAAAATGCAGAATACCTAAGGGTCGCGCCACAAAGCAGTGAATTTA
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AGGTCCTCCATCCCAACAGCCATGCCCGCATTAGCTCTTAGACCCACAGACTGGTTTTGCAACGTTTACACCGACT

AGCCAGGAAGTACTTCCACCTCGGGCACATTTTGGGAAGTTGCATTCCTTTGTCTTCAAACTGTGAAGCATTTACA
GAAACGCATCCAGCAAGAATATTGTCCCTTTGAGCAGAAATTTATCTTTCAAAGAG

4) The Human cDNA Sequence for the VEGF Gene, Showing the Primers and Amplification Area

AAGAGCTCCAGAGAGAAGTCGAGGAAGAGAGAGACGGGGTCAGAGAGAGCGCGCGGGCGTGCGAGCAGCGAAA
GCGACAGGGGCAAAGTGAGTGACCTGCTTTTGGGGGTGACCGCCGGAGCGCGGCGTGAGCCCTCCCCCTTGGGAT
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TCCGCGCGGGGGAAGCCGAGCCGAGCGGAGCCGCGAGAAGTGCTAGCTCGGGCCGGGAGGAGCCGCAGCCGGAG
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AGCCCTGGCGCTGAGCCTCTCTACCCCAGGTCAGACGGACAGAAAGACAAATCACAGGTTCCGGGATGAGGACAC
CGGCTCTGACCAGGAGTTTGGGGAGCTTCAGGACATTGCTGTGCTTTGGGGATTCCCTCCACATGCTGCACGCGCA
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TGGTCCTTCCCTTCCCTTCCCGAGGCACAGAGAGACAGGGCAGGATCCACGTGCCCATTGTGGAGGCAGAGAAAA
GAGAAAGTGTTTTATATACGGTACTTATTTAATATCCCTTTTTAATTAGAAATTAGAACAGTTAATTTAATTAAAGA
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TTGAAAGAGGGTTTCAATATACATCTACATACTATATATATATTGGGCAACTTGTATTTGTGTGTATATATATATAT
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CGTAGTACATATTTATTTTTAAACAACGACAAAGAAATACAGATATATCTTAAAAAAAAAAAA

Appendix L: Solutions

Chaps Lysis Buffer

1ml 1M Tris (pH 8) [final concentration 20mM]

1.875 ml 4M NaCl [final concentration 0.15M]

100µl 0.5M EDTA [final concentration 5mM]

310mg Chaps

1ml protease inhibitor

make up final volume 50ml with RO H2O

store at –4C

10% SDS solution

X % per 100g or 100ml

100g SDS up to 1L RO H20

Use the mask when dealing with SDS

Prepare 6 % SDS main Gel

2.5ml 1.5M Tris (pH 8.8) [18.165g Tris in 100ml H20]

2ml 30 % Bis/acrylamide 29:1 [pre-made in –4C]

100µl 10 % SDS

50µl 10 % ammonium persulphate [0.1g AP in 1ml, make fresh weekly at –4C]

10µl TEMED [in hood as neurotoxin]

5.35ml RO H2O

SDS Stacking Gel

1.25ml 0.5M Tris (pH 7.5)

0.65ml Bis/acrylamide 29:1

50µl 10 % SDS

25µl 10 % Ammonium persulphate

10µl TEMED (to be added when required)

3.05 ml RO H2O

Running Buffer x10 (use x1)

30.3g Tris

144g Glycine

100g SDS

RO H2O up to 1L

Blotting buffer

2.905g Tris

1.465g Glycine

0.5g SDS

100ml Methanol

RO H2O up to 1L

Primary wash buffer for Northern blot

6M Urea (360g)
0.4% SDS (4g)
0.5 x SCC (25ml 20xSCC)
to be kept at 2-8C.

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