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MODELLING THE GASTRIC EPITHELIUM FOR TESTING OF NEW  
CHEMICAL ENTITIES

KLAIRI MARIA KAVVADA  
Doctor of Philosophy

ASTON UNIVERSITY  
September 2004

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Aston University

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The surface epithelial cells of the stomach represent a major component of the gastric barrier. A cell culture model of the gastric epithelial cell surface would prove useful for biopharmaceutical screening of new chemical entities and dosage forms. A successful model should exhibit tight junction formation, maintenance of differentiation and polarity.

Conditions for primary culture of guinea-pig gastric mucous epithelial cell monolayers on Tissue Culture Plastic (TCP) and membrane inserts (Transwells) were established. Tight junction formation for cells grown on Transwells for three days was assessed by measurement of trans-epithelial resistance (TEER) and permeability of mannitol and fluorescein. Coating the polycarbonate filter with collagen IV, rather with collagen I, enhanced tight junction formation. TEER for cells grown on Transwells coated with collagen IV was close to that obtained with intact guinea-pig gastric epithelium *in vitro*. Differentiation was assessed by incorporation of [<sup>3</sup>H] glucosamine into glycoprotein and by activity of NADPH oxidase, which produces superoxide. Both of these measures were greater for cells grown on filters coated with collagen I than for cells grown on TCP, but no major difference was found between cells grown on collagens I and IV. However, monolayers grown on membranes coated with collagen IV exhibited apically polarized secretion of mucin and superoxide. The proportion of cells, which stained positively for mucin with periodic Schiff reagent, was greater than 95 % for all culture conditions.

Gastric epithelial monolayers grown on Transwells coated with collagen IV were able to withstand transient (30 min) apical acidification to pH 3, which was associated with a decrease in [<sup>3</sup>H] mannitol flux and an increase in TEER relative to pH 7.4. The model was used to provide the first direct demonstration that an NSAID (indomethacin) accumulated in gastric epithelial cells exposed to low apical pH.

In conclusion, guinea-pig epithelial cells cultured on collagen IV represent a promising model of the gastric surface epithelium suitable for screening procedures.

**Keywords:** stomach; mucous cell; Transwell; indomethacin; non-steroidal anti-inflammatory drug

*To my Parents*

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**Chapter 1**  
**General Introduction**



## 1.1 Background

Indisputably the oral route is the most common and convenient drug delivery pathway. As a consequence any novel technique that deals with the assessment of the rate, extent and mechanism of absorption of new chemical entities is very appealing. Thus, in an effort to develop novel strategies for delivery of drug candidates, arising from rational drug design and recombinant DNA technology, pharmaceutical scientists are developing cell culture techniques to study drug transport and metabolism at specific biological barriers (Audus *et al.*, 1990).

Important considerations in respect of the stomach are not so much the absorption of most drugs because the surface area for absorption in the small intestine is much larger but rather testing of novel pharmaceutical entities and dosage forms for effects on the gastric barrier. In addition specific classes of drug such as non steroidal anti-inflammatory drugs (NSAIDs) like indomethacin (refer to chapter 6) are weak acids and the low stomach pH will affect ionisation and possibly transfer.

## 1.2 Physiology and anatomy of the gastrointestinal tract

Information from the following references was used to write this section: Ito (1987), Allen *et al.* (1993) and Fox (1996).

### 1.2.1 Basic structure

The gastrointestinal tract exhibits the same basic structure throughout its entire length with specific modifications in certain regions to facilitate the digestive and absorptive process. The digestive tract can be divided into four major tissue layers: the mucosa, the submucosa, the muscularis and the serosa. The mucosa is created from a single layer of epithelial cells, the lamina propria (a subepithelial connective tissue that holds the epithelium in place and through which small blood capillaries, lymphatic vessels and nerves pass) and the muscularis mucosa

which is a thin layer of smooth muscle. The submucosa, is composed of dense connective tissue in which larger blood vessels and nerves including, the Meissner's plexus, and branching lymphatics are present. The muscularis propria consists of an inner circular layer, and an outer longitudinal layer of smooth muscle orientated at right angles to one another. Between these two muscular regions lie ganglion cell bodies and nerve trunks of the parasympathetic Auerbach's plexus. Finally, the outer region, the serosa, is a thin layer of loose connective tissue covered by a layer of squamous cells. The serosa secretes a fluid which lubricates and prevents friction between the digestive system and the surrounding area.

### **1.2.2 The stomach**

The stomach is an enlarged, specialised segment of the gastrointestinal tract between the oesophagus and small intestine that serves to store, as well as to process food, for absorption by the intestine. The stomach secretes acid and pepsin in order to reduce ingested food to chyme. Absorption is not a major function of the stomach, and water soluble substances are absorbed in limited quantities, although some fat-soluble compounds, such as ethanol, are absorbed readily and quickly. Invaginations of the gastric mucosa form the gastric gland.

The cardiac glands occupy the zone adjacent to the oesophagus where there is an abrupt transition from the stratified squamous epithelium to the simple columnar cells of the glandular mucosa. The surface and gastric pit cells of the cardiac glands are indistinguishable from those glands in the other regions in the stomach. The cardiac glands, however, are distinguished from the oxyntic glands by the absence of parietal and chief cells. Only mucous, undifferentiated, and endocrine cells are present.

A typical oxyntic gland (Figure 1.2.2) contains chief cells as the major cell type in the base of the gland; parietal cells are predominant in the isthmus and neck regions, and are

intermingled with mucous neck cells, undifferentiated cells, and a few chief cells. Surface mucous cells are present at the top of the gland. The endocrine cells occur as single cells sequestered between other cell types.

The pyloric antrum is adjacent to the oxyntic gland area of the corpus and is continuous with the pyloric sphincter, which joins the duodenum. The pyloric glands contain mucus secreting cells, which are similar to mucous neck cells in the oxyntic glands. The major source of gastrin is the G cells in the pyloric glands.

Gastric fundic glands are therefore composed of several cell types (see below). These functionally active cells come from multipotent stem cells that are found in the isthmus (Karam, 1993) of the gastric gland.



**Figure 1.2.2 Diagrammatically simplified tubular oxyntic gland from the corpus of a mammalian stomach. Diagram taken from Ito (1987).**



## **1.2.3 Cell types making up the stomach**

### **1.2.3.1 Surface mucous cells**

The ubiquitous surface mucous cell forms the free surface of the glandular stomach, lines the gastric pits, and intermingles with parietal cells in the isthmus of the gastric glands. Light microscope preparations of routine paraffin-embedded stomachs show surface mucous cells as columnar epithelial cells. The apical part of the cells is packed with mucous granules and the luminal surface of the mucous cells has short microvilli with a fuzzy surface coat (Figure 1.2.3.1). The amount of mucus stored in the cells is variable and depends on the stage of secretion or synthesis of mucus by the cell. Those cells on the surface usually contain more mucus granules than cells deeper in the gastric pits or isthmus of the glands. The nucleus of the surface mucous cell is located in the basal cytoplasm, may be extensively infolded, and contains a centrally located nucleolus. The cytoplasmic matrix has a moderate amount of rough-surfaced endoplasmic reticulum and free ribosomes. The mitochondria are smaller than, and not as numerous, as in parietal cells. Few lysosomes are present and endocytotic activity by these epithelial cells is limited.



**Figure 1.2.3.1 Surface Mucous Cell. Diagram taken from Ito (1987).**

### **1.2.3.2 Mucous neck cell**

In addition to the surface mucous cell, a second type of mucus-secreting cell is present in the gastric mucosa. The mucous neck cells are numerous in the neck and the isthmus region where there is transition from surface mucous cells to mucous neck cells. Mucous neck cells are frequently found adjacent to parietal cells, which tend to deform these mucous cells so that they have narrow or broad luminal surfaces and a slender central region. The mucous granules in the mucous neck cells differ in their fine structure from those in the surface mucous cells. The granules are larger and often found in the paranuclear region. They also contain mucin coded for by the MUC 6 gene rather than the MUC 5AC gene which is expressed in surface mucous cells (Ho *et al.*, 1995).

### **1.2.3.3 Parietal cells**

The most distinctive and characteristic gastric cell of the stomach is the parietal cell or oxyntic cell, named after its ability to secrete acid. Parietal cells are most numerous in the neck or isthmus of the oxyntic glands and are also present in the base of the glands, particularly in glands where chief cells are absent or scarce. Parietal cells are large oval to pyramidal cells measuring up to 25  $\mu\text{m}$  in their diameter with their bases bulging into the lamina propria. There are large numbers of mitochondria and on activation membrane structures called tubulovesicles fuse to form secretory canaliculi.

### **1.2.3.4 Gastrin (G) cells**

The G cell is the predominant cell type in the pyloric antrum. This type of cell secretes gastrin, which is important in the stimulation of gastric acid secretion. It is a medium sized cell and shares features common to other enteroendocrine cells. A typical basement lamina underlies the cell, and most cells have a narrow apical cell border with long microvilli.

### **1.2.3.5 Chief Cells**

The chief cell is a typical protein-secreting exocrine cell similar in morphology to the pancreatic acinar cell, but it synthesises, stores, and secretes by exocytosis pepsinogen the precursor of pepsin. Chief cells are predominately found in clusters at the base of the oxyntic gland and they are most abundant in the corpus of the stomach. Chief cells are absent in the cardiac glands and are rare in the pyloric glands.

#### 1.2.3.6 Argentaffin cells

Argentaffin cells are found in the oxyntic and pyloric glands. These cells which secrete serotonin and histamine, are about 300 nm in diameter, are flat, oval or crescent-shaped and have a narrow cytoplasmic extension to the gastric lumen.

#### 1.2.3.7 Other cell types

There are several additional cell types that have been described including the intraepithelial lymphocytes, globular leukocytes, caveolate cells and other endocrine cells.

#### 1.2.4 Mucus and bicarbonate

The continuous mucus gel, which lies over the epithelial surface, provides a viscoelastic unstirred layer which functions as physical barrier against the luminal contents and secreted pepsin. Three phases of gastric mucus can be identified: 1) presecreted mucus stored in the epithelial cells within intracellular vesicles, 2) a layer of mucus gel firmly adherent to the epithelial surface, and 3) mobile (largely soluble and partially degraded) luminal mucus mixed with the luminal contents. The distinct layer of adherent mucus gel provides a physical barrier and a stable unstirred layer between epithelial cell apical surfaces and the gastric lumen containing gastric secretions. Because luminal mucus is freely mobile and mixed with the gastric juice, it is unlikely that it has any mucosal protective role. (Allen *et al.*, 1993). The gastric mucosa secretes bicarbonate ( $\text{HCO}_3^-$ ) into the lumen. Secretion is stimulated by physiological stimuli such as the presence of acid in the gastric lumen and inhibited on stress induced sympathetic activation. The  $\text{HCO}_3^-$  secretion alkalinises the viscoelastic mucus gel adherent to the intact gastric mucosa and provides a first line of defence against luminal acid. Exposure of the gastric mucosa to damaging agents such as ethanol results in mucosal damage and induces



passive diffusion of interstitial fluid, including  $\text{HCO}_3^-$  across the mucosa. This passive diffusion of  $\text{HCO}_3^-$  alkalinises the fibrin mucoïd cap which forms over the damaged mucosa and is important in enabling the process of mucosal repair.

### **1.2.5 Proliferation and restitution**

Cells of the gastrointestinal tract have a rapid turnover rate, which makes the gastrointestinal mucosa one of the most rapidly proliferating tissues in the body, second to the skin (Davenport, 1982). The cell cycle for gastric mucous epithelial cells is 2 to 3 days with complete epithelia replacement in 4 to 6 days (Lipkin, 1987). This high proliferative rate contributes to the barrier function and the maintenance of the stomach in the face of noxious agents (Potten, 1998).

The gastric mucosa has a remarkable ability to repair damage. When the integrity of the superficial mucosa is breached, repair is dependent on the ability of the epithelial cells to migrate and subsequently to proliferate. Restitution is the process by which viable cells bordering the damaged area migrate to cover the denuded basal membrane. This process is rapid and *in vivo* can be accomplished within 15-60 min (Wallace and McKnight, 1990).

## **1.3 Transport of molecules across the gastrointestinal epithelia**

Information from the following references was used to write this section: Karacli (1989) and Artursson *et al.* (2001).



The transport of drugs across the epithelium may occur by: the paracellular and/ or the transcellular route. Transcellular transport is further divided into passive diffusion, carrier-mediated transport and transcytosis.

### **1.3.1. Paracellular transport**

Paracellular transport is the transport of molecules through the tight junctions bridging the intercellular spaces between the cells. Significant paracellular transport only occurs with very small ions (e.g.  $\text{Na}^+$  and  $\text{Cl}^-$ ) and small hydrophilic molecules like urea and mannitol. Hydrophilic molecules like mannitol show very limited transcellular transport and can therefore be used as indicators of paracellular permeability and thus tight-junction functionality. It is believed that new drugs discovered by combinatorial chemistry and high throughput pharmacological screening, could use the paracellular route as a means of transepithelial transport (Tavelin *et al.*, 1999).

### **1.3.2 Transcellular transport**

#### **1.3.2.1 Passive diffusion**

Passive diffusion refers to movement of a solute along its concentration and electrical gradient. As long as the diffusing molecule does not interact with the structural elements of the membrane, the driving force behind the diffusion of a molecule through the lipid bilayer and/ or the aqueous channels of the membrane is the chemical potential difference of the compound on the two sides of the membrane. Most approved drug products which are rapidly and completely absorbed following oral administration are transported by the passive transcellular route.

### **1.3.2.2 Carried mediated transport**

Carrier mediated transport can be either facilitated or active. Facilitated diffusion differs from passive diffusion in that the transport process is enhanced by proteins capable of reversibly binding specific substrates. The transport system can become saturated with the transported solute. In facilitated diffusion, the explicit provision of adenosine triphosphate (ATP), or an equivalent energy source, is not required since no transport against a concentration gradient takes place.

Active transport is similar to facilitated diffusion in that it also requires proteins in the membrane specific for ligands. Active transport, however is different from facilitated diffusion in that the solutes are transported against a concentration gradient and an energy source such as adenosine triphosphate (ATP) has to be supplied. Certain peptomimetic drugs use the di/tripeptide carrier mediated route for nutrients which is located in the apical membrane of small intestinal epithelial cells. 5-fluorouracil, biotin, cephalixin, bile salts and sugars use the active transport mechanism as an absorption route in the intestine.

Drugs that are slowly and incompletely passively absorbed, such as hydrophilic drugs (e.g. terbutaline and atenolol) and charged drugs, distribute poorly into cell membranes. It is therefore generally assumed that these drugs are transported through the paracellular pathway.

### **1.3.2.3 Transcytosis**

Receptor-mediated transcytosis involves the binding of membrane located receptors to macromolecules which are then transported across the cell in endocytosed vesicles. For example, cholesterol is transported into cells by binding to low density lipoprotein (LDL) at the apical membrane and is then transported via endocytosed vesicles.

## **1.4 Drug screening**

### **1.4.1 Models for screening for the transfer and toxicity of drugs in the gastrointestinal tract**

A key feature of any cellular *in vitro* model is that it can be grown as a monolayer in a compartmentalised system enabling study of the movement of solutes from an apical side to a basal compartment. Commercially-available cell culture inserts, with a variety of membranes on which cells are grown, are very useful in this context. A major supplier calls such inserts “Transwells” and this name will be used as a generic name in the thesis.

#### **1.4.1.1 Intestinal primary cultures**

Fully differentiated intestinal epithelial cells are very difficult to maintain in primary culture (Quaroni *et al.* 1979), and do not appear to have been grown in compartmentalised systems. Chew *et al.* (1998) have successfully cultured rat duodenocytes on Matrigel but monolayers were not formed.

#### **1.4.1.2 Cell lines**

There are three human intestinal adenocarcinoma cell lines that have gained prominence due to their capability to differentiate in culture, these are Caco-2, HT 29 and T 84 (Neutra & Louvard, 1989). All three form monolayers and have been used as transport models, although for T 84 cells, this is a minor application (Dias and Yatscoff, 1994). The Caco-2 cell line is a homogenous cell population derived from colon. It spontaneously differentiates in culture to resemble enterocytes of the small intestine. Although Caco-2 cells express microvillar hydrolases (Pinto *et al.*, 1983), the high TEER, ion conductances and permeability properties resemble colonic crypt cells (Grasset *et al.*, 1984). Caco-2 cells grown in Transwells can be used to



identify drugs with potential absorption problems, and to select drugs with optimal passive absorption characteristics (Artusson *et al.*, 2001). T 84 cells, while spontaneously forming well-polarised monolayers with high TEER, do not have a brush border membrane or hydrolyse activity (Neutra & Louvard, 1989). HT 29 cells do not display differentiated characteristics unless grown in the absence of glucose (Rousset, 1986). Under the appropriate growth conditions, HT 29 cells become highly polarised, express microvillar hydrolases and display phenotypes resembling enterocytes and goblet cells (Zweibaum *et al.*, 1985; Pinto *et al.*, 1982). Several subclones have been isolated from the multipotent HT 29-18 clone that differentiate as either absorptive or mucus-secreting cells (Huet *et al.*, 1987).

Although the Caco-2 model mimics well the dominant *in vivo* passive transcellular drug transport route, this model is not quantitatively predictive of passage by the paracellular route and does not necessarily reflect the various active drug transport, efflux and metabolism systems that are present in the various cells of the intestinal epithelium. (Lennernäs, H. *et al.*, 1996). It should be also noted that the Caco-2 cell line originates from the colon and some characteristics like paracellular transport in the Caco-2 cell line are much lower than is thought to be the case of the small intestinal epithelium. For this reason Tavelin *et al.* (1999) developed a cell culture model that mimicked the human tight-junctional jejunal permeability better than Caco-2 cells. The 2/4/A1 cell line is a rat intestinal epithelial cell line, conditionally immortalised with a temperature-sensitive mutant of the growth-promoting oncogene simian virus 40 (SV40). Monolayers form when the temperature is raised above the permissive temperature and can be used for studies of the paracellular barrier to drug transport (Tavelin *et al.*, 1999).

The rat small intestinal cell line, IEC-18 which is less differentiated than the Caco-2 cells, provides a size-selective barrier for paracellularly transported hydrophilic macromolecules and

could be a useful *in vitro* system for the prediction of passive transport across the intestinal barrier (Versantvoort *et al.*, 2002).

Caco-2, 2/4/A1 and IEC-18 monolayers have been grown on Transwells as models of the small intestinal epithelium suitable for drug permeability screening procedures. Automatic procedures for screening of drug transport in Caco-2 monolayers and other monolayers using robotics are routinely used in the pharmaceutical industry.

#### 1.4.1.3 Artificial Membranes

Parallel artificial membrane permeability assay (PAMPA) which was first introduced in 1998, can quickly provide information about passive transcellular permeability that is not complicated by other mechanisms (e.g. paracellular transport, active transport, metabolism). The procedure is used increasingly in pharmaceutical research (e.g. Kansy *et al.*, 1998; Ruell *et al.*, 2003). In PAMPA, a “sandwich” is formed with solutions of test compounds separated in a 96-well microtitre plate separated from a 96-well plate filled with buffer (initially free of test compounds), by a lipophilic microfilter. The microfilter is either impregnated with an alkane (e.g. hexadecane, dodecane) or a solution of 1–74% (w/v) phospholipid in alkane, forming a rate-limiting artificial membrane barrier across which test compounds can migrate by diffusion. Both sides of the artificial membrane barrier are in contact with an unstirred water layer, which becomes thinner with increased plate shaking. The lipid membrane and the unstirred water layer, each contribute a resistance to the movement of permeating species. For lipophilic molecules, this is the rate-limiting barrier to transport (Nielsen and Avdeef, 2004). This system can also be used with a pH gradient across the barrier. The method demonstrated excellent correlation with passive transport by Caco-2 cells (Wohnsland and Faller, 2001).



### 1.4.2 Stomach Preparations

These preparations are either intact sheets of tissue, which have limited survival *in vitro*, or monolayers constructed from cell lines or primary cultures.

#### 1.4.2.1 Tissue sheets *in vitro*

Intact sheets of gastric mucosa can be mounted in Ussing chambers in order to produce an *in vitro* model of the gastric mucosa. The effects of salicylates on the synthesis of proteins by gastric mucosal tissue (Spohn and McColl, 1980) and the first pass effect of 1-naphtol, which is a model drug for the assessment of glucuronidation and sulfation (Schwenk *et al.*, 1992), have been investigated with this *in vitro* model. This model can also be used as a restitution model by exposing the luminal surface to damaging agents. Thus [ $H^3$ ] mannitol flux was used as a paracellular marker to assess the role of nitric oxide (Yanaka *et al.*, 1995) and of epidermal growth factor (EGF) (Yanaka *et al.*, 2002) in restitution of injured gastric mucosa.

#### 1.4.2.2 Gastric cell lines

Most human gastric cell lines came from adenocarcinomas and are mainly used for screening anti-tumoral drugs. The human gastric cell line known as AGS has been characterised as being quite similar to gastric mucous cells, since cells react positively to periodic acid Schiff stain and have the ability to differentiate when postconfluent (Kokoska *et al.*, 1998). AGS monolayers have been used to quantitatively assess the adherence of *Helicobacter pylori* (Logan *et al.*, (1998) and the damaging effect of indomethacin in human gastric cells (Kokoska *et al.*, 1998).

MKN-28 is a well-differentiated human gastric epithelial cell line (Hojio, 1977). This cell line retains the characteristics of epithelial cells and gave similar quantitative and qualitative

results to human gastric epithelial cells in primary culture when exposed to damage by indomethacin or when acetaminophen was used to protect against damage by sodium taurocholate (Romano *et al.*, 1988).

MKN-28 and MKN-45 cell lines have also been used for the investigation of the effect of rebamipide, an anti-ulcer agent, on adhesion of *Helicobacter pylori* to gastric epithelial cells (Hayashi *et al.*, 1998). The manner of adhesion may differ between these cells and normal human gastric mucosal cells. However, normal human gastric cells are not readily available for laboratory adhesion assays and such cells, obtained from biopsied or surgical specimens, may show heterogenous characteristics (Hayashi *et al.*, 1998).

Other cell lines such as SGC-7901 cells, which is a moderate-differentiated gastric carcinoma cell line, have been used as models to determine the antitumor effects of salvicine in inhibiting cell growth and inducing apoptosis (Qing *et al.*, 2001).

The KATO III cell line originated from a human gastric tumour and grows in suspension. Cytologically, the cells have features which resemble cells in signet ring gastric carcinoma (Sekigushi *et al.*, 1978). A study on KATO III cells lead to the conclusion that gallic acid could be a candidate drug for digestive gut cancer treatment to overcome the resistance to chemotherapeutic drugs (Yoshioka *et al.*, 2000).

The need for non-transformed cell lines that mimic the *in vivo* physiological state of the gastric epithelial cells has become more apparent over the years. The best models displaying partially differentiated epithelial phenotypes so far are the mucous cell lines GMS 06 and RGM1 (Hollande *et al.*, 2001).

The first report on the establishment of gastric surface mucous cell lines from animals was by Sugiyama *et al.* (1993). The GMS 06 cell line is an *in vitro* model of gastric mucosa for physiological and pharmacological studies. It is derived from transgenic mice and harbours a

thermosensitive version of the SV40 large T antigen. One of the main advantages of this model is that cells can be grown at the permissive temperature of 33 °C and then the expression of the large T antigen can be repressed by raising the temperature to 39 °C which allows differentiation of the cells.

The RGM-1 cell line is a diploid and non-transformed epithelial cell line isolated from the gastric mucosa of a normal Wistar rat (Kobayashi *et al.*, 1996). This cell model is mainly used to study the effects of NSAIDs (e.g. Kusuvara *et al.*, 1998). Two cell damage models induced by acid (pH 3.5-5.0) and pepsin respectively were created by using RGM-1 cell lines. These cell damage models involving RGM-1 are useful for studying the mechanism underlying cell damage and for the screening of cytoprotective drugs (Furukawa *et al.*, 1997).

Wounds can be created in confluent monolayers of RGM-1 cells by scraping with a razor blade. Following wounding significant re-epithelialization occurs by 24 h. Re-epithelialization is a crucial factor in both gastrointestinal mucosal injury and ulcer healing. Thus this model was used to compare the effects selective and non-selective Cox-2 inhibitors on wound re-epithelialization (Giap *et al.* 2002).

A relatively novel non-tumoral gastric epithelial cell line IMGE-5 was obtained from the stomach of transgenic mice harbouring a thermosensitive version of the SV40 large T antigen and can be used for the studies of the proliferation and differentiation of gastric epithelial cells. This cell line is responsive to several hormones and growth factors and displays temperature-sensitive functional expression of adherens and tight junctional proteins (Hollande *et al.*, 2001).

One of the advantages of cell lines is that cytotoxicity or screening studies can be performed using different cell lines simultaneously. For example the effects of aspirin on gastric epithelial proliferation and cytokine expression was assessed on AGS, KATO III and RGM-1 cell lines or the tumoricidal effect of a novel carbazole topoisomerase poison was assessed not only



on human cancer gastric cell lines NS-3, HCG-27, MKN-28, and MKN-74 but as well as on human lung, colon and breast cancer cell lines (Nakamura *et al.*, 2003).

#### **1.4.2.3 Primary gastric cultures**

Most studies on the secretory or proliferative functions of gastric cells have been performed on cells in primary culture. Gastric epithelial cells grown in tissue culture offer a valuable tool to evaluate the intrinsic ability of gastric cells to resist exogenous injury. Furthermore, culture isolates cells from the input of the autonomic nervous system and the influence of endocrine and other regulatory cells that are normally resident in the gastric epithelium. This will permit the study of the factors that directly regulate gastric mucous cell functions (Terano *et al.* 2001). Cells in primary culture are more likely to resemble normal cells than gastric cancer cell lines.

However, the main disadvantage of primary cultures of gastric mucosal cells is that they cannot be maintained by subculturing over a long time because of factors such as fibroblast overgrowth (Terano *et al.*, 1982).

##### **1.4.2.3.1 Human**

Primary culture gastric epithelial cells have been prepared from biopsy specimens (Terano *et al.*, 1983) or from surgical specimens from adult humans (Rutten *et al.*, 1996). Terano *et al.* (1983) described a technique for the culture of normal human gastric mucosal cells using specimens obtained by routine endoscopic biopsy. Under phase contrast microscopy, all cells had epithelial characteristics. Ninety percent of cells contained periodic acid Schiff reaction-positive mucous granules and two percent of the cells were identified as parietal cells (Terano, 1983). However, biopsy specimens yield small amounts of tissue although there is minimal risk to the

donor. Rutten *et al.* (1996) have successfully isolated human gastric epithelial cells from gastric tissue obtained from *Helicobacter pylori* free patients undergoing surgical gastrectomy. The monolayers were cultured on 0.45 µm pore filters. This system has been used for the identification of a functional  $\text{Ca}^{2+}$ -sensing receptor in normal human gastric mucous cells (Rutten *et al.*, 1999) and for the examination of the effects of *Helicobacter pylori* on intracellular signalling (Marlink *et al.*, 2003). Yet provision of surgical specimens is dependent on patient consent, timing of operations, and cooperation of surgical and pathology departments.

Basque *et al.* (1999) established a primary culture which is representative of the human gastric epithelium. The monolayers, prepared from 17-20 week-old foetuses, were cultured on Tissue Culture Plastic (TCP) and maintained in culture for at least seven days, at which stage they comprised of 60 % mucus-secreting cells, 25 % chief cells, 5 % parietal cells and a small amount of mitotic precursors (Basque *et al.*, 1999). This model was used to assess the effect of extracellular matrix components such as laminins and the growth factor TGF- $\beta$  on cell polarity and functionality (Basque *et al.*, 2002). A potential application of this primary culture system would involve studying the pathogenesis of infection by *Helicobacter pylori*.

#### **1.4.2.3.2 Rabbit**

The first primary rabbit gastric epithelial cell cultures were established using foetal tissue. Logson *et al.* (1982) cultured foetal rabbit gastric epithelial cells on floating collagen gels that were subsequently mounted in Ussing chambers. Although the persisting cell-type comprising the monolayers were columnar, it seems unlikely that these cells were fully differentiated gastric epithelial cells. Another example of a foetal gastric epithelial monolayer was that produced by Matuoka *et al.* (1983). The culture consisted mainly of surface mucous cells (50%-60% of the



total cell population) and produced prostaglandins (predominantly I<sub>2</sub>). The addition of acetylsalicylic acid to the culture medium caused marked inhibition of prostaglandin production by the cultured gastric epithelial cells.

Cultured cells from adult animals are preferable, since they should reflect more accurately the physiological features of cells in mature animals. Thus in 1990, Ota *et al.*, developed a method for the culture of gastric mucous cells from adult rabbits. The monolayer was mainly composed of mucous neck cells (92%) and other cell-types such as parietal cells, chief and surface mucous cells were also present at a smaller percentage. A modification of this model has been used to study on the effect of hepatocyte growth factor (HGF) on cell proliferation and migration in primary gastric epithelial culture (Takahashi *et al.*, 1995). The modification involved the production of round-wounds with a custom-made scraper on confluent monolayers grown on TCP. A method by Watanabe *et al.* (1994) for the culture of rabbit gastric mucosal monolayers has been extensively used as a wound restitution model. Most of the cultured cells comprising the monolayers were morphologically epithelial-like, and 80 % to 90 % of the cells were periodic acid Schiff reaction-positive mucous cells. Examples of the use of this model include the effect of platelet-derived growth factor on gastric epithelial restoration (Watanabe *et al.*, 1996) and the investigation of extracellular matrix components, such as collagen I, collagen IV and laminin, in attachment, migration and repair of wounded cultured gastric cells (Fujiwara *et al.*, 1995). Furthermore, the effects on gastric cell restitution of anti-ulcer drugs such rebamipide (Watanabe *et al.*, 1998) and teprenone (Watanabe *et al.*, 1998) were studied. Instead of using a silicon tip to produce the wound on confluent monolayers, an *in vitro* model of gastric epithelial cell injury based on the protocol of Watanabe *et al.* (1994) could also be produced by the addition of NSAIDs such as aspirin (Watanabe, 2002) or indomethacin (Takahashi and Okabe, 1996).



#### 1.4.2.3.3 Rat

Rat gastric mucosal cell cultures have been successfully used to study the effect of damaging and protective agents on gastric mucosa. Terano *et al.* (1982) established a method for cell culture of the gastric mucosa from newborn rats. The cells formed confluent monolayers with 90 % of cells being surface mucous and mucous neck cells, 5 % parietal cells and less than 5 % fibroblasts. This method has proved successful and it allows the evaluation of the effects of anti-ulcerogenic agents and the direct measurement of cytokine production as well as enzyme activities in the mucous cells. The system has also been used to investigate the roles of free radicals or scavengers in gastric cell damage or its prevention (Hiraishi *et al.*, 1987; Hiraishi *et al.*, 1994, Terano *et al.*, 1989). Thus a role of intracellular glutathione against ethanol induced damage has also been demonstrated using this system (Mutoh *et al.*, 1991). Terano's cell culture has proved to be a useful *in vitro* model for ulcer repair and restitution. The effect of HGF and other growth factors was studied using this model as a gastric epithelial restitution model. As with rabbit gastric epithelial monolayers, confluent monolayers grown on tissue culture plates can be wounded with a custom-made scraper (Horie-Sakata *et al.*, 1998). Damage could also be induced by taurocholate in order to assess the cytoprotective effect of acetaminophen (Ota *et al.*, 1988) or induced by hydrogen peroxide or ethanol in order to investigate the antioxidant effects of a novel anti ulcer agent polaprezinc (Hiraishi, 1999). Even though the method developed by Terano *et al.* (1982) has been widely adopted, it should be remembered that the preparation comes from newborn rats and may therefore not accurately reflect the functions of adult gastric cells.

Ota *et al.* (1988) modified Terano's technique of newborn rat gastric cell culture in order to culture gastric cells from adult rats for a study on the effect of salicylate on these particular cells. Although the cells formed monolayers on collagen coated cultured dishes, and 94 % of the

cells contained periodic acid Schiff reaction-positive mucous cells, this technique has not subsequently been used by the group or by any other researchers. Thus, the success of this technique and the ability of gastric epithelial cells from adult rats to adhere on culture dishes and to reach confluence should be accepted with reservation.

#### **1.4.2.3.4 Canine**

One of the first primary canine mucous cell cultures was developed to study the synthesis, processing, and secretion of gastric mucin (Boland *et al.*, 1990). The cells did not form monolayers but the preparation consisted of dispersed mucous epithelial cells. The same system was used to study the synthesis and prostaglandin E<sub>2</sub>-induced secretion of surfactant phospholipid by isolated gastric mucous cells (Scheiman *et al.*, 1991). Canine chief cell monolayers, grown on collagen gels and mounted in Ussing chambers, have been used to study the acid-barrier function of chief cells (Sanders *et al.*, 1985). The monolayers were able to resist apical acidification down to pH 2.5.

With the exception of the human cell preparation of Rutten *et al.* (1999) none of the above primary cultures from a variety of species have been grown on Transwells. Chen *et al.* (1994) developed a monolayer, cultured on Transwells, which was composed of 65 % pepsinogen-secreting chief cells and 24 % of acid-secreting parietal cells. This preparation is therefore a model for the interior of the gastric gland rather than the surface of the epithelium. These monolayers were also able to resist apical acidification down to pH 2.5.

#### **1.4.2.3.5 Guinea pig**

In 1985 Rattner *et al.* (1985) developed a method for isolation and separation of guinea-pig gastric mucous cells. This was the first monolayer of adult gastric mucous cells grown in



collagen cups (Rattner *et al.*, 1985). The monolayer comprised 65 % mucous cells, 25 to 30 % chief cells, and 5 to 10 % parietal cells, a few endocrine cells and there were no identifiable fibroblasts. This system was used to study the electrophysiology of gastric epithelial cell monolayers (Rutten *et al.*, 1985) and a modification of this method has been used in order to examine the role of extracellular matrix constituents such as collagen IV on epithelial cell adhesion (Rhodes *et al.*, 1994). Furthermore Rattner's *et al.* (1985) technique has been used to isolate and culture guinea-pig gastric epithelial cells on tissue culture plastic and investigate the mitogenic effects of growth factors such as epidermal growth factor (EGF), transforming growth factor alpha (TGF- $\alpha$ ) or insulin on gastric epithelial cells (Rutten, *et al.*, 1991, Rutten *et al.*, 1993). The effects of the above growth factors on proliferation of guinea-pig gastric mucous epithelial cells have also been studied by Sakamoto *et al.* (1994), Matsuda *et al.* (1996), Ogihara *et al.* (1996), and Rokutan *et al.* (1998).

The isolation method used in this current study is based on a method by Nakamura *et al.* (1991) which is a development of the original work of Rattner *et al.* (1985), and which was used to study the induction of heat shock proteins and their role in protection against ethanol induced damage. The preparation contained more than 95 % of cells with periodic acid Schiff reaction-positive mucous granules and less than 5 % were identified as parietal cells (Nakamura *et al.*, 1991). The main difference from Rattner *et al.* (1985) was the finding that enrichment of mucous cells prior to plating was unnecessary in order to obtain a monolayer, which after 3 days, consisted predominantly of mucous cells. Nakamura *et al.* have successfully used this gastric epithelial model to examine a variety of cellular functions.

Mucous cell apoptosis has been extensively studied using guinea-pig gastric epithelial monolayers mainly for two reasons. Firstly, accelerated apoptosis is a feature of gastric mucosa



infected with *Helicobacter pylori* (Moss *et al.*, 1996) and secondly it has been demonstrated that apoptosis is increased in the stomach of human patients taking NSAIDs (Zhu *et al.*, 1998). Thus, the role of exogenous nitric oxide on apoptosis in guinea-pig gastric mucous cells (Potter and Hanson, 2000) and the role of superoxide, which is produced by surface mucous cells, in regulation of growth and apoptosis on cultured mucosal cells (Teshima *et al.*, 2000) has been investigated. Moreover, the spontaneous apoptotic DNA fragmentation in cultured guinea pig gastric mucosal cells (Tsutsumi *et al.*, 2000) and *Helicobacter pylori*-induced apoptosis in cultured guinea pig gastric mucosal cells (Kawahara *et al.*, 2001) have been demonstrated.

The model based on Nakamura's initial isolation and culture method has also been used to investigate the effects of various NSAIDs on apoptosis in guinea-pig gastric epithelial mucous cells. Thus, the effects of flubiprofen (Johal and Hanson, 2000), ibuprofen and diclofenac (Ashton and Hanson, 2002); and aspirin, indomethacin and cycloheximide (Tomisato *et al.*, 2001) on apoptosis in guinea pig gastric mucosal cells has been elucidated.

As it must have been made clear from the sections above, guinea-pig gastric epithelial cell preparation are by far the best characterised and studied gastric epithelial cells from adult animals, but their use in a compartmental system has been limited and they do not appear to have been grown on Transwells.

## **1.5 Conclusion**

Monolayers of gastrointestinal cell lines such as Caco-2 (Artursson *et al.*, 2001), 2/4/A1 (Tavelin *et al.*, 1999) and IEC-18 (Versantvoort *et al.* 2002) have been grown on Transwells and used as models for intestinal permeability. However, orally administered drugs and dosage forms first come into contact with the gastric epithelium, the surface epithelial cells of which represent a major component of the gastric barrier. In the acid-secreting (fundic) region of the stomach,

the mucous epithelial cells which cover the gastric surface and extend downwards into the gastric pits, are structurally and functionally distinct from the intestinal cells represented by the cell lines mentioned above. We sought to use Transwells to develop a primary culture model of the gastric surface suitable for biopharmaceutical screening of new chemical entities and dosage forms. In particular there is a need for a preparation suitable for screening compounds and dosage forms for their effects on the integrity of the gastric barrier and that will assess the effects of such agents on cellular viability and on gastric paracellular permeability. A critical feature on any such preparation is that it should function in the presence of a reduced luminal pH to mimic the *in vivo* situation, since resistance to acid will enable examination of the effect of the low gastric pH on drug uptake, particularly the possibility of intracellular trapping of drugs with a low pKa.

## **1.6 Aims and Objectives**

### **1.6.1 Stage 1**

a) To investigate the attachment and growth of guinea-pig gastric epithelial cells on TCP and to determine the time-scale of cell survival in culture; b) to compare growth on TCP with growth on various biodegradable polymeric surfaces and on Transwells. The ultimate aim of stage 1 was to optimise the conditions of culture in order to produce a confluent cell monolayer on Transwells with fully developed tight junctions suitable to use as an *in vitro* model.

### **1.6.2 Stage 2**

To assess the effect of the matrix used to coat the Transwells on: a) the proportion of mucous cells; b) tight junction formation as assessed by apical to basal transfer of fluorescein and [<sup>14</sup>C]mannitol, and from the trans-epithelial electrical resistance (TEER) of the monolayers; c) the differentiation and polarity of the gastric epithelial monolayers as assessed by the synthesis

and secretion of mucin and superoxide; and d) to employ confocal microscopy on monolayers grown on collagen IV to indicate the intracellular location of mucin granules.

### **1.6.3 Stage 3**

To assess the ability of gastric epithelial cells grown on Transwells to resist acidification for 30 min by measuring [<sup>14</sup>C] mannitol flux and TEER for monolayers cultured on collagen IV coated Transwells.

### **1.6.4 Stage 4**

To examine the effect of the pH of the apical incubation medium on the transfer of the acidic drug indomethacin (pKa 4.5) across a gastric mucous epithelial cell monolayer, and to determine whether indomethacin accumulates in cells exposed to a low apical pH.



**Chapter 2**  
**General methods**

## **2.1 Introduction**

This chapter describes methodology which was used throughout the project. Methodologies, which were used only in association with work described in a particular chapter, are described in the relevant chapter.

## **2.2 Cell culture**

All cell culture procedures were carried out under aseptic conditions in a Gelaire, biohazard level II, laminar flow cabinet from ICN (Thame, Oxfordshire, UK).

### **2.2.1 Animals**

Dunkin-Hartley guinea-pigs of 200-400g body weight (3-8 weeks-old) were obtained from B&K Universal Limited, Grimston, East Yorkshire and were fed on SDS Economy guinea-pig diet supplied by Lillico, Betchworth, Surrey, UK.

### **2.2.2 Culture medium**

Complete culture medium was used in culturing gastric epithelial cells in TCP, Transwells and on polymeric supports. Complete culture medium was RPMI 1640 containing 10 % foetal calf serum (FCS), 100 u/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml of amphotericin B. The medium was stored at 4 °C and made up fresh for each cell preparation.

### **2.2.3 Culture medium in presence of 10ng/ml EGF**

After a series of pilot experiments (please refer to Chapter 3), it was concluded that the 10 ng/ml EGF would be present throughout culture.

#### 2.2.4 Cell preparation procedures

The cell isolation was based upon a method previously described by Ashton and Hanson (2002). The stomach was removed from non-fasted guinea-pigs which had been anaesthetised with intramuscular “hypnorm” (1.0 ml/kg body weight) and intraperitoneal diazepam (3.75 mg/kg). Fundic mucosa was scraped off and minced with curved scissors in 4 ml of RPMI 1640 containing 2g/l bovine serum albumin (isolation medium). The mucosal pieces were incubated with 45 ml of isolation medium containing 0.5 mg/l pronase for 20 min at 37 °C, shaking at 120 cycles/min and gassing with 95 % O<sub>2</sub> 5 % CO<sub>2</sub>. The mixture was then centrifuged at 250 x g for 2 min at 15 °C. Unless described to the contrary the resulting pellet was resuspended in 45 ml of isolation medium containing 0.4 mg/l collagenase, and incubated, in a flask with shaking for 40 min at 37 °C as described above. The contents of the flask were filtered through a 150 µm nylon mesh, centrifuged as previously and were resuspended under sterile conditions in complete culture medium. The resulting cell suspension was centrifuged at 200 x g for 2 min at 15 °C, and then resuspended in culture medium in the presence of 10 ng/ml EGF at approximately 10<sup>6</sup> cells per ml under normal circumstances. Experiments to assess monolayer formation were performed with Transwells fitted with polycarbonate membranes of 24 mm diameter, 3 or 0.4 µm pore size (1.5 ml of cell suspension per well). Cells grown on Transwells were compared with those grown on six-well plastic culture plates (Gibco, 2 ml cell suspension per well) to establish the impact of apical and basal compartments on the incorporation of [<sup>3</sup>H]glucosamine into glycoprotein and on the production of superoxide. 12 mm Transwells with 0.4 µm polycarbonate membranes (0.5 ml of cell suspension per well) were used for experiments on the effects of acidification of the apical medium and for screening. The medium was changed at 24 and 48 h.



At 72 h cells were fully confluent, and unless described to the contrary, experiments were initiated at that time.

## **2.2.5 Collagen coating**

### **2.2.5.1 Preparation of stock solution of collagen I (100 µg/ml)**

250 ml of 0.1M acetic acid was prepared by adding 1.5 g of glacial acetic acid to 250 ml of double distilled water. Then in a 100 ml sterile glass bottle, 100 ml of the 0.1 M acetic acid was added together with 10 mg collagen (Type I Rat Tail). The mixture was stirred with a magnetic stirrer bar for 1 to 3 h until all of the collagen had dissolved. In order to sterilize the collagen solution, 10 ml of ANALAR chloroform was added. The biphasic solution which must not be stirred or shaken was left at 4 °C overnight. The sterile collagen was removed under aseptic conditions and subsequently stored at 4 °C.

### **2.2.5.2 Preparation of stock solution of collagen IV (100 µg/ml)**

In non-sterile conditions 0.25 % acetic acid was made by adding 0.25g of glacial acetic acid to 100 ml of double distilled water. In order to make a collagen IV stock solution, 5 mg of collagen IV from human placenta was added to 5 ml of 0.25 % acetic acid. The resulting solution was left to dissolve for approximately five h at 2 °C with very gentle stirring. To ensure that the collagen fully dissolved, the solution was left at 2 °C overnight but without any stirring. In order to produce a 100 µg/ml collagen IV solution, 5 ml of the stock solution were added to 45 ml of 0.25 % acetic acid.

### **2.2.5.3 Preparation of stock solution of collagen IV plus laminin**

10 µg/ml mouse laminin in phosphate-buffered saline (138 mM NaCl, 2.7 mM KCl, 10 mM phosphate pH 7.4) was added to the 100 µg/ml collagen solution to produce a laminin concentration of 6.23 µg/ml .

### **2.2.5.4 Collagen I coating procedure for tissue culture plastic**

In the culture hood 500 µl collagen solution (100 µg/ml) was added to each well of a 12 well plate. The plates were left for 6 h at 37 °C. Any excess fluid was removed by suction and plates were then left to dry overnight in the hood under UV illumination with the lids off. About 1 hour before use coated wells were filled with 1 ml complete culture medium and the latter was removed prior to seeding.

### **2.2.5.5 Collagen coating (collagen I, collagen IV or collagen IV plus laminin) of Transwells**

In the culture hood 2.6 ml collagen solution was added to each basal well and 1.5 ml collagen solution was added to the apical well for 24 mm diameter Transwells, and 1.5 ml collagen solution to each basal well and 0.5 ml collagen solution was added to each apical well for 12 mm Transwells. The plates were left for 6 h at 37 °C. Any excess fluid was removed by suction and plates were then left to dry overnight in the hood under UV illumination with the lids off. About 1 h before use basal wells were filled with 2.6 ml complete culture medium and Transwells with 1.5 ml for experiments with 24 mm Transwells. For experiments with 12 mm diameter Transwells 1.5 ml of complete culture medium was added to the basal well and 0.5 ml to the Transwell. The culture medium was removed just before use.

### **2.3 Removal of cells by use of Trypsin/EDTA**

The principle behind the disaggregation treatment with Trypsin/EDTA (0.2 g/l EDTA.4Na and 0.5 g/l trypsin in HBSS with phenol red) is that trypsin hydrolyses the extracellular matrix and EDTA, by chelating  $\text{Ca}^{2+}$ , breaks intercellular junctions. After the Trypsin/EDTA treatment the cell suspension was added to an equal volume of complete culture medium in order to halt digestion as FBS contains a trypsin inhibitor.

For TCP, medium was removed from the wells of a 12 well plate in the culture hood and 1 ml Trypsin/EDTA at 37 °C was added to each well. After 15 min of incubation at 37 °C, the plates were transferred to the culture hood, where medium was removed from each well and transferred to 2 ml microfuge tubes to which 1 ml of culture medium had been added previously. Samples were left on ice prior to cell counting.

For 24 mm diameter Transwells, both apical and basal media were removed by gentle suction and were replaced by 1 ml per apical well and 1.7 ml per basal well of Trypsin/EDTA solution. After 20 min of incubation at 37 °C, the plates were transferred to the culture hood, where 1 ml of the apical Trypsin/EDTA was removed with a pipette and vigorously dispensed over the surface of the Transwell. 1 ml of complete medium was dispensed into the Transwell, the Transwell was then swirled around, and the contents then transferred to a 2 ml microfuge tube. Samples were left on ice prior to cell counting.



## **2.4 Cell counting**

A haemocytometer was used as a routine for initial cell concentration determination before plating of cells or for quantification of the cells comprising the monolayers at different time points during culture.

With the cover slip in place, 10  $\mu$ l of cell suspension, acquired by Trypsin/EDTA treatment, was transferred to one chamber of the haemocytometer. Carefully the edge of the cover slip is touched with the pipette tip to allow the chamber to fill by capillary action. The chamber must not be overfilled or under-filled. The large central square (1 mm<sup>2</sup>) in a standard haemocytometer is divided by double lines into 5 x 5 squares; each of which is composed of 5 x 5 smaller squares. Regulation of cell counting was achieved by counting only the cells which touched the top and left lines of each subdivided square. Each square of the haemocytometer, with the cover slip in place, represents a total volume of 0.1 mm<sup>3</sup> or 10<sup>-4</sup> cm<sup>3</sup>. Since 1 cm<sup>3</sup> is equivalent to approximately 1 ml, the subsequent cell concentration and hence the total cell number was determined using the following calculation:

$$\text{Number of cells/ml} = \text{the average count per square} \times 1 \times 10^4$$

where 1 x 10<sup>4</sup> is the volume of the large square. Multiplication by 2 then gives cells/well.

## **2.5 Protein Assay**

### **2.5.1 Preparation of standards**

Standards (please refer to table 2.5.1.) were prepared from 0.8 mg/ml bovine serum albumin (BSA) stock solution.

**Table 2.5.1**

<b>Standard</b>	<b>Stock (0.8 mg/ml)</b>	<b>Buffer*</b>	<b>Protein Conc<sup>n</sup></b>
	<b>µl</b>	<b>µl</b>	<b>mg/ml</b>
1	5	45	0.08
2	10	40	0.16
3	15	35	0.24
4	20	30	0.32
5	25	25	0.40
6	30	20	0.48
7	35	15	0.56
8	40	10	0.64
9	45	5	0.72
10	Undiluted	0	0.80

\*The buffer used to make the standards was the same as that used to dissolve the cells, so for section 4.6.2, 0.1 M NaOH/ 1% SDS was used, for section 4.7, PBS/ 1% Triton was used, and for section 4.8.1 sample buffer with distilled water in a 1: 3 ratio was used.

### **2.5.2 BCA assay**

The protein content of monolayers was determined using the Pierce bicinchoninic acid (BCA) protein assay kit. Protein concentrations were determined by this method for cell pellets and for samples prepared for gel electrophoresis.

The reagent system combines the reaction of protein with  $\text{Cu}^{2+}$  in an alkaline medium (yielding  $\text{Cu}^{1+}$ ) with a sensitive and selective detection reagent for  $\text{Cu}^{1+}$ , namely BCA. The BCA

chelates with  $\text{Cu}^{1+}$  ion, where one  $\text{Cu}^{1+}$  interacts with two molecules of BCA, to give a water soluble purple reaction product which exhibits strong absorbance at 562 nm. This allows the spectrophotometric quantitation of protein in aqueous solutions.

#### **2.5.2.1 Assay procedure**

Samples (unknowns) containing tissue protein were thawed. In a 96-well plate 10  $\mu\text{l}$  duplicate samples of buffer, standards and unknowns were added. Using a multi-channel pipette 200  $\mu\text{l}$  of a solution consisting of 50 parts of Reagent A (BCA detection agent) and 1 part of Reagent B (4 % (w/v) copper sulphate solution) were added to each well of the 96-well plate. After one hour of incubation at 37  $^{\circ}\text{C}$  absorbance measurements were taken at 570 nm. A standard curve was prepared by plotting the absorbances of the standards against protein concentration using the GraphPad PRISM software. Then employing non-linear regression curve-fitting conducted by GraphPad PRISM, the equivalent protein concentration of the unknowns was calculated from the standard curve. The results were expressed as mg of protein per ml.

### **2.6 Crystal Violet Assay**

Crystal violet is a basic dye that can bind to any negatively charged molecules in the cell (i.e. proteins and DNA) and thus stains the cells purple. When cells proliferate they increase the amount of proteins and DNA on the plate and thus an increase in the amount of staining represents an increase in cell number. Measuring the absorbance of a cell extract provides a quick and simple method for quantifying cell number/ growth.



In non-sterile conditions, culture medium was removed from all wells and vials by gentle suction. Then 500  $\mu\text{l}$  of 0.4 % (w/v) crystal violet in 30 % methanol (v/v) was added to each well. After 20 min at room temperature, the dye was removed by suction and the fixed cells were washed 3 times with 1 ml of single distilled water. The final water wash was then removed by suction and the fixed cells were left to dry overnight. 800  $\mu\text{l}$  of SDS 1 % (w/v) was added to each well and the cells were left on a slowly agitating plate mixer for 40 min at room temperature. From each well 100  $\mu\text{l}$  of extract was placed into 2 wells of a 96-well plate. The absorbance of each well was then read at 570 nm on a plate reader.

## **2.7 Transepithelial Electrical Resistance (TEER)**

Open circuit membrane potential and membrane resistance are often used to characterise the structure and behaviour of epithelial monolayers. The EVOM epithelial voltohmmeter, a millivolt and ohmmeter, was used. Membrane resistance has usually been measured by passing DC current through a membrane sample and measuring the resulting voltage gradient across the membrane. The current and voltage electrodes are in the form of miniature dual “chopsticks”. Each stick has an outer and an inner electrode. The outside electrodes are small silver pads for passing current through the membrane sample. Inside electrodes are small Ag/AgCl voltage sensors (Figure 2.7.1).

The “chopsticks” were sprayed with IMS and as soon as the IMS had evaporated, one stick was dipped inside the Transwell and the second stick electrode in the receiving well. This was possible since the external stick was slightly longer than its companion. Each measurement was repeated 3 times and an average value was taken which is expressed in Ohms. Resistance was

expressed as transmembrane resistance ( $\text{ohms. cm}^2$ ) after subtraction of the intrinsic resistance of cell free inserts.

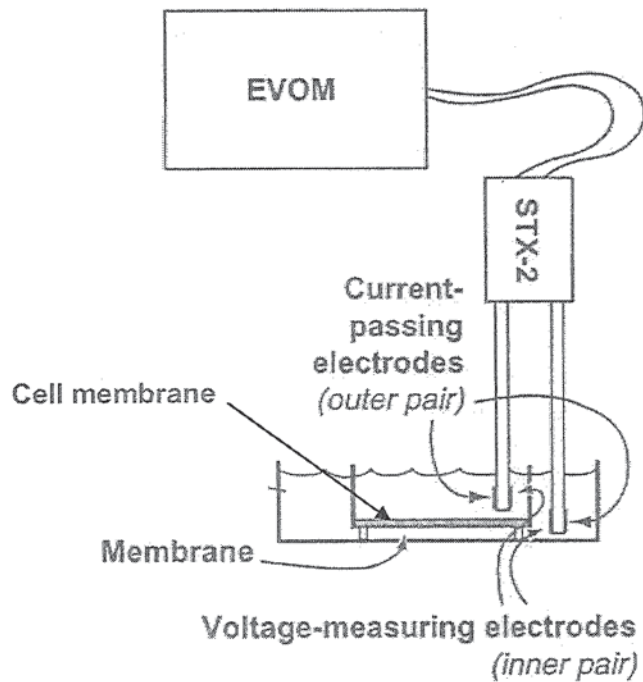


Figure 2.7.1 The EVOM volttohmmeter with “chopstick” electrodes (taken from the manufactured manual)

## 2.8 Measurement of permeability of monolayers

### 2.8.1 Fluorescein transfer

The Transwells were removed from the incubator and in non-sterile conditions the culture medium was removed from both Transwells and the basal wells and was replaced by warm ( $37^{\circ}\text{C}$ ) Hank's Balanced Salt Solution (HBSS). HBSS was then removed and the experiment was initiated by adding to each of the basal wells 2.6 ml of HBSS ( $37^{\circ}\text{C}$ ) and to all of the apical wells 1.5 ml of HBSS ( $37^{\circ}\text{C}$ ) containing  $10\ \mu\text{g/ml}$  fluorescein. Immediately the plates were transferred onto a gently shaking ( $1.5\ \text{cycles/min}$ ) platform of a hybridisation oven at  $37^{\circ}\text{C}$ . The inserts were being transferred into fresh HBSS ( $37^{\circ}\text{C}$ ) at 5 min intervals over a period of 30 min.

Fluorescein was determined by measurement of the fluorescence of 150  $\mu$ l basal samples in 96-well plates using a Victor <sup>2</sup> multilable counter. In order to obtain a calibration curve, 10  $\mu$ g/ml fluorescein stock in HBSS was serially diluted in HBSS to give standards of ( $\mu$ g/ml) 1, 0.1, 0.01, 0.001, 0.0001.

### 2.8.2 [<sup>14</sup>C] mannitol transfer

The Transwells were removed from the incubator and in non-sterile conditions the culture medium was removed from both Transwells and the basal wells and was replaced with warm HBSS (37 °C). HBSS was then removed and to each of the basal wells 2.6 ml of HBSS (37 °C) was added and in all of the Transwells 1.5 ml of HBSS (37 °C) containing [<sup>14</sup>C] mannitol (0.45  $\mu$ Ci/ml, 7.75  $\mu$ M) was added. Immediately the plate containing the Transwells was transferred onto a gently shaking platform (1.5 cycles/min) of a hybridisation oven at 37 °C. At 5 min intervals over a period of 30 min Transwells were moved to fresh HBSS (37 °C). Subsequently 750  $\mu$ l samples of basal medium were removed for assay of [<sup>14</sup>C] mannitol. The 750  $\mu$ l samples were transferred to counting vials containing 5 ml of Optiphase HiSafe 3. Radioactivity measurements were then made using a scintillation counter.

### 2.8.3 P<sub>app</sub> Apparent permeability

The apparent permeability (P<sub>app</sub>) was calculated from the equation

$$P_{app} = \text{Rate of transfer} \times \frac{1}{A} \times \frac{1}{C_0}$$

Where transfer is in amount per sec, A, the surface area of the filter, is in cm<sup>2</sup>, and C<sub>0</sub>, the initial apical concentration, is in amount/ml. The units of P<sub>app</sub> are therefore cm.sec<sup>-1</sup>.



## **2.9 Liquid scintillation counting**

Beta emitting radionuclides [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] were quantified using liquid scintillation counting. Optiphase Hisafe 3 (5ml) was added to every sample and mixed before being counted on a Hewlett Packard TRiCarB2000 CA liquid scintillation analyser (10 minutes per sample). In all cases counts per minute (CPM) were converted into disintegrations per minute (DPM) by comparison with standard quench-correction curves.

**Chapter 3**  
**Pilot Experiments**

### **3.1 Aims**

The main aims of this chapter were: 1) to investigate the attachment and growth of guinea-pig gastric epithelial cells on Tissue Culture Plastic (TCP) and to determine the time-scale of cell survival in culture; 2) to characterise growth of guinea-pig gastric epithelial cells on various biodegradable polymeric surfaces; and 3) to culture guinea-pig gastric epithelial cells on Transwells and to optimise conditions of culture in order to produce a confluent cell monolayer with fully developed tight junctions.

### **3.2 Introduction- Experimental Design**

#### **3.2.1 Factors affecting growth of cells on Tissue Culture Plastic**

##### **3.2.1.1 Enrichment prior to plating**

As mentioned in the general introduction Rattner *et al.* (1985) developed a method for growing confluent primary cultures of guinea-pig gastric cells on collagen cups. The monolayers were produced by first enriching the cell preparation on a Percoll density gradient to obtain a higher percentage of mucous before plating. The silica colloid Percoll has nearly ideal physical characteristics, including low osmolarity, for use in separating cells, organelles, viruses, and other subcellular particles. A crude digest of guinea-pig gastric mucosa before enrichment is made up of about 40 % mucous, 20 % chief, 35 to 38 % parietal, and 2 to 3 % endocrine cells (Rattner *et al.*, 1985). Fibroblasts were found only rarely in the crude cell fraction. After enrichment there were 65 % mucous, 25 to 30 % chief, and 5 to 10 % parietal cells, a few endocrine cells and no identifiable fibroblasts. Hence, it was thought useful to examine the effects of enriching the cell preparation of this study using the Percoll technique.



### 3.2.1.2 Growth Factors and Serum

Under normal conditions, cell populations within the gastrointestinal tract are maintained in a dynamic steady state because of the cell loss, through exfoliation of surface cells (resulting from frequent exposure of the gastric mucosa to substances, with a wide range of pH, osmolarity, and temperature) and also to apoptosis, is balanced by a continuous cell renewal (Hall *et al.*, 1994). Important in this regulation are several growth factor peptides including epidermal growth factor (EGF). EGF is the prototypic member of a family which has been recognized to encompass at least seven different peptides, four of which have been found to be expressed within the GI tract. EGF was initially isolated by Cohen (1962) from mouse submaxillary glands as the factor responsible for promoting eyelid opening in neonatal mice which was a reflection of epidermal proliferation. The preparation was found to promote epithelial cell proliferation in a wide variety of tissue and organ culture systems from different species. Hence, EGF was named originally for its stimulatory effects on proliferation and keratinisation of the epidermis. EGF is a 53-amino acid polypeptide that regulates the proliferation and differentiation of a variety of cell types, including polarised epithelial cells. EGF is present in salivary glands, duodenal Brunner's glands, and mucosal epithelial cells after damage (Berseth, 1987; Goodland and Wright, 1995; Barnard *et al.*, 1995). In addition, EGF is relatively stable in gastric juice, although some degradation occurs to smaller forms that retain moderate physiological activity (Playford, 1995). EGF is an important regulator of aspects of mucosal defence and repair (Barnard *et al.*, 1995; Goodland and Wright, 1995). The related EGF family member, transforming growth factor- $\alpha$  (TGF- $\alpha$ ), is present in gastric mucosa like other agents, hepatocyte growth factor, insulin, and insulin-like growth factor I (IGF-I), has been shown to stimulate proliferation of primary cultures of gastric epithelial cells from rats (Terano *et al.*, 1982), dogs (Boland *et al.*, 1990; Chen *et al.*, 1991; Chen *et al.*,

1993), rabbits (Takahashi *et al.*, 1995) and guinea-pigs (Rutten *et al.*, 1991, Rutten *et al.*, 1993, Ogihara *et al.*, 1996).

Historically, fetal bovine serum has been the most widely used growth supplement for cell culture media because of its high content of embryonic growth-promoting factors. The sera used in most tissue culture are calf, foetal calf, horse and human serum. Calf (CF) and foetal calf serum (FCS) are the most widely used, the later particularly for more demanding cell lines and for cloning. When used at appropriate concentrations serum supplies many defined and undefined components that have been shown to satisfy specific metabolic requirements for the culture of cells *in vitro*. In general, primary cultures of guinea-pig epithelial cells require a high concentration of FCS (Hirakawa *et al.*, 1996; Rokutan *et al.*, 1996; Rokutan *et al.*, 1994.). The range of FCS supplementation in culture media usually ranges from 5 to 20 % and thus in this study the effect of serum at two different concentrations (10 % and 20 %) was investigated. Unless stated otherwise all experiments were performed on 6-well cluster plates (wells 35mm in diameter).

### **3.2.2 Growth of cells on polymeric matrices used in tissue engineering**

There are a number of advantages making polymers ideal candidates for tissue engineering and as supports for cell growth. Firstly, polymers are readily available commercially and secondly, polymeric matrices can be engineered to promote optimal growth and compatibility with the tissue of interest. (Devins *et al.*, 1996). Finally and most importantly, due to its biodegradable nature, removal of the polymeric implant is not required (Ibim, 1997).

The use of polymers as scaffolds to grow cells *in vitro* is a relatively new idea. Almost forty years ago, whole organ transplantation was the new concept. The many problems associated with organ transplantation, rejection (immunologic attack of the allograft), organ scarcity, major traumatic surgery, and cost have contributed at least in part to the development



of mechanisms that enable cell transplantation. Cell transplantation occurs when a polymer matrix (scaffold, fibre, patch) is laden with many cells of one specific type and allowed to grow *in vitro* for a specific amount of time and then can be implanted into a test subject. The polymer matrix slowly degrades as the cells grow into identifiable tissue structures *in vivo*.

The use of artificial biomaterials, which resorb over a period of time, offers the possibility of the growth and development of normal tissue in the body and results in a reduced risk due to the long-term presence of a foreign material (Freed and Vunjak-Novakovic, 1998). Only biodegradable polymers with resulting byproducts that are non-toxic are good candidates. Natural polymers (e.g. collagen) have been the first choice for research in this area, but have poor mechanical properties and variable sources of protein in the matrices. By contrast synthetic polymers can be readily produced and the physical properties can be easily changed. For example the surface area could be optimised for cell attachment, for exposure to nutrients or to alter the porosity of the polymer. The advantage of changing the pore size is that it can increase or decrease the matrix's strength, elasticity and compressibility.

In this work the effect of a variety of matrices on cell growth was investigated. Of the polymers used in tissue engineering the following were chosen. Poly (L-lactide-co-glycolide) (PLGA) which is produced by copolymerisation of 50 % racemic lactide and 50 % glycolide and forms a poly ( $\alpha$ -hydroxy ester) (Coombes and Meikle, 1994). PLGA has a MW of 34,000, a low melt temperature of 205 °C and a low processing temperature, low crystallinity and crystallizability, and high strength retaining in a biological environment. Its major advantage is biocompatibility although it degrades fully in 6-12 weeks.

Poly ( $\epsilon$ -caprolactone) (PCL) (MW 12,000) is biodegradable semi crystalline aliphatic polyester, with a low melting point of about 60°C (Lin, 1999). It has a long *in vivo* degradation time ranging from 1 to 2 years (Pitt, 1981) due to its high crystallinity and hydrophobicity.



Poly (L-lactide) (PLA) and PLGA are among the few polymers that are approved by the Food and Drug Administration (FDA) for specific clinical uses such as wound repair and bone fixation pins (Cohen *et al.*, 1994 ;Ibim *et al.*, 1997).

If a monolayer of the present preparation would be formed on any of the above polymeric surfaces then by mounting it in a chamber a model of the gastric epithelium might be created.

### **3.2.3. Growth of gastric epithelial cells on Transwells**

#### **3.2.3.1 Advantages of Transwells**

The opportunities provided by Transwells for interaction, stratification, polarisation and differentiation have made them a popular culture system with significant advantages over solid substrates. Cellular functions such as transport, adsorption and secretion can also be studied since cells grown on permeable supports provide convenient, independent access to apical and basolateral plasma membrane domains. The development of polarity and functional integrity was exhibited by thyroid epithelium explanted onto a collagen coated filter in a specially constructed mount (Chambard *et al.*, 1983). Transwells have been used to generate stratified epidermis (Kondo *et al.*, 1997) and polarised intestinal (Halleux and Schneider, 1994) and kidney (Mullin *et al.*, 1997) epithelium. Others have used them to study invasion by granulocytes or malignant cells (Brunton *et al.*, 1997). Only one primary culture of gastric cells appears to have been grown on Transwells and to have been fully characterised (Chen *et al.*, 1994). Canine gastric mucosal cells formed a monolayer with a resistance of greater than  $1000 \Omega \cdot \text{cm}^2$ , but the monolayer was composed of 65 % pepsinogen-secreting chief cells and 24 % of acid-secreting parietal cells and therefore is a model for the interior of the gastric gland rather than the surface of the epithelium.

Cell attachment and growth can be improved by pretreating the support (filter) in a variety of ways. Treatment with denatured collagen improves the attachment of many types of cells, including epithelial cells, and it may be necessary for the expression of differentiated functions. Collagen is an important cell adhesion molecule for most cells and it is known to contain adhesion domains which bind to a number of integrin receptors ( $\alpha1\beta1, \alpha2\beta1, \alpha3\beta1$  and  $\alpha4\beta1$ ) (Hubbell, 1995). In the first series of experiments collagen type I from rat tail was used to coat the filters in order to promote monolayer formation and differentiation.

Transwells come in different pore sizes ranging from 0.01  $\mu\text{m}$  pore to 12 $\mu\text{m}$  pore and the membranes, made of polycarbonate, polyester or polyethylene terephthalate, range in diameter from 6.6 mm (24-well plate) to 24 mm (6-well plate). In the current study, both polyester and polycarbonate filters were used and 2 different pore sizes (3  $\mu\text{m}$  and 0.4  $\mu\text{m}$ ) were tested in order to establish the best membrane to produce a confluent cell monolayer. Filters with pore sizes of 3 and 0.4  $\mu\text{m}$  are recommended for studies involving tissue remodelling and re-epithelialisation, cell matrix interactions, drug transport across epithelial barriers and establishment of cell polarity. Larger pore sizes of 5, 8 and 12  $\mu\text{m}$  are usually used for chemotaxis/motility studies and for studies involving metastatic potential and invasion.

### **3.2.3.2 Effect of extracellular $\text{Ca}^{2+}$ concentration on growth of gastric cells on Transwells**

Epithelial cells are joined by a junctional complex which is comprised of three separate structures: tight junctions, intermediate junctions, and desmosomes. The integrity of these structures is dependent on  $\text{Ca}^{2+}$  (Madara and Trier, 1987). In human gastric mucous epithelial cells the predominant localization of the calcium receptor (CaR) is to the basolateral membrane however weak CaR immunofluorescence was present at the apical membrane. These results would suggest that the CaR would be primarily involved in “sensing” changes



to serum  $\text{Ca}^{2+}$  concentrations that could regulate growth or secretion. However, another possibility is that damage to the luminal surface mucous cells could produce an influx of luminal  $\text{Ca}^{2+}$  across the damaged gastric epithelium to activate the basolateral CaR of underlying surface mucous cells to aid in cell proliferation and gastric mucosal repair (Rutten *et al.*, 1999). In addition, increases in extracellular  $\text{Ca}^{2+}$  stimulate acid and bicarbonate secretion by guinea pig gastric surface epithelium and hence these observations suggest that surface epithelial cells are capable of sensing changes in extracellular  $\text{Ca}^{2+}$  (Flemstrom and Garner, 1980; Garner and Flemstrom, 1978). Thus, the effect of extracellular calcium concentration in the culture medium on the formation of tight junctions was examined, as it was reported by Artusson and Magnusson (1990) that extracellular calcium concentration has an effect on the formation of tight junctions of Caco2 cell monolayers.

### **3.2.3.3 Effect of conditioned medium on growth of cells on Transwells**

Puck and Marcus (1955) found that the survival of low-density cultures could be improved by growing the cells in the presence of feeder layers. This effect was probably due to a combination of effects, including conditioning of the substrate and conditioning of the medium by the release of small molecular metabolites and growth factors into the media (Takahashi and Okada, 1970). Hauschka and Konigsberg (1966) illustrated that the conditioning of culture medium was necessary for the growth and differentiation of myoblasts, due to collagen released by the feeder cells. Using feeder cells and conditioning the medium with embryonic fibroblasts or other cell lines, remains a valuable method of culturing difficult cells. Conditioned medium contains both substrate modifying matrix constituents, like collagen, fibronectin, and proteoglycans, metabolites and growth factors, such as fibroblast growth factor (FGF), insulin-like growth factors (IGF-1 and IGF-2), platelet derived growth factor (PDGF), and several others. However, conditioned medium adds



undefined components to the medium. In the original guinea-pig gastric mucous cell monolayers produced by Rattner *et al.* (1985), the culture medium comprised 45 % conditioned medium derived from gastric mucosal fibroblast cultures mixed with culture medium. In order to optimise the growing conditions to produce the ultimate cell monolayer, conditioned medium was mixed with culture medium in a 1:1 ratio.

### **3.2.4 Measurement of cell number**

Direct cell counting remains a very economical and popular method of cell quantitation. It involves the enzymatic disaggregation of the cells adherent to the support into a cell suspension by Trypsin/EDTA treatment. The principle behind the disaggregation lies in the combination of both trypsin to hydrolyse extracellular matrix and EDTA to break the cell-cell adhesion and cell-matrix contacts. Adhesion of cells is mediated by cell adhesion molecules some of which are calcium dependent (cadherins) and hence are sensitive to chelating agents such EDTA. Integrins, which bind to the RGD motif of the extracellular matrix, also have calcium binding domains and are affected by  $\text{Ca}^{2+}$  depletion. The concentration of cells in a suspension can be determined by placing the cells in a chamber haematocytometer under a microscope. The cell number within a defined area of known depth is counted and the cell concentration derived from the count.

Another very economical and widely used assay for quantitation of cell number is the use of crystal violet. It has been shown on Hela cells that the number of viable cell counted, correlated with the crystal violet absorbance values (Chiba *et al.*, 1998).

### **3.2.5 Cryopreservation**

The adoption of newly developed cell lines or imported cell lines into regular use implies an investment in time and resources that increases, often exponentially, with

continuous use. Thus the cell line becomes a valuable resource replacement which would be expensive and time-consuming. In addition cell lines in continuous culture are prone to variation, due to selection in early passage culture, senescence in finite cell lines and genetic instability and/ or contamination in continuous use. Consequently cryopreservation of cell lines is very common practice.

Cryopreservation would also have been appealing for the primary culture. The initial cell preparation is extracted from a live animal and thus freezing surplus cells would save the use of a guinea-pig. In addition, due to strict Home Office procedures animal handling can only be carried out by an entitled project licensee and thus the cell preparation is not only restricted to licensed persons but it can only be carried out in the designated premises. Frozen cells could however be used by non-licensed personnel.

### **3.3 Methods- Cell culture**

#### **3.3.1 Additional culture information**

For the studies investigating cell concentration at plating, the effect of epidermal growth factor (EGF 10 ng/ml) on the number of cells attached to wells of the TCP previously coated with collagen (type I) and for cell culture on polymers, the cell isolation procedure was followed as described in section 2.2, but cells were treated for 20 min with 0.4 mg/ml of collagenase instead of 40 min. In addition, where stated in the text, cells were cultured for 4 and 7 days. Medium change was performed daily.

#### **3.3.2 Increasing the concentration of Foetal Calf Serum (FCS)**

From 30 ml of freshly prepared isolated gastric cell suspension in complete culture medium (around  $10^6$  cells/ml), 15 ml was poured into a universal 30 ml tube containing 1.5 ml of complete culture medium, resulting in a FCS concentration of 10 % in the cell suspension. The remaining 15 ml was poured into a second 30 ml universal tube containing 1.5 ml of undiluted foetal calf serum which gave a total of 18 % FCS in this cell suspension. 1 ml of the appropriate cell suspension was added to wells of a twelve-well plate.

#### **3.3.3 Calcium Supplementation**

Under aseptic conditions, 14.7 g of calcium chloride ( $\text{CaCl}_2$ ) were added to 100 ml of sterile double distilled water in order to produce 1M  $\text{CaCl}_2$  solution. Since 100 ml of complete medium contains 0.042 millimoles of  $\text{Ca}^{2+}$ , in order to obtain a 2 mM  $\text{Ca}^{2+}$  (0.2 millimoles/100 ml), 0.158 ml of 1M ( $\text{CaCl}_2$ ) solution was added to 100 ml of complete medium. For the purpose of the calcium supplementation investigation the resulting 2 mM  $\text{Ca}^{2+}$  was referred to as high



calcium culture medium whereas the standard complete medium was referred to as low calcium medium.

### **3.3.4 Preconditioning the Transwells**

Under sterile conditions, 24 h prior to plating, 1.5 ml per apical well and 2.6 ml per basal well of complete culture medium in the presence of 10 ng/ml EGF were added to each well of a 24 mm Transwell plate. The Transwells were transferred to the cell culture incubation oven and were left for 24 h. Just before plating the medium was removed from both apical and basal wells.

### **3.3.5 Preparation of conditioned medium**

Epithelial cells were isolated and plated at 1 ml per well and at approximately  $1.3 \times 10^6$  cells/ml (12-well TCP) according to standard procedures but in the absence of 10 ng/ml EGF. The culture medium was changed daily with 1 ml per well in the absence of EGF for 6 days, after which the culture medium was changed every 3-4 days with 3 ml per well. 26 days after the initial plating, the cells remaining exhibited the appearance of fibroblasts. The fibroblasts were then treated with 1 ml Trypsin/EDTA solution per well and were incubated for 20 min at  $37^\circ\text{C}$ . The contents of two wells (i.e.  $2 \times 1$  ml of Trypsin/EDTA-fibroblast suspension) were then mixed with 18 ml of complete medium. The resulting fibroblast suspension which contained  $5 \times 10^6$  cells/ml was then passaged to a  $75\text{ cm}^2$  flask. The same procedure was followed for the remaining 10 wells. Two days later when the fibroblasts had almost reached confluence, the media were removed and filtered through a  $0.4\ \mu\text{m}$  pore sterilisation filter unit, in order to be used as conditioned media. The medium used for culturing epithelial cells on Transwells in the presence of conditioned medium was made by mixing conditioned medium with complete medium in a 1:1 ratio in the presence of 10 ng/ml EGF. The unused conditioned media were stored at  $-20^\circ\text{C}$ .

### **3.3.6 Enrichment of cells on a Percoll density gradient**

Cell types from the crude cell suspension can be partially separated by use of a self-forming Percoll gradient. Fractionation involves resuspension in Percoll-containing medium and centrifugation to produce a high density enriched in mucous cells (Brown *et al.*, 1992).

Under sterile conditions, “complete Percoll” was made by mixing 10.8 ml Percoll (Sigma cell culture tested) with 1.2 ml 10 x RMPI medium, and to which 24 mg of NaHCO<sub>3</sub> was added. Then 10 ml of a freshly isolated gastric epithelial cell suspension was loaded into two 15 ml centrifuge tubes each containing 5 ml of “complete Percoll” mixture and the contents mixed. Tubes were centrifuged at 100 x g for 15 min at 4 °C. The resulting combined pellets, which are enriched in mucous cells, were resuspended in 8 ml of complete tissue culture medium in order to give around 10<sup>6</sup> cells per ml. Enriched cells were then plated at 1 ml per well (TCP, 12 well plate) and at 1.5 ml per Transwell.

## **3.4 Methods- Cell Freezing**

The cells were isolated according to the isolation protocol but in the absence of 10 ng/ml EGF. The cells were placed into five cryovials labelled A to E containing 1 ml of 2 x 10<sup>6</sup> cells/ml each in the presence of 10 % DMSO. The cryovials were individually wrapped in tissue towel for insulation and placed in a polystyrene box at -80 °C. A day later the vials were stored into a liquid nitrogen storage unit at -140 °C .

### **3.4.1 Resuscitation of cells from frozen stocks**

Cryovials were removed from the liquid nitrogen storage unit and were immediately transferred to 37 °C water bath, shaking occasionally. The cells were thawed until the point

when the last crystal melted. Under sterile conditions the cryovials which were previously labeled A to E were treated as follows:

Vial A: The DMSO concentration in the cell suspension derived from this vial was reduced to 1 %, thus 100 µl of the contents of vial A were mixed with 1 ml of complete medium in a 2 ml microfuge tube. This was repeated another 3 times in order to produce 4x 2 ml microfuge tubes. The final cell concentration was  $2 \times 10^5$  cells/ml. 1 ml of each suspension was plated to 4 wells of a 12 well TCP. For two other wells 200 µl of vial A were mixed with 1 ml of complete medium in order to obtain a final concentration of 2 % DMSO (Figure 3.4.1).

Vial B: The whole contents of vial B which contained 1ml of cell suspension in the presence of 10 % DMSO were plated on a well of a 12 well TCP (Figure 3.4.1).

Vials CDE: Cells in vials C, D and E were pooled into a 15 ml centrifuge tube giving a final volume of cell suspension of 3 ml. 7 ml of complete medium was added to the centrifuge tube and the whole mixture was gently mixed. The resulting suspension was then centrifuged at  $200 \times g$  at  $15^\circ C$  for 5 min. The resulting pellet was then gently resuspended in 4 ml of complete culture medium. 1 ml of the resulting suspension was then added to three wells of the 12 well TCP (Figure 3.4.1).

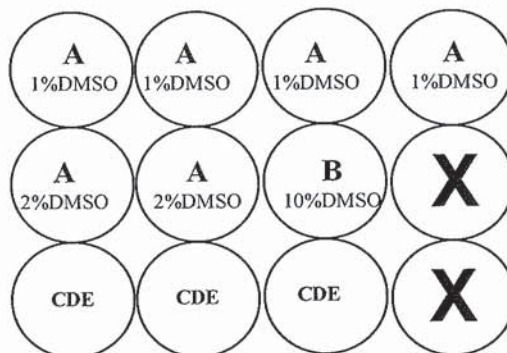


Figure 3.4.1 Layout of plate used to examine the effects of freezing cells



Once the cells were plated, the medium was changed at 24 h and 48 h. At 72 h medium was removed and the cells were stained with crystal violet after which a photograph was taken.

### **3.5 Methods- Polymer casting**

The poly (L-lactide co-glycolide) [PLGA], poly (L-lactide) [PLA] and poly (  $\epsilon$ -caprolactone) [PCL] films were produced using a solvent casting technique. Polymer stock solutions were prepared by dissolving a known mass of polymer in a specific volume of dimethylchloride. The concentrations for PCL and PLA were 1 % (wt/vol.) and for PLGA 2 % (wt/vol.). From the stock solutions 1 ml, 2 ml and 4 ml of each polymer was dispensed into glass vials. The dimethylchloride was allowed to evaporate, leaving a polymer film in the bottom of the vial.

### **3.6 Methods- Initial transfer experiment protocol for studies on polyester Transwells only**

The Transwells were removed from the incubator and in non-sterile conditions the culture medium was removed from both Transwells and the basal wells. To each of the basal wells 2.6 ml of warmed (37 °C) HBSS was added and in all of the Transwells 1.5 ml of warmed HBSS containing 10  $\mu$ g/ml fluorescein was added. Immediately the plate containing the Transwells was transferred onto a gently shaking platform of a hybridisation oven at 37 °C.

At 0, 5, 10, 15, 20, 25, 30, 35 and 40 min after the onset of the experiment, 150  $\mu$ l samples were taken from the basal well. An insert without cells (blank) was used to determine the maximal transport of the fluorescent marker during the same period of time.

### **3.7 Methods- Fixation and staining of Transwells**

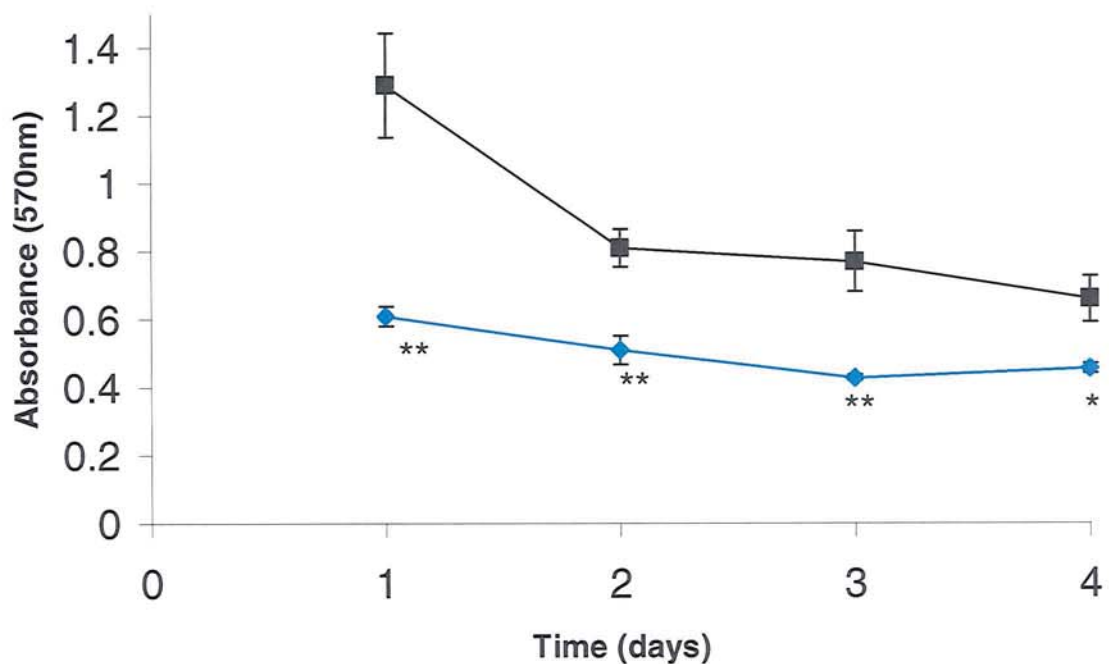
This method permitted the visualisation of the appearance of the cell monolayers formed on Transwells following the transfer experiments. The monolayer was firstly fixed by 1 % (v/v) glutaraldehyde in PBS so that no cell detachment would occur in response staining with crystal violet.

At the end of the transfer experiment, the plates were transferred into a fume cupboard hood and medium was removed by gentle suction from both apical and basal wells and replaced with 1.5 ml per apical well and 2.6 ml per basal well of 1 % (v/v) glutaraldehyde in PBS. This solution was produced by adding 2 ml of 50 % glutaraldehyde stock solution into 98 ml PBS. After glutaraldehyde treatment for one hour at room temperature, the glutaraldehyde was removed by gentle suction and the fixed monolayers were washed 3 times with 1.5 ml per apical well and 2.6 ml per basal well of PBS. After the last wash, the PBS was removed and the plates were then transferred onto the laboratory bench. The plates were filled with 1.5 ml per apical well and 2.6 ml per basal well of 0.4 % (wt./vol.) crystal violet in 30 % methanol (v/v). After 20 min at room temperature, the dye was removed by suction and the fixed cells were washed 3 times with 1.5 ml per apical well and 2.6 ml per basal well of single distilled water. The plates were then ready for photography.

### 3.8 Results- Growth of cells on Tissue culture plastic

#### 3.8.1 Effect of cell concentration at plating

Figure 3.8.1 suggests that with an initial high cell concentration a significant loss in cell number occurred with time in comparison with the control cell concentration. By 4 days there were still, however, more cells attached to the wells of the plate when plated at high concentration.



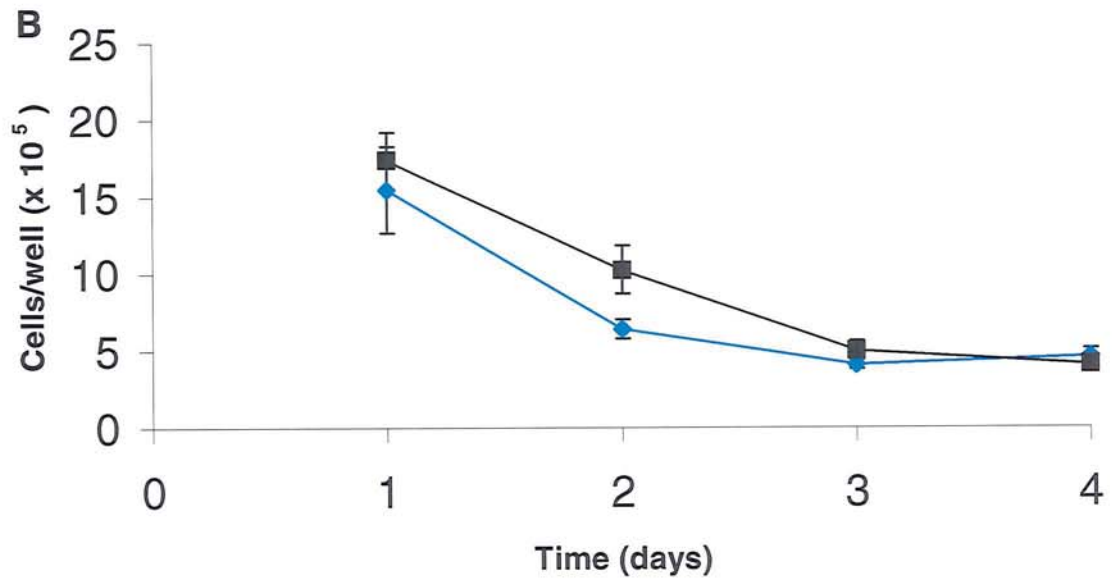
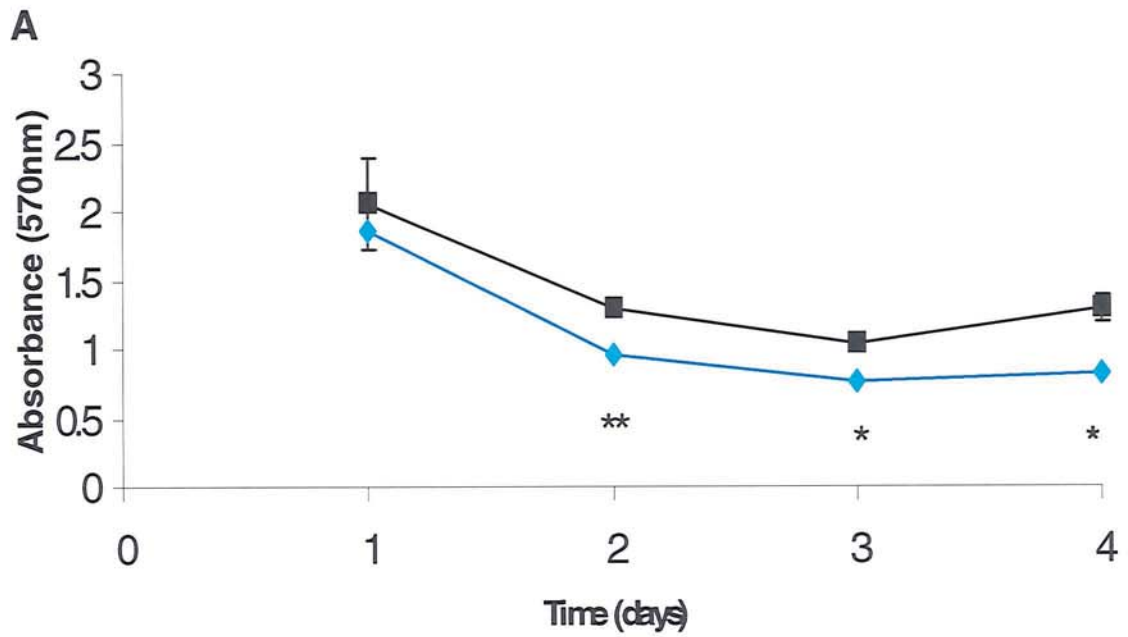
**Figure 3.8.1 Effect of cell concentration on the number of cells (Crystal violet assay, absorbance at 570nm) attached to wells of the tissue culture plate.**

Results are means  $\pm$  S.E.M. of mean from four wells on each day. Squares: cells plated at  $2.82 \times 10^6$  cells/well; diamonds: cells plated at  $1.55 \times 10^6$  cells/well. \* $P < 0.05$ , \*\* $P < 0.01$  for effect of plating concentration by unpaired t-test.



### **3.8.2 Effect of epidermal growth factor (10 ng/ml) on the number of cells attached to the plate**

Figure 3.8.2 shows that with both control cells and EGF-treated cells, cell loss occurred over the initial 36 h of incubation whether the crystal violet assay or direct cell counting was employed. Cell number assessed by crystal violet assay was higher in the presence of EGF in comparison to control cells on days 2, 3 and 4. However no such effect of EGF was apparent if trypsinization and direct cell counting was used to assess cell number.

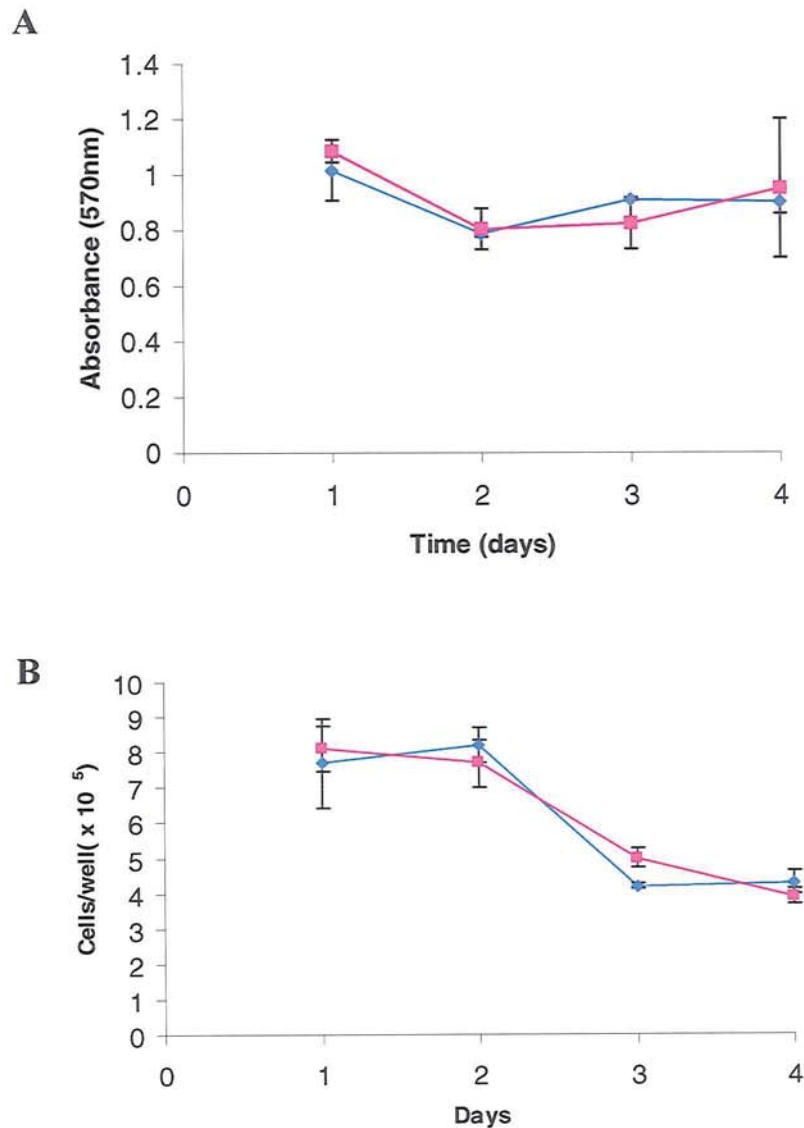


**Figure 3.8.2** Effect of epidermal growth factor (10 ng/ml) on the number of cells attached to wells of the tissue culture plate assessed by (A) crystal violet assay and (B) trypsinization and direct cell counting.

Results are means  $\pm$  S.E.M. of mean from 3 wells on each day, diamonds are for control cells and squares for cells incubated with 10 ng/ml EGF. \* $P < 0.05$ , \*\* $P < 0.01$  for effect of EGF by unpaired t-test.

### 3.8.3 Effect of coating the wells of the tissue culture plate with collagen (type I)

Figure 3.8.3 shows that collagen coating had no significant effect on cell number in comparison to control wells whether the crystal violet assay or direct cell counting was used to assess cell number. Coating the wells with collagen did not appear to prevent cell-loss with time.



**Figure 3.8.3** Effect of coating the wells of the tissue culture plate with collagen (type I) on the number of cells attached to the wells of the tissue culture plate assessed by (A) crystal violet assay and (B) trypsinization and direct cell counting.

Results are means  $\pm$  S.E.M. of mean from three wells on each day, diamonds are for cells grown on collagen-coated wells and squares for cells cultured on control wells. No difference between conditions by unpaired t-test.



### 3.8.4 Effect of EGF (10 ng/ml) on an estimate (crystal violet assay) on the number of cells attached to wells of the tissue culture plate previously coated with collagen (type I)

In this experiment cells were plated at a lower density than usual  $6.3 \times 10^5$  cells per well. Under these conditions (Figure 3.8.4.1) the presence of EGF clearly increased cell number relative to control wells on days 3, 5 and 7. Microscopic examination of cells seeded on collagen-coated TCP in the presence of EGF (10 ng/ml) showed that after 24 h cells seemed to be in present both as flat layers and as clumps (dark blue) and that after 2 days cells seemed to spread and flatten and the clumps disappear. After 4 days a near monolayer was formed but after 7 days some deterioration of the culture with possible overgrowth or de-differentiation of cells was visible (Figures 3.8.4.2 to 3.8.4.5).

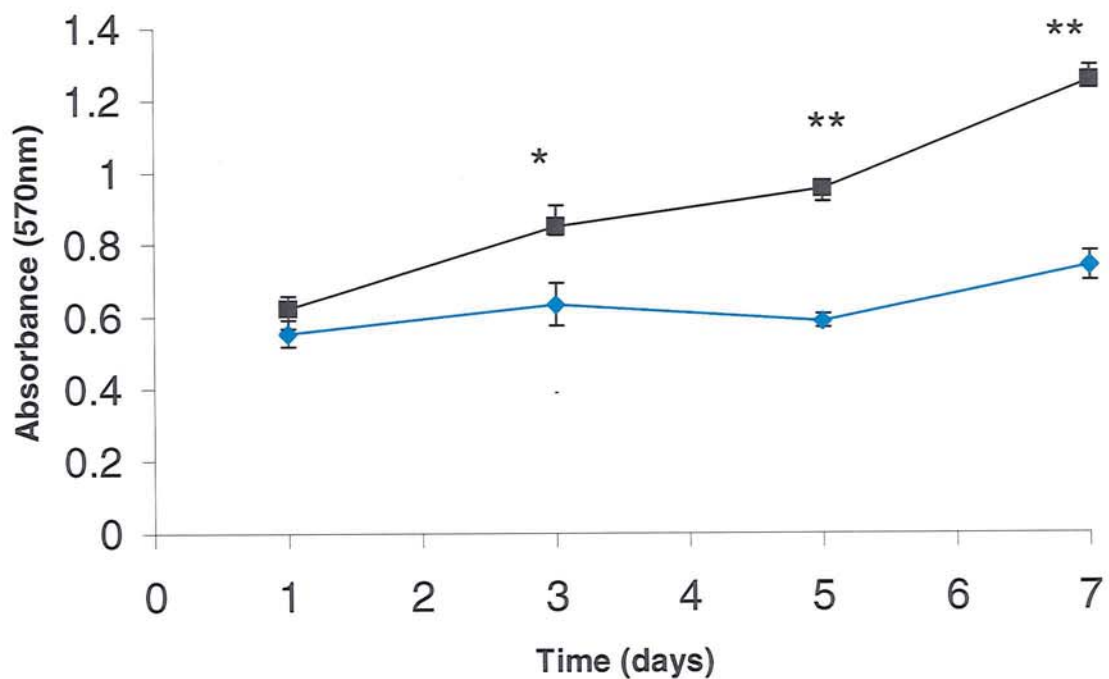
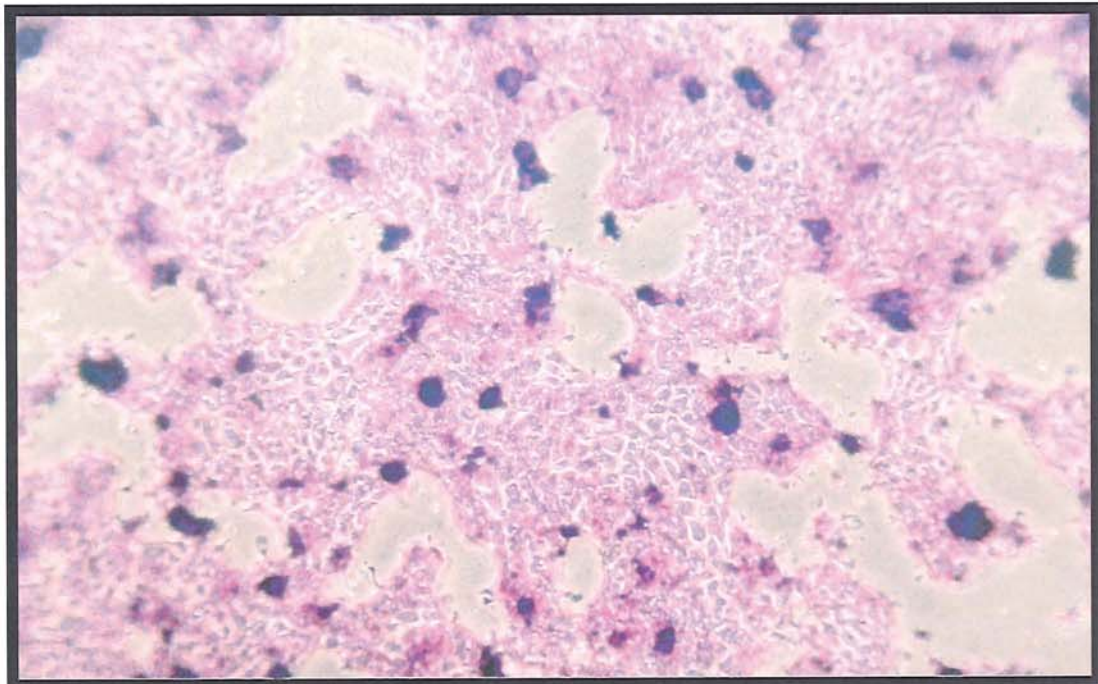
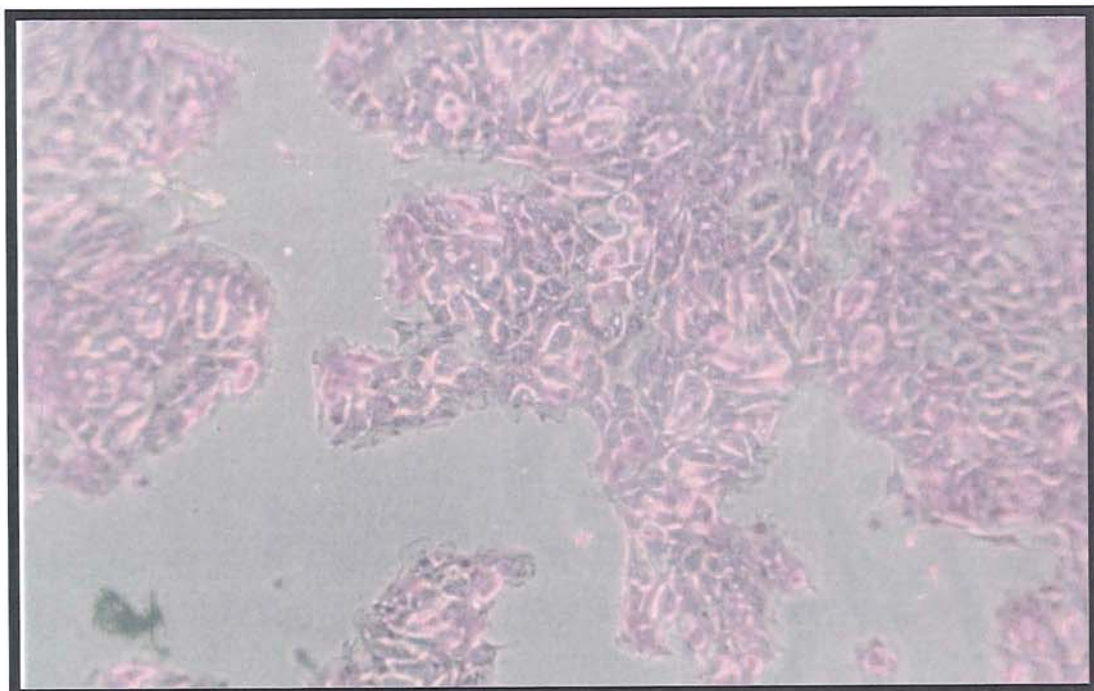


Figure 3.8.4.1 Effect of 10 ng/ml EGF on the number of cells attached to wells of the tissue culture plate previously coated with collagen (type I) assessed by staining with crystal violet (absorbance at 570nm). Results are means  $\pm$  S.E.M. of mean from six wells on each day, diamonds are for control cells and squares for cells cultured in the presence of 10 ng/ml EGF.\* $P < 0.05$ , \*\* $P < 0.01$  for effect of EGF by unpaired t-test.

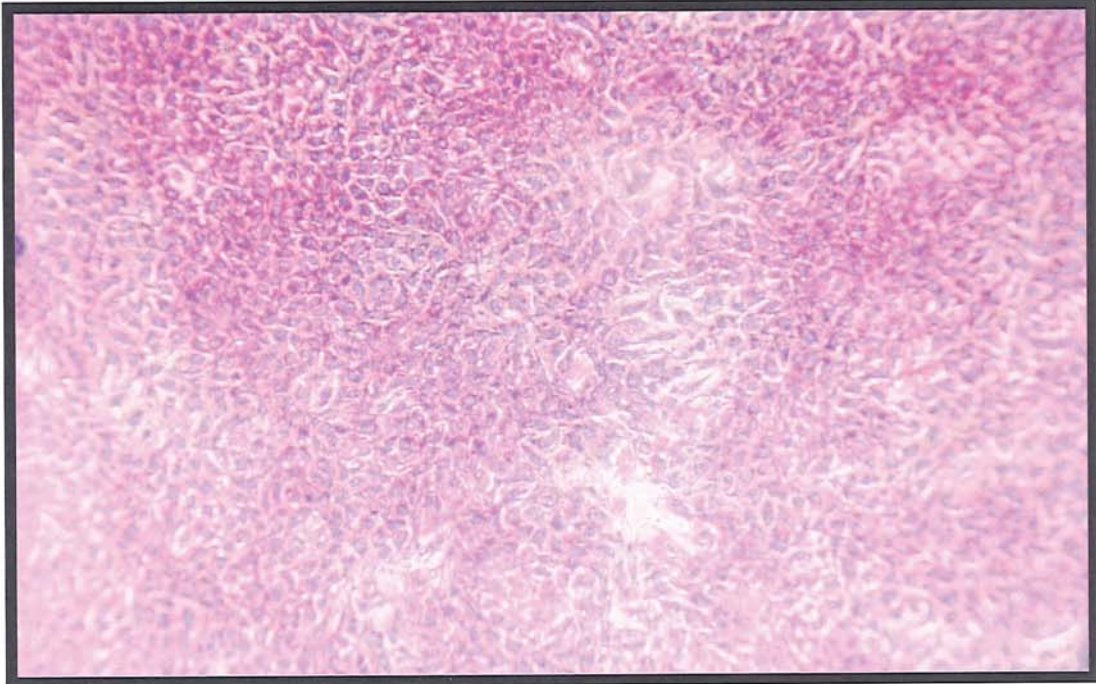


**Figure 3.8.4.2** Appearance of cells attached to the wells of the tissue culture plate previously coated with collagen type I.  
Cells were cultured for 24 h with 10 ng/ml EGF and stained with crystal violet



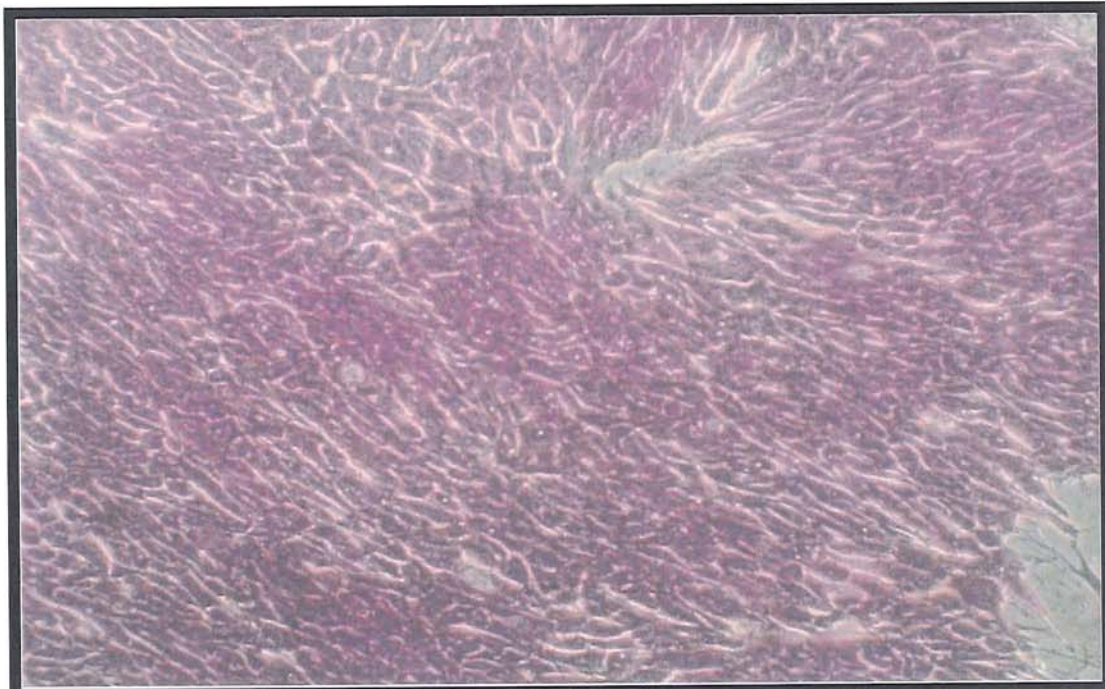
**Figure 3.8.4.3** Appearance of cells attached to the wells of the tissue culture plate previously coated with collagen type I.  
Cells were cultured for 48 h with 10ng/ml EGF and stained with crystal violet





**Figure 3.8.4.4** Appearance of cells attached to the wells of the tissue culture plate previously coated with collagen type I.

Cells were cultured for 72 h with 10 ng/ml EGF and stained with crystal violet



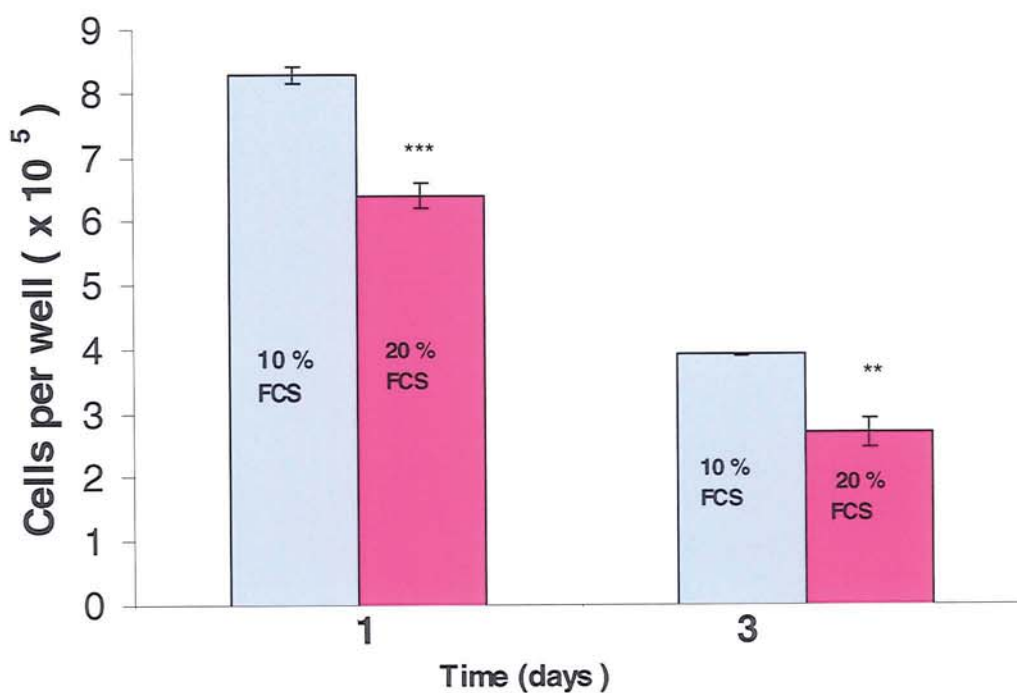
**Figure 3.8.4.5** Appearance of cells attached to the wells of the tissue culture plate previously coated with collagen type I.

Cells were cultured for 168 h with 10 ng/ml EGF and stained with crystal violet



### 3.8.5 Effect of FCS concentration on cell attachment and growth

Direct cell counting (Figure 3.8.5) shows that with both control cells (10% FCS) and with cells grown in the presence of 20% FCS, cell loss occurs over the initial 72 h of incubation. However the number of cells attached to the tissue culture plate was significantly higher when cells are grown with 10 % FCS (control) than with 20 % FCS both at 24 and 72 h of culture.



**Figure 3.8.5** Effect of foetal calf serum (FCS) concentration present in the culture medium on the number of cells attached to the wells of the tissue culture plate assessed by trypsinisation and direct cell counting on days 1 and 3 of culture.

Results are means  $\pm$  S.E.M. of means from 4 wells on each day. Cells were plated at  $12.5 \times 10^5$  cells/ well and culture plates were agitated 1h after plating. \*\*P<0.001, \*\*\*P<0.0001 for a difference between 10 % and 20 % FCS by unpaired t-test.

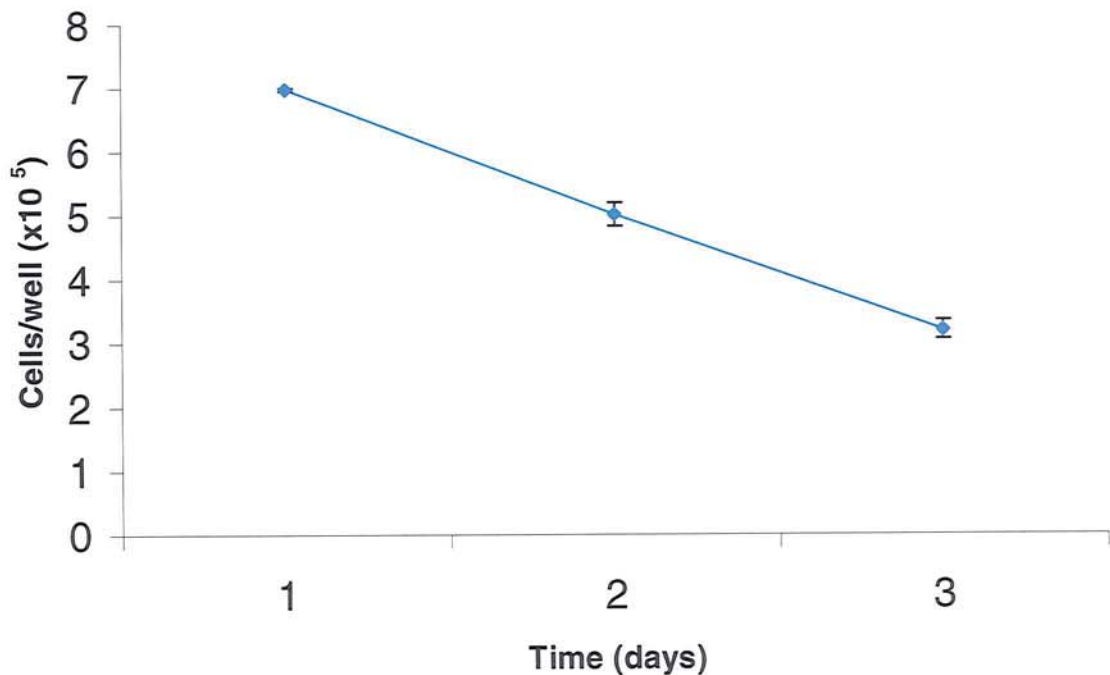
### 3.8.6 Enrichment of mucous cells on Percoll density gradient

#### 3.8.6.1 Cell loss still occurred

Microscopic examination of enriched cells grown on TCP (results not shown) revealed that when cells are enriched they tend to congregate and form a single “island” on the culture well instead of spreading. When enriched cells grown on TCP were agitated one hour after initial plating, although they spread better on the well, trypsinisation and direct cell counting showed that cell loss still occurred over the initial 72 hours of culture (Figure 3.8.6.1)

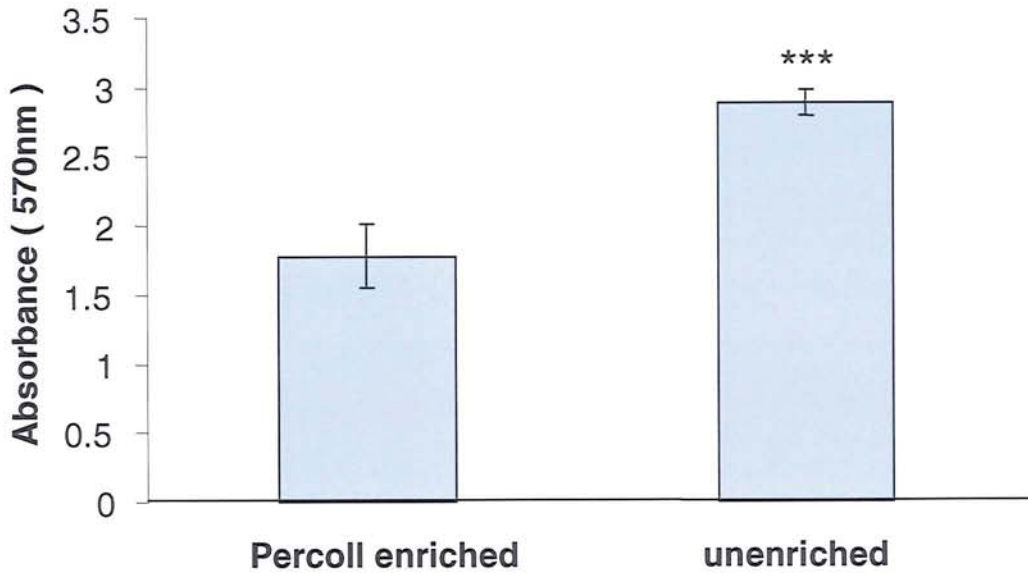
#### 3.8.6.2. Growth was poorer after 72h if cells were enriched before plating

When cell number was assessed after 3 days by the crystal violet assay (Figure 3.8.6.2), there were more cells when unenriched cells were plated in comparison with enriched cells.



**Figure 3.8.6.1** Effect of enriching the cells on a Percoll density gradient on the number of cells attached to the wells of tissue culture plate (TCP) assessed by trypsinisation and direct cell counting.

Results are means  $\pm$  S.E.M. of mean from 5 wells on each day.

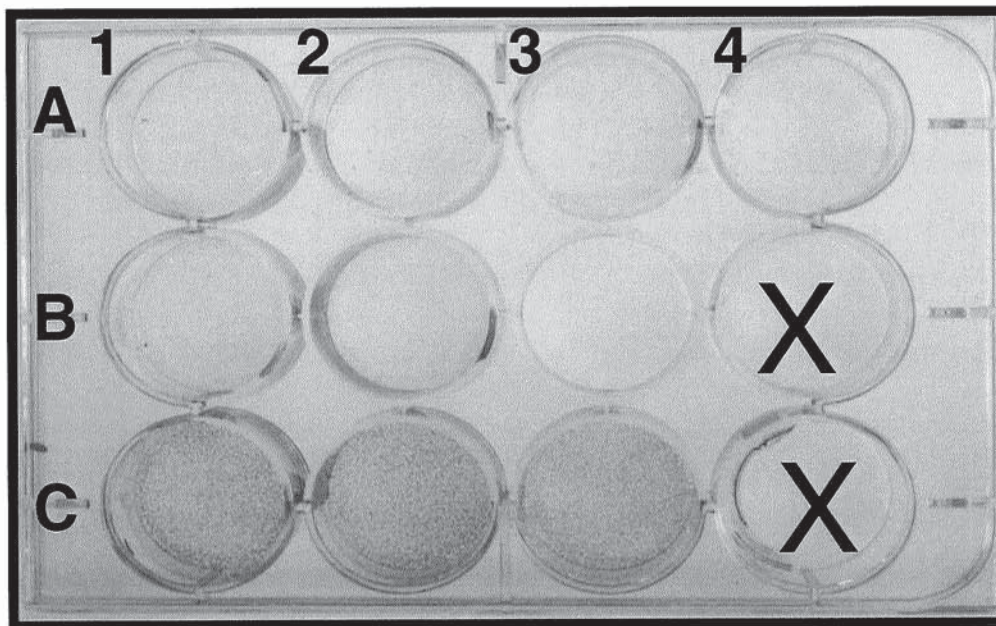


**Figure 3.8.6.2** Effect of enriching the cells on a Percoll density gradient on the number of cells attached to the wells of tissue culture plate (TCP) after 72 h of initial incubation as assessed by the crystal violet assay. Results are means  $\pm$  S.E.M. of mean from 3 wells for Percoll enriched cells and 6 wells for unenriched cells (control cells) respectively. \*\*\* $P < 0.001$  for a difference between cell number of Percoll enriched and unenriched cells by unpaired t-test.

### 3.8.7 Cryopreservation

Photographic examination (figure 3.8.7 shows that when plated in the presence of DMSO at two different concentrations (1 % and 10 %), cells did not attach or survive. When DMSO was removed by centrifugation and the cell pellet resuspended in fresh medium before plating, the cells were able to attach and grow. However, growth was poor compared to fresh cultures and thus cells were unable to form a monolayer. These results indicate that cells do not fully recover after cryopreservation and this could be due to the sensitivity of the cells to DMSO.





**Figure 3.8.7 Effect of DMSO upon retrieval of gastric epithelial cells after cryopreservation**

Cells were cultured for 72 h post thawing.

Lane A (wells 1-4) represents cells plated in the presence of 1 % DMSO

Lane B (wells 1-2) represents cells plated in the presence of 2 % DMSO

Lane B (well 3) represents cells plated in the presence of 1 % DMSO

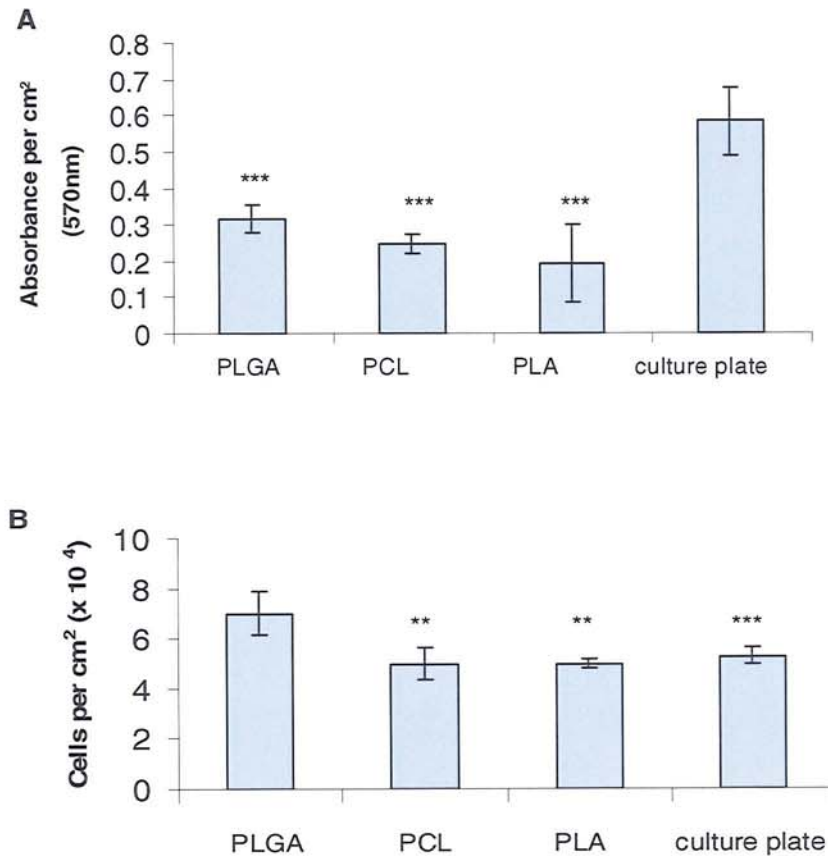
Lane C (wells 1-3) represents cells plated post-centrifugation in the absence of DMSO

### **3.9 Results- Growth of gastric epithelial cells on biodegradable polymer matrices**

When cell number was assessed after 3 days by trypsinisation and direct cell counting more cells were attached to PLGA than to the other surfaces Figure 3.9B. Note that to enable comparison with the 12 well plate cell numbers were expressed per unit surface area with the assumption that the polymer surfaces were flat.

However, microscopic examination after 3 days of culture showed, that coverage was less for the PLGA matrix than for tissue culture plastic, with cells on PLGA existing as ‘islands’. A different result was obtained when the crystal violet assay was used to assess cell number Figure 3.9A. Results were variable with this procedure for the polymeric support and it is unclear whether the ‘blank’ correction for dye absorption in the absence of cells was

appropriate. The results obtained from direct cell counting suggest that initially cell attachment was less on PLGA than TCP but that cell loss from TCP was greater such that after 3 days more cells were on PLGA.



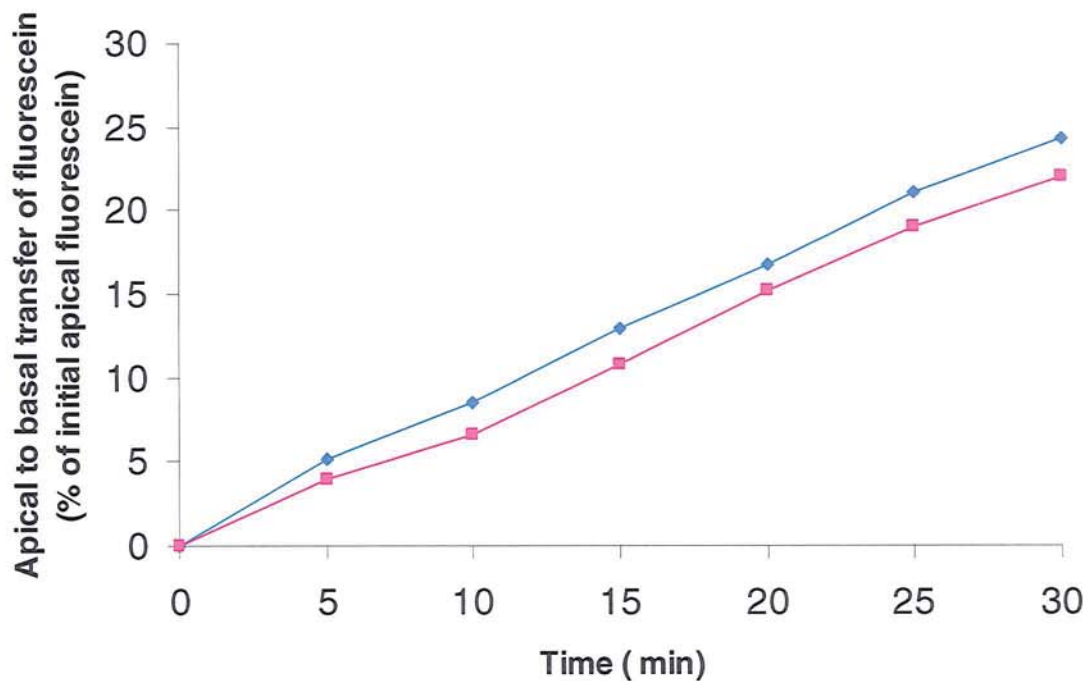
**Figure 3.9 Effect on cell number after 72 hours of culture on four different surfaces (PLGA (poly (L-lactide co-glycolide), PCL (poly(  $\epsilon$ -caprolactone), PLA( poly (l-lactide)) and TCP.**

Cell number was assessed by (A) crystal violet assay and (B) trypsinisation and direct cell counting. Cells were plated at  $13.8 \times 10^5$  cells per ml with 1.4 ml in each well of a 12 well plate ( $5.1 \times 10^5$  cells/cm<sup>2</sup>) and 0.5 ml in the vials ( $5.1 \times 10^5$  cells/cm<sup>2</sup>). Both sets of data have been expressed per cm<sup>2</sup> of vessel surface area to normalise for the difference in size between the wells and vials. (A) Data are expressed as means  $\pm$  S.E.M. of three vials containing polymer and twelve wells of the culture plate. \*\*\*P<0.001 for a difference between the culture plate and the other treatments after single factor ANOVAR and a Newman-Keuls test. All other comparisons were not significantly different. Note: A correction was made for adsorption of crystal violet dye to the polymers by using results obtained with vials containing polymer but no cells. This correction was variable and may not have been the same in the presence of cells. (B) Data are expressed as means  $\pm$  S.E.M. of three vials containing polymer and eight wells of the culture plate. \*\*, P<0.01, \*\*\*P<0.001 for a difference between PLGA and the other treatments after single factor ANOVAR and a Newman-Keuls test. All other comparisons were not significantly different.

### 3.10 Results- Growth of gastric epithelial cells on Transwells

#### 3.10.1 Effect of increased digestion time with collagenase for cells cultured on polyester Transwells (3 $\mu\text{m}$ pore)

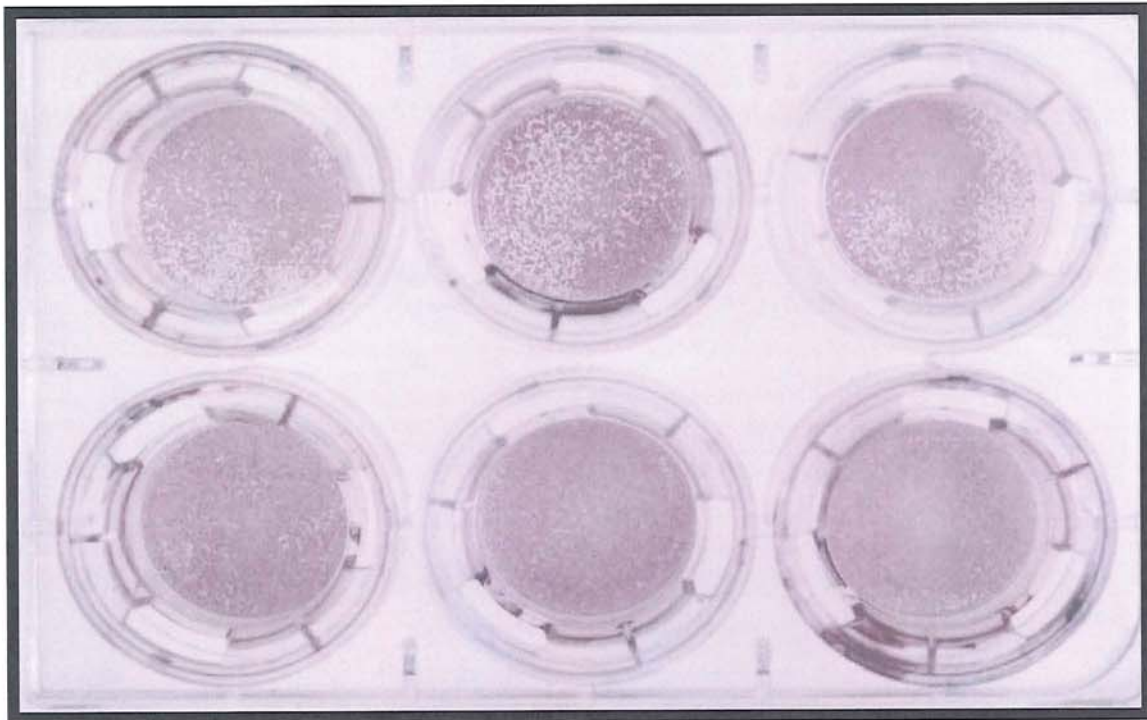
Figure 3.10.1.1 shows that increasing the time of digestion in collagenase to reduce cell clumps did not affect transfer of fluorescein from the apical to the basal chamber for cells grown on polyester Transwells. Microscopic examination (Figure 3.10.1.2) suggested that in neither treatment was the production of a cell monolayer achieved, but a possible improvement in coverage was seen after digestion for 40 min.



**Figure 3.10.1.1** Effect of time of treatment of cells with collagenase on monolayer formation as assessed by fluorescein transfer from the apical to basal chamber of polyester Transwells after the culture of guinea-pig gastric mucosal cells for 4 days.

Results are means  $\pm$  S.E.M. of means from 3 Transwells for each time of digestion. Squares are cells treated with collagenase for 20 min and diamonds are cells treated with collagenase for 40 min.





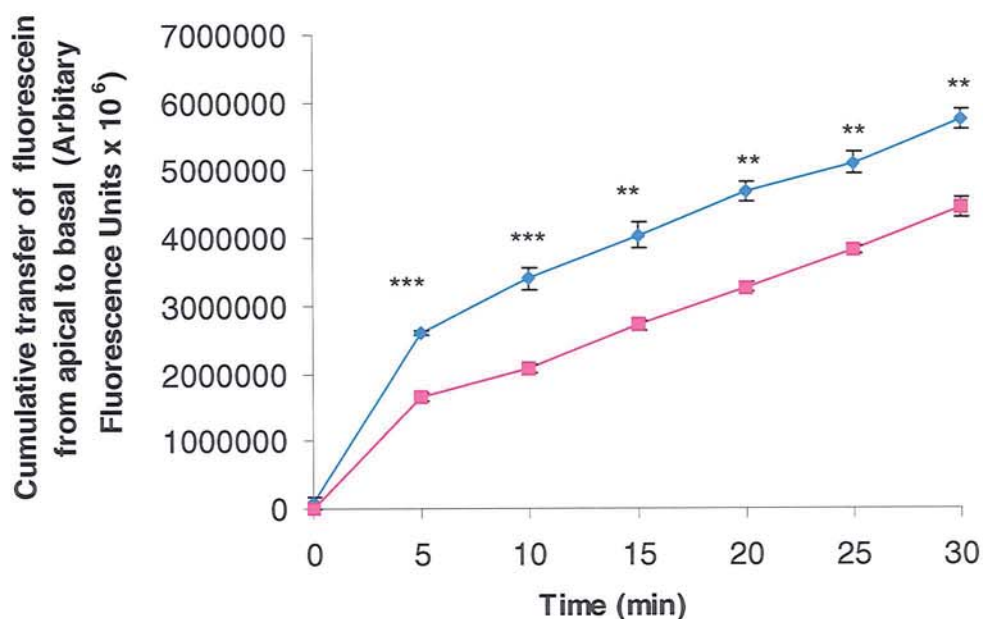
**Figure 3.10.1.2** Effect of time of treatment of tissue with collagenase on subsequent monolayer formation on polyester Transwells during the culture of guinea-pig mucosal cells for 4 days

The photograph shows gastric epithelial cells after 4 days of culture on polyester Transwells. The top three wells were plated with cells treated for 20 min with collagenase, whereas the three bottom wells were plated with cells treated for 40 min with collagenase. The violet colour is due to staining of the cells with crystal violet after post to fixation with 4% paraformaldehyde.

### **3.10.2 Effect of increased time of digestion in collagenase for cells cultured on polycarbonate Transwells (3 $\mu\text{m}$ pore)**

The cumulative transfer of fluorescein from the apical to the basal chamber was apparently lower for Transwells plated with cells obtained after digestion for 40 min with collagenase compared to Transwells seeded with cells obtained after digestion for 20 min with collagenase (Figure 3.10.2.). Microscopic examination (results not shown) suggested that after 4 days in culture cells prepared using increased time of digestion in collagenase spread more evenly over the filter in comparison to cells prepared with shorter exposure to collagenase.

Subsequently all the studies that followed were performed with cells treated with collagenase for 40 min and seeded on Transwells fitted with a polycarbonate membrane.

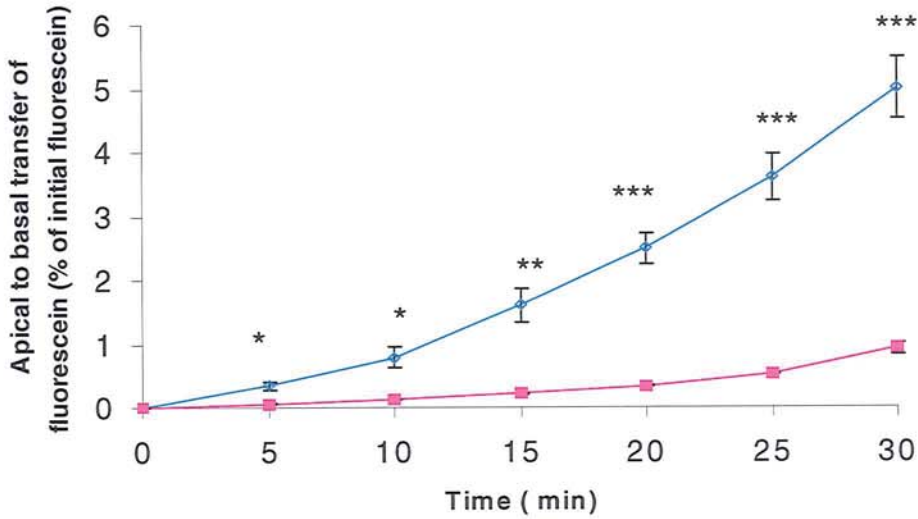


**Figure 3.10.2 Effect of time of digestion of tissue with collagenase on monolayer formation after the culture of guinea-pig gastric mucosal cells for 4 days as assessed by fluorescein transfer.**

Results are means  $\pm$  S.E.M. of means from 3 Transwells in each case. Results are expressed in arbitrary units because a satisfactory calibration curve was not obtained on this occasion. Diamonds are tissue treated with collagenase for 20 min and squares are tissue treated with collagenase for 40 min. \*\* $P < 0.01$ , \*\*\*  $P < 0.001$  for a difference between collagenase treatment by unpaired t-test.

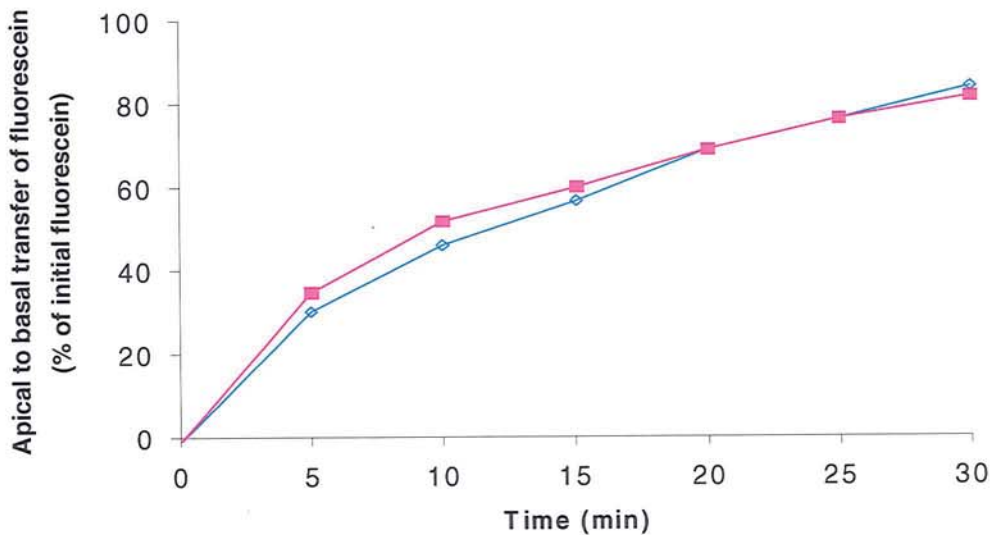
### 3.10.3. Effect of coating Transwells with collagen (3 $\mu\text{m}$ pore, polycarbonate, 10 ng/ml EGF)

Transfer of fluorescein from apical to basal for cells grown on collagen-coated filters (Figure 3.10.3.1) is very much lower in comparison to cells grown on uncoated filters. Collagen coating of the Transwells (Figure 3.10.3.2) does not affect the amount of fluorescein that is transferred across the filters in the absence of cells. Photographic examination of the Transwells (Figure 3.10.3.3) clearly indicates the formation of a more complete monolayer when the cells are plated on collagen-coated filters by comparison with the uncoated filters.



**Figure 3.10.3.1** Effect of coating Transwells (3.0  $\mu\text{m}$  pore) with type I collagen on the formation of monolayers after the culture of guinea-pig gastric mucosal cells for 3 days as assessed by fluorescein transfer.

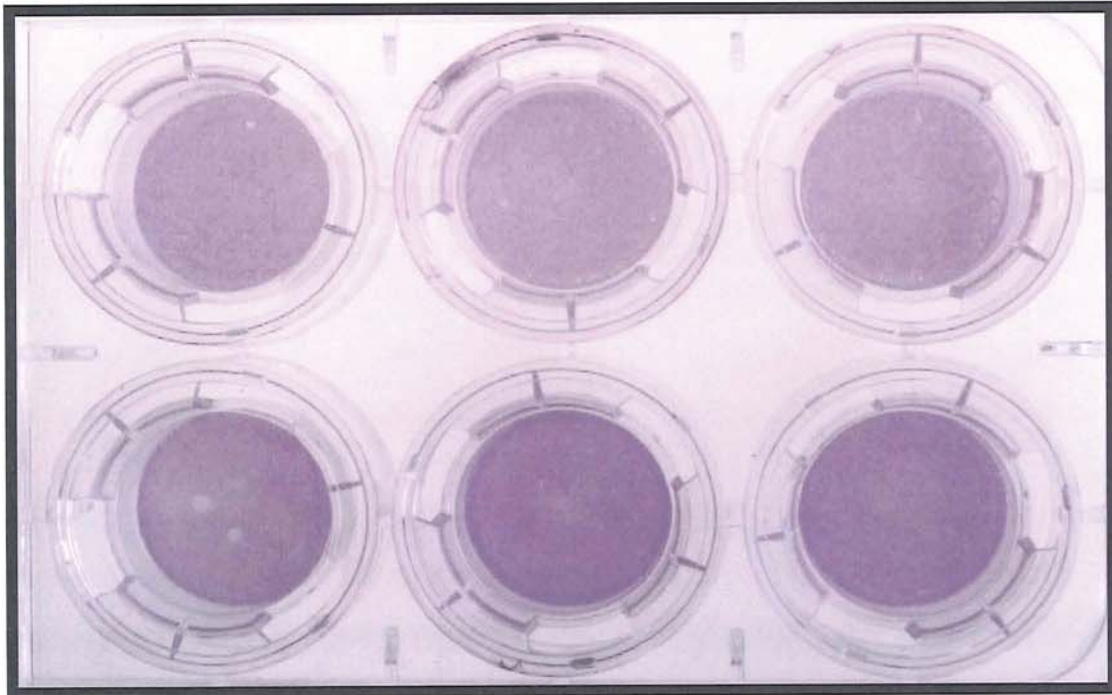
Results are means  $\pm$  S.E.M. of means from 3 Transwells. Diamonds are cells plated on control Transwells and squares are cells plated on Transwells coated with type I collagen. \*P< 0.05, \*\*P< 0.01, \*\*\* P< 0.001 for a difference between uncoated and coated Transwells by unpaired t-test.



**Figure 3.10.3.2** Effect of coating Transwells (3.0  $\mu\text{m}$  pore) with type I collagen on fluorescein permeability through cell free Transwells.

Diamonds represent the uncoated Transwell and squares the coated Transwell. No difference between conditions by unpaired t-test.



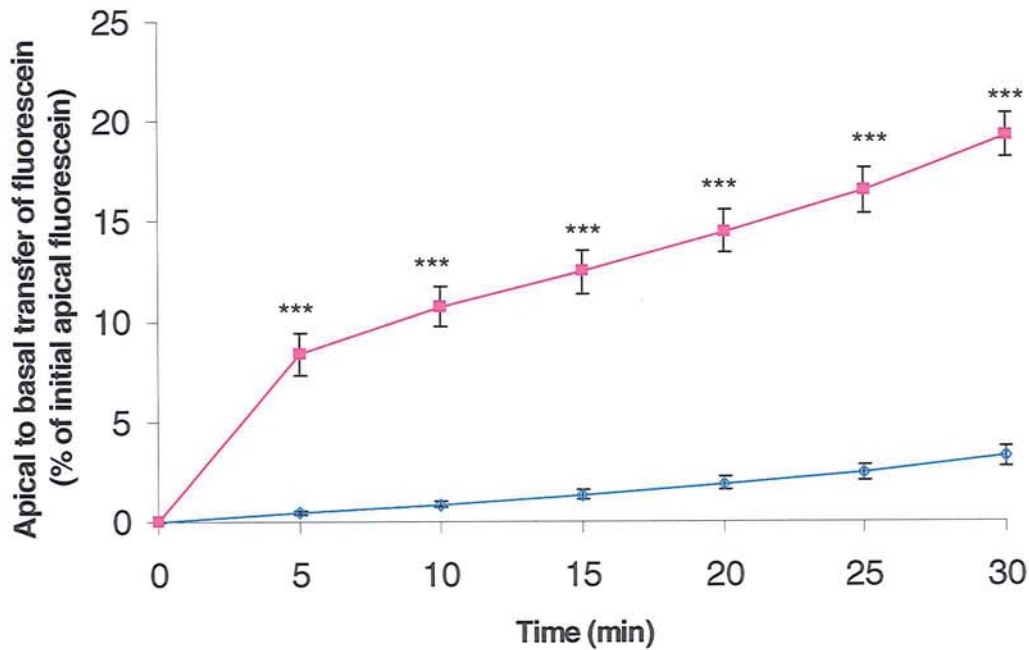


**Figure 3.10.3.3 Effect of coating Transwells with collagen type I on the formation of cell monolayer.**

The photograph shows gastric epithelial cells after 3 days of culture. The top three wells represent monolayers on uncoated Transwells, whereas the three bottom wells represent monolayers formed on collagen coated Transwells. The blue colour is due to staining of the cells with crystal violet post fixation 1% glutaraldehyde.

### **3.10.4 Effect of pore size on monolayer formation (polycarbonate) (3 $\mu\text{m}$ pore, collagen I coated Transwells , 3 days, 10 ng/ml EGF)**

Decreasing the pore size of the filter from 3  $\mu\text{m}$  to 0.4  $\mu\text{m}$  effected a 76 % reduction in the  $P_{\text{app}}$  of fluorescein ( $P < 0.001$ ) when the pore size was smaller (Figure 3.10.4.1). Thus the  $P_{\text{app}}$  for cells cultured on 3  $\mu\text{m}$  pore size Transwells was  $1.31 \times 10^{-5} \pm 3.6 \times 10^{-7}$  ( $\text{cm} \cdot \text{sec}^{-1}$ ) whereas for cells cultured on 0.4  $\mu\text{m}$  pore Transwells was  $3.2 \times 10^{-6} \pm 5.35 \times 10^{-7}$  ( $\text{cm} \cdot \text{sec}^{-1}$ ) ( $P < 0.001$ ) for triplicate wells from a single experiment). The transepithelial electrical resistance (TEER) for cells on 3 $\mu\text{m}$  pore support was much lower at  $150 \pm 36$  ohms. $\text{cm}^2$  than for cells on 0.4  $\mu\text{m}$  pore with a TEER of  $281 \pm 23$  ohms. $\text{cm}^2$ . There was no difference between the resistance of 3 and 0.4  $\mu\text{m}$  pore Transwells in the absence of cells.

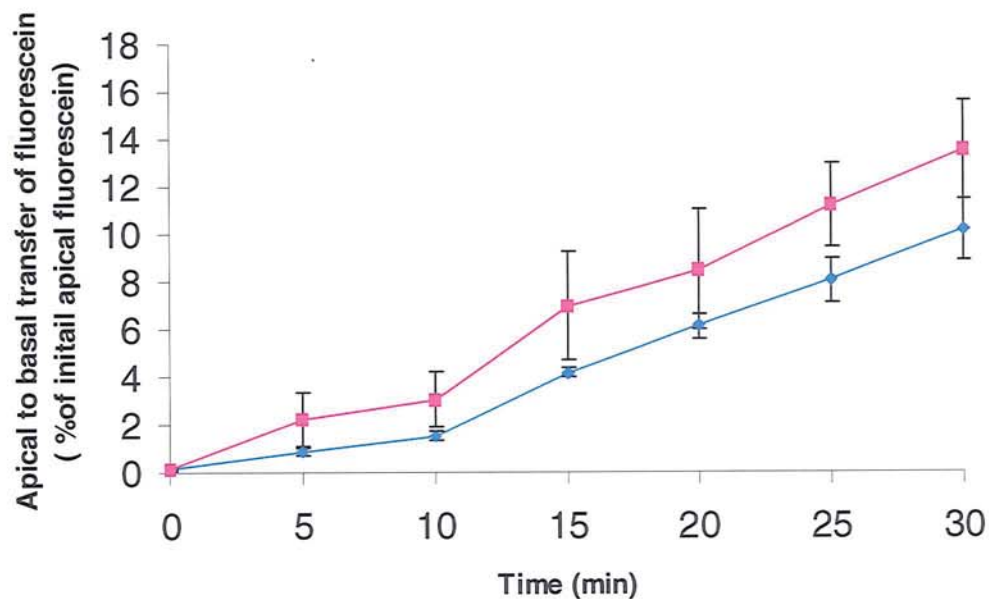


**Figure 3.10.4** Effect of pore size of collagen I coated Transwell on fluorescein permeability through guinea-pig gastric mucous cells cultured for 3 days.

Results are presented as means S.E.M. of a single cell preparation. \*\*\*  $P < 0.001$  for a difference between Transwell pore size by unpaired t-test. Squares symbols represent results for 3  $\mu\text{m}$  pore size and diamonds results for 0.4  $\mu\text{m}$  Transwells.

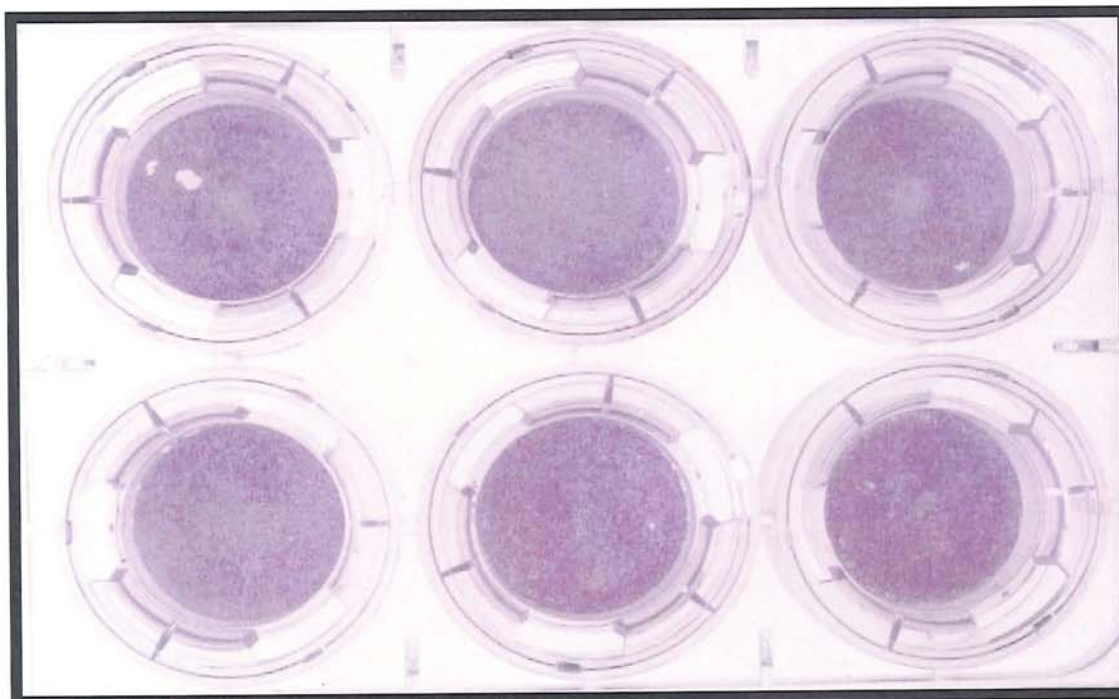
### 3.10.5 Effect of conditioning Transwells with complete culture medium (3 $\mu\text{m}$ pore, 3 days, 10 ng/ml EGF)

Conditioning the filters with culture medium did not significantly affect fluorescein transfer from apical to basal in comparison to control filters (Figure 3.10.5.1).  $P_{\text{app}}$  values for control filters were  $1.40 \times 10^{-5} \pm 2.09 \times 10^{-6}$  ( $\text{cm}\cdot\text{sec}^{-1}$ ) and for conditioned filters was  $1.75 \times 10^{-5} \pm 5.04 \times 10^{-6}$  ( $\text{cm}\cdot\text{sec}^{-1}$ ), with no difference between conditions by unpaired t-test. Photographs of the Transwells (Figure 3.10.5.2) showed no obvious difference between the quality of the monolayers on the control and conditioned filters respectively.



**Figure 3.10.5.1** Effect of preconditioning the Transwells with culturing medium on the formation of monolayers after the culture of guinea-pig gastric mucosal cells for 3 days as assessed by fluorescein transfer.

Results are means  $\pm$  S.E.M. of means from 3 Transwells. Diamonds are cells plated on control transwells (i.e. not preconditioned) and squares are cells plated on preconditioned transwells. Cells were plated at  $15.8 \times 10^5$  cells /well in the presence of 10 ng/ml EGF. No difference between conditions by un-paired t-test.



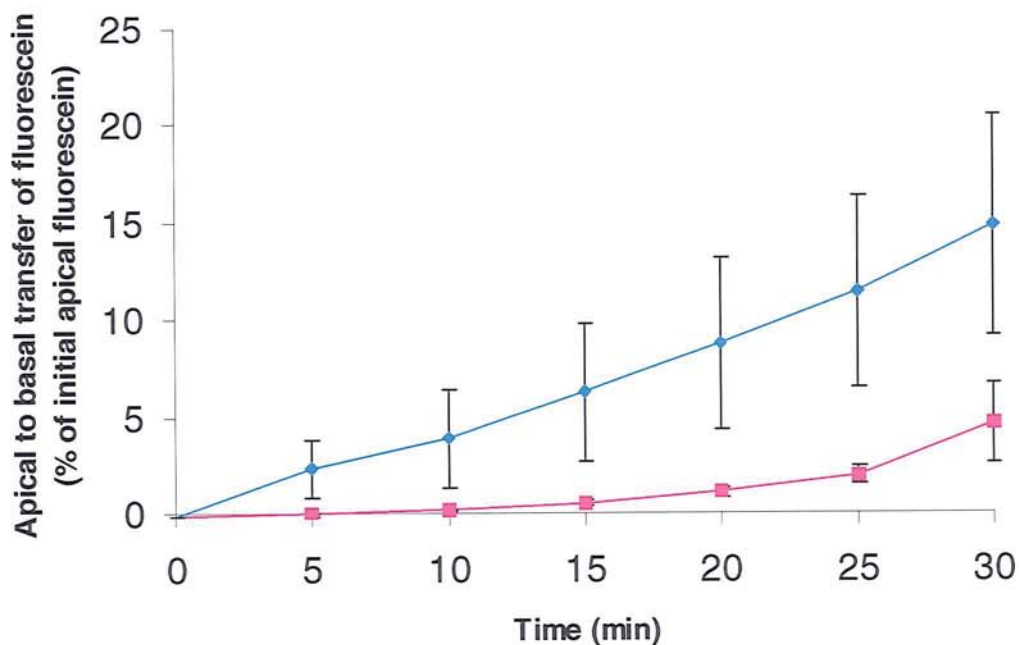
**Figure 3.10.5.2** Effect of conditioning Transwells with medium on monolayer formation

The photograph shows gastric epithelial cells after 3 days of culturing on polycarbonate Transwells (3.0  $\mu$ m pore size). The top three wells are control wells, whereas the three wells on the bottom were conditioned with culturing medium 24h prior seeding. The blue colour is due to staining of the cells with crystal violet post fixation with 1% v/v glutaraldehyde in PBS.



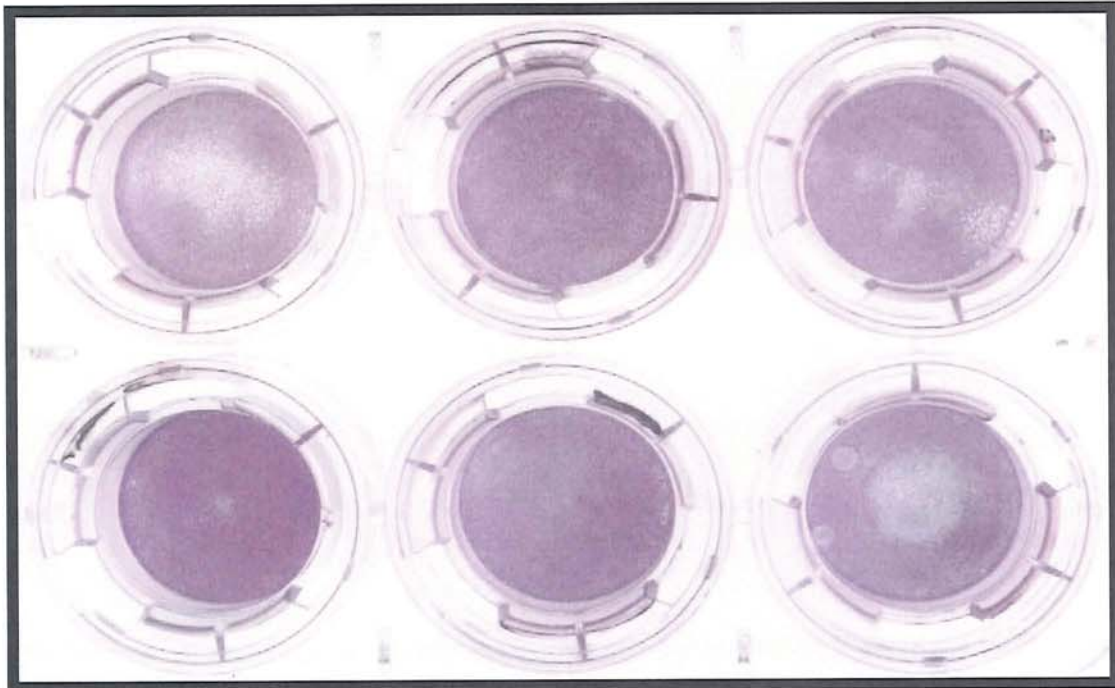
### 3.10.6 Effect of enrichment of mucous cells on monolayer formation (3 $\mu\text{m}$ pore size, 10 ng/ml EGF)

The formation of monolayers was not achieved when enriched cells were plated onto collagen I coated filters (3  $\mu\text{m}$  pore size, 10 ng/ml EGF) (Figure 3.10.6.1) and this observation is supported by the increased transfer of fluorescein from the apical to the basal chamber for filters seeded with enriched cells in comparison with the low percentage of fluorescein transfer for collagen coated filters seeded with unenriched cells (Figure 3.10.6.1).



**Figure 3.10.6.1** Effect of enrichment of mucous cells by Percoll density gradient on the formation of monolayers during the culture of cells for 3 days as assessed by fluorescein transfer.

Diamonds are enriched cells and squares are control (unenriched) cells. Results are means  $\pm$  S.E.M. of means from 3 wells.

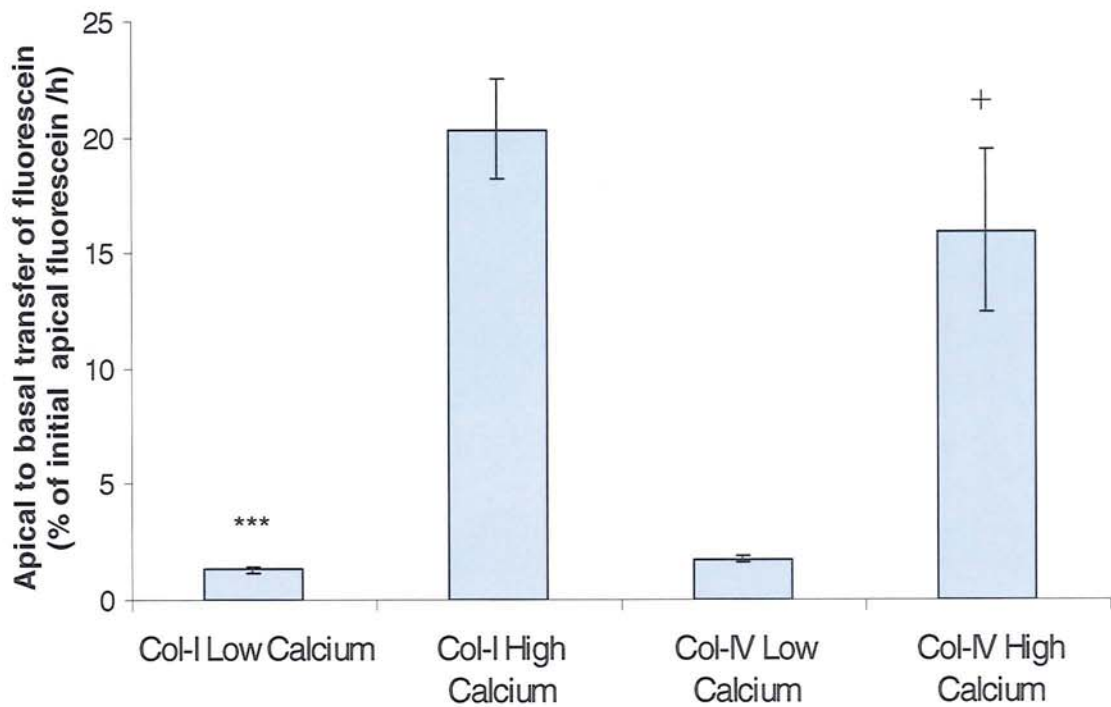


**Figure 3.10.6.2 Effect of enrichment of mucous cells before plating on monolayer formation.**

The photograph shows gastric epithelial cells after 3 days of culture on collagen type I coated Transwells (3  $\mu\text{m}$  pore size). The top three wells are seeded with enriched cells, whereas the three wells on the bottom are seeded with unenriched cells. The blue colour is due to staining of the cells with crystal violet post to fixation with 1 % glutaraldehyde.

### **3.10.7 Effect of calcium supplementation on the monolayer formation as assessed by transfer of fluorescein across collagen I and collagen IV coated Transwells (0.4 $\mu\text{m}$ pore, 10 ng/ml EGF)**

Calcium supplementation of the culture medium 24-72 h after plating clearly prevented the formation of monolayers whether the Transwells were coated with either type of collagen as evidenced by the increased values of fluorescein transfer when the medium was supplemented with additional calcium (Figure 3.10.7).



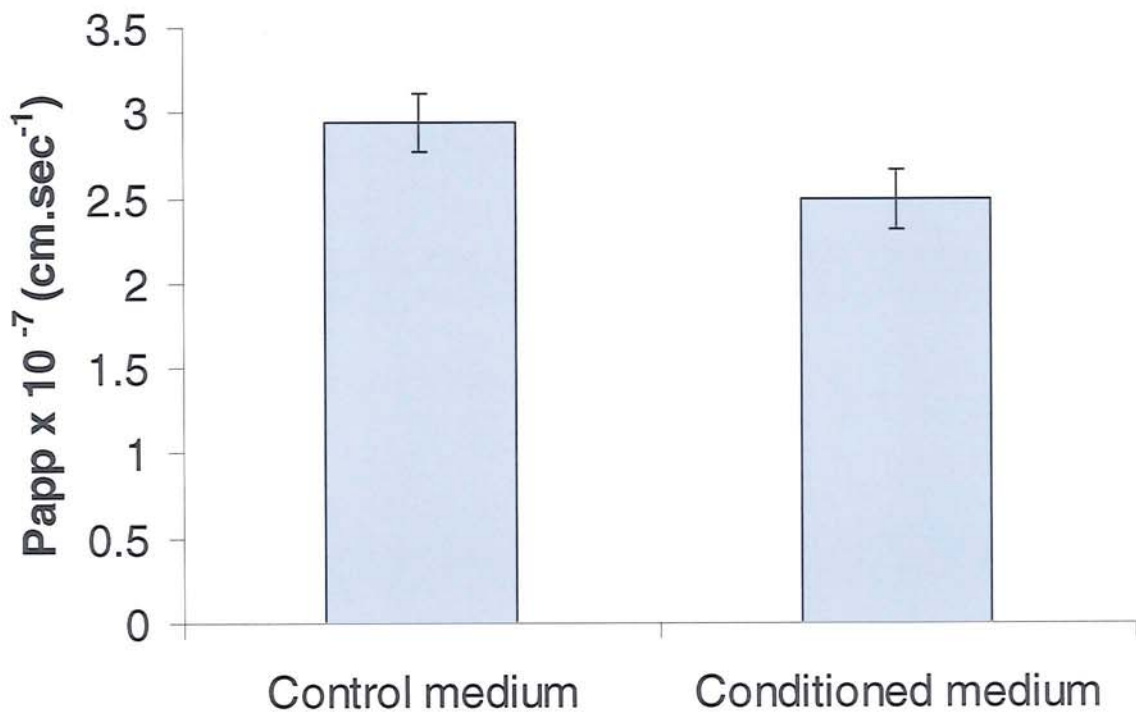
**Figure 3.10.7 Effect of extracellular calcium concentration on the transfer of fluorescein across collagen I and collagen IV coated Transwells.**

Data are from single cell preparation and are means  $\pm$  S.E.M. of 3 wells in each case. Cells were cultured for 72h on polycarbonate Transwells with 0.4  $\mu$ m pore and were coated with collagen I and collagen IV respectively. For low calcium, the culture medium was changed at 24 and 48 h with standard 0.4 mM  $\text{Ca}^{2+}$  culture medium, whereas for high calcium, culture medium was changed at 24 and 48 h with 2mM  $\text{Ca}^{2+}$  supplemented culture medium. \*\*\*  $P < 0.001$  for a difference between collagen I low calcium and high calcium, and †  $P < 0.05$  for a collagen IV low calcium and high calcium.

### 3.10.8 Effect of conditioned medium

Replacement of half the culture medium with conditioned medium from 24-72 h had no subsequent effect on fluorescein permeability (Figure 3.10.8) or TEER compared to cells grown with control medium. The TEER value was  $699 \pm 30$  ohms. $\text{cm}^2$  for cells cultured in the presence of conditioned medium, while for control cells the TEER was  $560 \pm 9.8$  ohms. $\text{cm}^2$ .





**Figure 3.10.8 Effect of conditioned culture medium on apparent permeability of fluorescein across collagen-I coated Transwells.**

Results are from a single cell preparation, and are presented as means  $\pm$  S.E.M. of 3 wells in each case. Cells were cultured for 72 h on polycarbonate Transwells with 0.4  $\mu$ m pore which were coated with collagen-I. For control medium, culture medium was changed at 24 and 48 h with standard culture medium, whereas for conditioned medium, culture medium was changed at 24 and 48 h with conditioned medium (50:50)(refer to section 3.3.5) culture medium. No difference between conditions by unpaired t-test.

**3.10.9 The overall findings of the above pilot experiments are summarised below (Table 3.10.9)**

**Table 3.10.9**

<i>Factors which promoted monolayer formation</i>	<i>Factors which had no effect or impaired monolayer formation</i>
Increasing digestion time in collagenase from 20 to 40 min	Prior enrichment of mucous cells on a Percoll density gradient
Reducing the pore-size of polycarbonate membranes from 3 to 0.4 $\mu\text{m}$	Preincubation of Transwells with tissue culture medium for 24 h
Coating Transwells with collagen I compared to untreated Transwells	Adding conditioned medium from gastric mucosal fibroblasts in 1:1 ratio with cultured medium for 24 –72 h
Epidermal growth factor at 10 ng/ml throughout culture	Increasing medium $\text{Ca}^{2+}$ from 0.4 to 2.0 mM for the period 24-72 h Culture for longer than 72 h

### 3.11 Discussion

The results obtained from these experiments indicate that conditions can be obtained under which isolated guinea pig gastric epithelial cells attach, grow and form almost confluent cell monolayers on tissue culture plastic (TCP) and on polycarbonate Transwells after 72 h of culture. Cell adhesion to materials is mediated by cell surface receptors (such as integrins) interacting with cell adhesion molecules on the material substrate. The initial cell attachment is accompanied by spreading and flattening of the cell, clustering of cell adhesion receptors in the cell membrane and assembly of cytoskeletal complexes on these clusters. This process eventually leads to integration of the extracellular matrix with the intracellular cytoskeleton and the cell signalling cascades that control proliferation and differentiation (Hubbell, 1985). In reality, cells do not directly attach to the material substrate but it is the proteins of the culture medium that interact with them and eventually form a coat. The cells then secrete a matrix that contains type IV collagen and laminin. Integrins on the cell surface bind to collagen and to the laminin and as a result the cells become firmly attached to the TCP, the polymeric surfaces, or the Transwells.

The essential factors in serum include (1) adhesion factors such as fibronectin; (2) growth factors such as platelet derived growth factor (PDGF), EGF, and TGF- $\beta$ , that regulate growth and differentiation; (3) essential nutrients, such as minerals, vitamins, fatty acids, and intermediary metabolites; and (4) hormones such as insulin, hydrocortisone, oestrogen, and triiodothyronine, that regulate membrane transport, phenotypic status and the constitution of the cell surface. Our results show that 10 % FCS produced greater cell attachment and growth on TCP as evidenced in comparison to 20 % FCS. This agrees with a study by Matsuda *et al.* (1996) where 10 % FCS had a significant stimulatory effect in comparison with 20 % on the growth of guinea pig gastric mucous cells on collagen I coated TCP. Matsuda *et al.* (1996) also showed that the isolated gastric mucous cells from guinea-pigs did not sufficiently attach



to TCP when FCS was omitted. Increasing, the FCS concentration to 20 % suppressed cell proliferation and attachment in comparison to 10 % FCS. As well as its growth-promoting activity, serum contains growth inhibiting activity, and although stimulation usually predominates, the net effect of the serum is an unpredictable combination of both. While substances such as PDGF may be mitogenic to fibroblasts, other constituents of serum such as TGF- $\beta$  can be cytostatic (Freshney, 2000). In fact, TGF- $\beta$ , completely inhibited EGF induced proliferation of guinea-pig gastric epithelial cells, and TGF- $\beta$  treated cells did not replicate and remained in an immature stage (Rokutan *et al.*, 1998). Different gastric epithelial cell cultures require different FCS concentrations. Thus the rat RGM-1 (Katao *et al.*, 2003) gastric epithelial cell line requires 20 % FCS, however the rat (Takao *et al.*, 1993) and guinea pig (Kawahara *et al.*, 2001) primary epithelial cells require 10 % FCS, and the AGS human gastric adenocarcinoma cell line requires 5 % (Logan, *et al.*, 1998). In retrospect, it might have been interesting to examine effects of reducing the FCS content to 5 % or below especially on collagen-coated supports.

EGF (10 ng/ml) probably aided monolayer formation of cultured guinea pig mucous cells by promoting cell growth (Sakamoto *et al.*, 1994). Studies by Matsuda *et al.* (1996), showed that high affinity EGF receptors were present on the guinea-pig gastric mucous cells and that EGF was involved in the stimulation of DNA synthesis by the cells. Functional apical and basolateral EGF receptors exist in primary cultures of canine gastric chief/parietal cells and these receptors can regulate paracellular permeability (Chen *et al.*, 2001). Protein tyrosine phosphorylation mediates activation of the EGF receptor (EGFR), a process initiated by autophosphorylation of EGFR itself (Pai and Tarnawski, 1998). The intracellular cascade that is produced by the receptor phosphorylation induces cellular responses such as cell proliferation, differentiation and cell migration. Our results suggest that EGF (10 ng/ml) had a significant effect on growth of the cells seeded on TCP in comparison to control cells

especially if cells were seeded at low density, and for that reason EGF (10 ng/ml) was used almost throughout the project to promote the formation of confluent monolayers. Increasing EGF concentration from 10 ng/ml to 20 ng/ml was not examined since such a change did not increase cell number of guinea-pig gastric epithelial cells grown on TCP (Rokutan *et al.*, 1998). 10 ng/ml EGF was used throughout any subsequent culture.

Direct cell counting and the crystal violet assay revealed that significant net cell loss occurred over time in culture, however after 3 days a monolayer was formed comprising polygonal-shaped cells. Initially cell digests were plated as individual cells and as isolated clumps of cells from which they spread to form a monolayer with epithelial characteristics. That is, a polygonal shape and sheet-like organised growth pattern (Romano *et al.*, 1988). The net loss of cells was due to the disappearance of clumps. An increase of time in collagenase digestion medium will break up the cell clumps and thus monolayer formation should be more attainable. This was confirmed by the photographic examination of cells after 4 days in culture prepared using increased time in digestion collagenase. Cells spread more evenly on the filter in comparison to cells prepared with shorter exposure to collagenase. In addition, gentle agitation of cultures one hour after plating was successful because a more uniform spread of attached cells was achieved. Thus these results generated two improvements in our initial method of cell isolation. Neonatal rat gastric epithelial cells initially attach in clumps to TCP and this is followed by rapid cell growth for 3-4 days, after which time they reach confluency and cease growing. This monolayer also comprised of polygonal shape and sheet-like organised growth (Terano *et al.*, 1982, Terano *et al.*, 2001).

Culturing of cells on biodegradable polymeric supports appeared appealing both for tissue engineered constructs and also an alternate support for cell monolayer formation. In this project the number of cells grown on polymeric baskets composed of respectively PLGA, PLA and PCL were compared with the number of cells attached on TCP. Data obtained by



crystal violet staining were considered unreliable because of uncertainties over appropriateness of the correction for staining of polymers in the absence of cells. Direct cell counting after 72h of culture revealed that more cells attached to PLGA than any of the other surfaces. However microscopic examination showed that cells grown on TCP formed a near monolayer whereas cells cultured on PLGA formed 'islands'. A possible explanation could be that cells use the morphology of the material for orientation and migration and in particular epithelial cells are very sensitive to the morphology of the materials. Thus, variations in surface texture of a material can effect their cellular response. Ouyang *et al.* (2002) showed that bone marrow stromal cells attach and proliferate on high molecular weight PLGA (50:50) to a greater extent than on PCL and PLA. This could also be explained by the fact that the hydrophobicity of substrates affects biological responses, such as cell proliferation. Due to the presence of the extra methyl group in lactic acid, PLA is more hydrophobic than PLGA so that a lower percentage of cells attached to PLA as compared with PLGA. Again, PCL has a high olefinic characteristic and is more hydrophobic than PLGA. (Ouyang *et al.*, 2002). However the advantage of PLGA over TCP was marginal, cells formed islands, and difficulty of maintaining cell monolayers grown on PLGA in chambers caused no further work with these polymers as means of supporting cell culture to be performed.

An essential feature of all epithelial cells is their polarity. Elevation of the culture in a filter well (Chambard *et al.*, 1983) may be advantageous, particularly for certain epithelia, as it provides access to the basal surface to nutrients and ligands. Culture on Transwell also enables the opportunity to establish polarity. A method of assessing monolayer integrity is by the use of a fluorescent or labelled marker molecule which is passively transported across the monolayers. Passive transport of molecules across epithelial monolayers can either occur across the cell membranes (transcellular route), or through the tight junctions bridging the intercellular spaces between the cells (paracellular route). It has been demonstrated that the



transport of hydrophilic molecules across monolayers of intestinal epithelial (Caco-2) cells is by the paracellular pathway whereas lipophilic molecules travel across the monolayers of intestinal epithelial (Caco-2) cells by the transcellular pathway (Artusson & Magnusson, 1990). Thus, the permeability of the hydrophilic fluorescein molecule should be minimal in confluent monolayers with fully developed tight junctions.

TEER gives an evaluation of the ionic paracellular permeability of the monolayer. TEER measurements of cell monolayers grown on permeable supports give information about the resistance to ion flux across the monolayer. Provided that the ion flux across the cell membranes is small compared with that through the tight junctions of the paracellular spaces, TEER should reflect the integrity of the tight junctions between the cells (Tavelin, *et al.*, 2002).

The nature of the support on which the cell monolayers are cultured is fundamental for the achievement of a monolayer with fully developed tight junctions. In the first series of experiments using Transwells, polyester Transwells were used (3  $\mu\text{m}$  pore). However their utilisation was discontinued since staining of Transwells showed that cells did not grow well, and in an attempt to improve cell growth on Transwells polyester Transwells were replaced with polycarbonate Transwells (3  $\mu\text{m}$  pore). Photographic examination (compare 3.10.1.2 bottom row with 3.10.3.3 top row) suggested that the cells adhered and grew better on polycarbonate filters. A study by Artusson, *et al.* (1990), showed that mixed cellulose ester membranes were unsuitable for epithelial transport of drugs in cell culture because these membranes absorbed significant amounts of radiolabelled drugs, when compared to polycarbonate membranes, and thus any subsequent transport studies by those authors were performed with polycarbonate filters. The dramatic role the pore size plays on the quality of the monolayer was observed when reducing the pore size of the Transwells from 3  $\mu\text{m}$  to 0.4  $\mu\text{m}$  which effected a reduction of 76 % on the apparent permeability of fluorescein. Reduction

in the pore size had the inverse effect on TEER, giving an almost 50 % increase in TEER when cells were cultured on 0.4  $\mu\text{m}$  pore compared with the larger pore. This was expected as theoretically the higher the TEER the more established is the monolayer. The above conclusions made the smaller pore size, the pore of choice for the subsequent cell cultures. For over a decade the Artusson group *have* been using Caco-2 cell lines and for half a decade intestinal epithelial cell lines 2/4/ A1, cultured on polycarbonate 0.4 $\mu\text{m}$  pore Transwells as models of small intestinal epithelium. Due to the size of epithelial cells, with a 7nm wide basolateral aspect a smaller pore size could facilitate cell attachment, cell-cell interaction and formation of fully developed tight junctions.

Supports such as TCP and Transwells can be coated with attachment matrices. Coating TCP with collagen type I from rat tail proved to have no effect on promotion of cell growth as evidenced by our results. For the production of a rabbit gastric epithelial cultured cell model, collagen type I was an essential factor (Watanabe *et al.*, 1996). Whether the TCP was coated with collagen I or not, net cell loss with time still appeared, due to the loss of clumps.

Unlike data obtained with TCP, coating polycarbonate Transwells with collagen type I had a major impact on monolayer and tight junction formation. This was evidenced by the significant decrease of fluorescein transfer in comparison to uncoated Transwells which indicated the formation of monolayers with better developed tight junctions when cells were cultured on collagen coated filters. It could have been speculated that the significant decrease of fluorescein transfer when Transwells were pre-coated with collagen could have been due to collagen blocking the filter pores. However this was not the case since our results showed that collagen coating of the Transwells did not affect the amount of fluorescein that was transferred across the Transwells in the absence of cells.



According to Steele *et al.* (1985) by soaking the filter in culture medium overnight, epithelial cells grow better since attachment factors in the medium permeate the filter and thus allow better cell attachment. However, this did not happen in our case as indicated by the similar rate of fluorescein transfer for both cells seeded on conditioned and unconditioned filters. Unlike the preparation of Rattner *et al.* (1985) enriching mucous cells in the current preparation by centrifugation on a Percoll density gradient had a negative effect on cell attachment and proliferation both on TCP and on Transwells.

The enrichment of guinea-pig mucous cells before plating is not necessary to produce gastric epithelial monolayers on TCP, after 3 days comprised of over 90 % of cells with PAS-positive mucous granules in the cytoplasm (Tsutsumi, 2000) (see also later chapters of this thesis). The detrimental effect of Percoll-density gradient centrifugation prior to plating experienced in this work may have been derived from loss of cell viability or extensive mucus release effecting clumping of the cells on the density gradient.

In order to examine the effect of extracellular  $\text{Ca}^{2+}$  on tight junction formation, the culture medium used in this study, RPMI 1640 which contained a low  $\text{Ca}^{2+}$  concentration (0.4 mM) was supplemented with  $\text{Ca}^{2+}$  in order to produce a concentration of 2 mM. Artusson *et al.* (1990) observed a gradual decrease in TEER, when Caco-2 monolayers were exposed to medium containing low  $\text{Ca}^{2+}$  (0.4 mM) which was reversed when medium containing high  $\text{Ca}^{2+}$  concentration (1.8 mM) was added. This study thus suggested that a high extracellular  $\text{Ca}^{2+}$  decreased tight junction permeability. The results obtained from the current study however showed a detrimental effect on monolayer formation. This results could agree with a study by Rutten *et al.* (1999) on human primary gastric epithelial cells where it was shown that an extracellular  $\text{Ca}^{2+}$  concentration of 0.5mM produced a stimulation of cell growth rates over a 3-day period, however concentrations of extracellular  $\text{Ca}^{2+} > 2$  mM produced no further increase in cell proliferation but actually produced a slow decrease



in cell growth compared with the lower  $\text{Ca}^{2+}$  concentrations. In other cases a combination of low and high  $\text{Ca}^{2+}$  concentrations was required for attachment and formation of confluent monolayers on polycarbonate Transwells. For example, for immortalized human proximal tubule HK-2 cells and primary human proximal tubule cells, initially a low medium  $\text{Ca}^{2+}$  concentration of 5  $\mu\text{M}$  is required for the first 4 h post seeding which is then replaced with a  $\text{Ca}^{2+}$  concentration of 1.26 mM until the monolayers reach confluence (Whitin *et al.*, 2002).

Despite the high expectations on the effect of conditioned medium (50:50) on monolayer formation, our results suggested that there was no subsequent difference in the quality of the monolayer in the presence or absence of conditioned medium (50:50), as assessed by fluorescein transfer and the TEER of the monolayer. Although gastric mucosal fibroblasts are thought to secrete hepatocyte growth factor, the present experiments with conditioned medium derived from fibroblasts do not support a role for this factor in promoting monolayer formation by gastric mucosal cells.

Recovery of cells from cryopreservation was difficult. When DMSO was present in the culture post thawing, the cells did not recover and did not attach and grow on the TCP. Much of the cellular damage in freezing is caused by formation of ice crystals within the cells. Use of DMSO and a controlled rate freezing minimises crystal formation and thus it is not possible to freeze cells in the absence of DMSO. Technical support material of Biowhittaker suggests not to centrifuge after thawing to remove the cryoprotectant (DMSO) since this action is generally more damaging than the effect of DMSO itself. This was not so our case since cells only grew upon cryoretrieval when they were centrifuged in order to remove the cryoprotectant. Although a monolayer was not formed, this might have been expected, since successfully recovered cells should probably have a reduced plating efficiency. Cryopreservation for primary cultures is not however impossible as cryopreservation of freshly isolated hepatocytes is considered a standard procedure for the

long-term storage of liver cells. Although both necrosis and apoptosis does occur in primary hepatocytes following isolation and cryopreservation (Fu *et al.*, 2001).

This current system, like rat and other guinea-pig gastric cell cultures, cannot be maintained by subculturing over a long term because of factors such as fibroblast overgrowth (Terano *et al.*, 1982; Gilbert and Migeon, 1975; Teshima *et al.*, 1998). Microscopic examination of our preparation showed that after 5 days in culture the number gastric mucosal cells began to decrease, and a large number of another cell type probably fibroblasts appeared on day 7. Two weeks later, fibroblasts became predominant. Terano, *et al.* (1982) could prevent overgrowth by collagenase treatment or by using culture medium supplemented with D-valine. However after 14 days (two passages) the epithelial cells stopped growing and were covered with fibroblasts.

### **3.12 General conclusions**

The results obtained from the preliminary experiments show that near monolayers are achievable by 72h of cell culture on TCP. Cell number drops with culture time, as cells spread and divide and clumps are lost. 10 ng/ml EGF facilitated cell growth. Preliminary methods for cryopreservation of this primary culture proved to have limited success.

Growth of gastric cells on polymeric surfaces was not such an improvement over TCP to merit further work. It was concluded that it was possible to attain a confluent monolayer of gastric epithelial cells with tight junctions on Transwells when membranes were polycarbonate Transwells with a pore size of 0.4  $\mu\text{m}$  which had been previously coated with collagen type I from rat tail.

## **Chapter 4**

**Effect of the matrix used to coat Transwells on tight-junction formation, differentiation and polarity of gastric epithelial cells**



## 4.1 Aims

The main aims of this chapter were: 1) to examine the effect of culturing the cells on TCP, Transwells, collagen I coated Transwells and collagen IV coated Transwells, with regard to the proportion of mucous cells; 2) to investigate the effect of coating the Transwells with collagen I, collagen IV and collagen IV plus laminin on tight junction formation as assessed by apical to basal transfer of fluorescein and [<sup>14</sup>C]mannitol, and from the TEER of the monolayers; 3) to consider how the matrix used to coat the transwells affects the differentiation and polarity of the gastric epithelial monolayers as assessed by the synthesis and secretion of mucin and superoxide; 4) to employ confocal microscopy on monolayers grown on collagen IV to indicate the intracellular location of mucin granules.

## 4.2 Introduction - Epithelial organisation and polarity

### 4.2.1 Polarity

Epithelial cells are polarized cells, which participate in endocytosis, exocytosis, and vesicle transport. In epithelial cells, the apical surface faces the lumen and is the site of secretion or absorption. The basolateral surfaces refer to basal areas, which contain hemidesmosomes and interact with the extracellular matrix (ECM). The lateral sides interconnect neighbouring cells via gap junctions and desmosomes. Secretory and membrane proteins are transported in membrane-bound vesicles from the endoplasmic reticulum to the Golgi network and are then sorted in the *trans*-Golgi network to the plasma membrane or other organelles. Basolateral sorting is mediated by signals (such as specific amino acid sequences) that reside within the protein to be transported. There are also apical signals (such as N glycosylation and glycosylphosphatidylinositol anchor) and late endosome sorting signals (Keller and Simons, 1997). The three major activities of digestive epithelial cells, namely, secretion, digestion and absorption, require the establishment and maintenance of cellular polarity and intracellular transport, all functions intimately linked to the cytoskeleton.

Other important roles for the cytoskeleton in digestive and nondigestive-type epithelia, include involvement in mitosis, protection from environmental stresses, cell and intracellular organelle anchorage, gene regulation, and motility during migration, differentiation, and wound repair.

#### **4.2.2 Contacts between cells and between cells and extracellular matrix**

Epithelial cells form highly specialised membrane structures to facilitate cell-cell and cell-ECM contacts, which may be required in the maintenance of cell morphology and tissue integrity. All these contacts consist of transmembrane proteins that interact with neighbouring cells or ECM via their extracellular portions, and with cytoplasmic adaptor molecules via their intracellular domains. Many of the cytoplasmic adaptor proteins interact with the actin cytoskeleton to strengthen these contacts. Examples of important epithelial actin-associated membrane structures include:

- 1) Tight junctions, which cross-link cells to form a “fence” and are the gate-keepers that regulate the paracellular permeability pathway. Tight junctions (also called zonula occludens) consist of two families of four-transmembrane domain proteins, namely occludin (Furuse *et al.*, 1993) and claudin (Morita *et al.*, 1999), and one single transmembrane domain protein, JAM (Martin-Padura *et al.*, 1998), along with associated cytoplasmic proteins, including ZO-1, ZO-2, and ZO-3. The ZO proteins associate with each other via their PDZ domains (named for postsynaptic density protein 95, disks large, ZO-1), bind actin via the ZO-1 proline-rich tail or via  $\alpha$ -catenin, and bind occludin via their guanylate kinase domains. The precise function of the ZO-1/ ZO-2 complex in epithelia is not known.

- 2) Adherens junctions, which include the homotypically binding transmembrane protein E-cadherin, help to form a continuous adhesion belt around each interacting cell in the epithelial sheet, and are another important epithelial actin-associated membrane structure. Epithelial cells contain a continuous band of cadherin molecules, usually located near the



apical surface just below the tight junction, that connect the lateral membranes of epithelial cells. The adherens junction is associated with  $\alpha$  and  $\beta$ -catenins that link E-cadherin in the plasma membrane to the circumferential belt of actin and myosin filaments. Other associated cytoplasmic components include the actin-binding proteins vinculin,  $\alpha$ -catenin,  $\alpha$ -actinin, paxillin, talin, vasodilator-stimulated phosphoprotein, and adaptor proteins such as vinxin, - and plakoglobin.  $\alpha$ -Catenin plays a critical role in the transmembrane anchorage of cadherins, since deletion of  $\alpha$ -catenin inactivates cadherin-mediated cell adhesion, resulting in a nonadhesive phenotype (Imamura *et al.*, 1999).

3) Gap junctions, which connect neighbouring cells by intercellular channels, consist of connexins, a family of four-transmembrane domain proteins with >14 different genes in mice. Molecules less than approximately one kDa, including ions, metabolites and messengers, pass freely through these channels. The role of gap junctions in digestive epithelia is important for coordinating tissue behaviour, but their interaction with cytoskeletal elements is not clear.

4) Focal adhesions (i.e., focal contacts) connect the actin cytoskeleton to the ECM through the integrins and their associated cytosolic actin-binding proteins. The ectodomain of integrins binds to ECM, whereas their intracellular domain binds to many actin-binding proteins such as talin, vinculin, paxillin, and  $\alpha$ -actinin, which in turn interact with actin (Giancotti and Ruoslahti, 1999). The integrins are a large family of  $\alpha$ ,  $\beta$  heterodimeric transmembrane cell-adhesion receptors that recognise both cell-surface and extracellular matrix molecules. At present, 24 different integrin receptors have been identified in higher vertebrates, each drawn from a collection of 18  $\alpha$  subunits and eight  $\beta$  subunits (Hyne, 1992; Humphries, 2000). The cellular distribution of integrins varies considerably, with the  $\beta$ 1 and  $\beta$ 3 integrins generally having a widespread distribution, with the  $\beta$ 2 integrins being restricted to leukocytes (Springer, 1990).



In the absence of extracellular contacts, single epithelial cells exhibit very few structural characteristics of polarised cells. Extracellular contacts between single cells and the ECM, or between cells in the absence of ECM, are sufficient to initiate segregation of membrane and cytoskeletal proteins between contacting and non-contacting surfaces of cells. E-cadherin-mediated adhesion is sufficient to initiate the segregation of apical membrane proteins into the non-contacting (free) membrane and basolateral membrane proteins into the contacting membrane (Wang *et al.*, 1990).

#### **4.2.3 The extracellular matrix**

The extracellular matrix has three major protein components: highly viscous proteoglycans, which cushion cells; insoluble collagen fibres, which provide strength and resilience; and soluble multiadhesive matrix proteins, which bind components to receptors on the cell surface. Different combinations of these components tailor the strength for different purposes. Proteoglycans are macromolecules that are distributed almost everywhere in the body. Their size and structure vary enormously. The basic structure of all proteoglycans includes a core protein and at least one, but frequently more (up to tens or hundreds) carbohydrate chains, so called glycosaminoglycans. Proteoglycans can be found intracellularly, on the surface of the cells and from the extracellular matrix (Lodish *et al.*, 2000).

The collagens constitute a superfamily of extracellular matrix proteins with a structural role as their primary function (Table 4.2.3). The collagen superfamily can be divided to several subfamilies.

a) Fibrillar collagens which include collagen I, II, III, V, and XI are the triple-helical products of procollagen processing, which polymerise to form fibrils that serve as stabilising scaffolds in extracellular matrices (Kühn, 1987). Within the fibrils, the 300 nm long rod-like molecules overlap with their ends by about 30 nm and are arranged in quarter-staggered

arrays. The fibrils therefore have a periodic structure. Each period is 67 nm long and consists of a “hole” zone with more loosely packed molecules and an overlap “hole” zone with more densely packed molecules.

b) The FACIT (fibril associated collagens with interrupted triple helices) group of collagens which includes collagens IX, XII, XIV and XIX are composed of seven distinct polypeptide chains (Shaw and Olsen, 1991). This group of proteins may serve as molecular bridges between fibrillar collagens and other extracellular matrix components. Their structure is strikingly different from that of other collagens in that their molecules contain two, three, or more relatively short triple-helical domains connected by non-triple-helical sequences.

c) Short chain collagens which include types VIII and X are composed of three chains  $\alpha 1$  (VIII),  $\alpha 2$  (VIII), and  $\alpha 1$  (X) and form the subgroup of short chain collagens, so named because of their subunits are short (about 60 kDa) as compared with fibrillar collagen chains. These collagens are related in structure and assembly, though their distributions and biological functions are distinct (Hulmes, 1992). Type VIII collagen is the major constituent of the hexagonal lattice observed in Descemet’s membrane (Kahsai *et al.*, 1997). Type X collagen is the most specialised of the collagens, and it is the product of hypertrophic chondrocytes in the deep calcifying zone of the cartilage (Gordon and Olsen, 1990).

d) Basement membrane collagens. Basement membranes are thin (30-40 nm), rather amorphous structures that are found in the extracellular matrix adjacent to plasma membranes and which line epithelial and endothelial cell layers, at the interface with mesenchyme, or surround particular cell types (e.g. muscle fat, nerve). Their functions are to provide support for cell layers, to serve as molecular sieves (e.g. in the glomerulus), to act as selective barriers to the passage of inflammatory and tumour cells and to provide substrates for cell adhesion and differentiation. Type IV collagen, is the major collagenous component of basement membranes, forming a network structure with other basement membrane components



(laminin, nidogen, heparan sulphate proteoglycan). Type IV collagen forms the basic fibrous two-dimensional network of all basal laminae. Three type IV collagen chains form a 400-nm-long triple helix with large globular domains at the C-termini and smaller ones of unknown structure at the N-termini. The helical segment is unusual in that the Gly-X-Y sequences are interrupted about 24 times with segments that cannot form a triple helix; these nonhelical regions introduce flexibility into the molecule. Lateral association of the N-terminal regions of four type IV molecules yields a characteristic tetrameric unit that can be observed in the electron microscope. Triple-helical regions from several molecules then associate laterally, in a manner similar to fibril formation among fibrous collagens, to form branching strands of variable but thin diameters. These interactions, together with those between the C-terminal globular domains and the triple helices in adjacent type IV molecules, generate an irregular two-dimensional fibrous network (Lodish *et al.*, 2000).

e) Multiplexins. These non-fibrillar collagens, type XV and type XVIII, are broadly expressed in many tissues, but are present at particularly high levels in internal organs. They contain multiple short triple-helical domains, separated and flanked by non-triple helical regions. The two members of this class of proteins, have been given the name multiplexins (Oh *et al.*, 1994) because they both contain multiple-triple-helix domains with interactions.

f) The MACIT (membrane-associated collagens with interrupted triple-helices) group.

g) Other collagens. This is a heterogenous group of proteins that on the genetic basis do not belong to one of the defined collagen families. This group comprises collagen VI and collagen VII. Type VI is broadly expressed in different tissues as the major component of beaded microfibrils (Timpl and Engel, 1987). Collagen type VII is the major collagenous component of anchoring fibrils associated with the basement membranes under stratified squamous epithelia.



**Table 4.2.3**

TYPE	TISSUE DISTRIBUTION
<b>I</b>	Most connective tissues e.g. bone, tendon, skin, lung, cornea, vascular system
<b>II</b>	Cartilage, vitreous humour, embryonic cornea
<b>III</b>	Extensive connective tissues, e.g. skin, lung, vascular system.
<b>IV</b>	Basement membranes
<b>V</b>	Tissues containing collagen I, quantitatively minor component
<b>VI</b>	Most connective tissues, including cartilage
<b>VII</b>	Basement membrane-associated anchoring fibrils
<b>VIII</b>	Product of endothelial and various tumour cells
<b>IX</b>	Tissues containing collagen II, qualitatively minor component
<b>X</b>	Hypertrophic zone of cartilage
<b>XI</b>	Tissues containing collagen II qualitatively minor component
<b>XII</b>	Tissues containing collagen I, qualitatively minor component
<b>XIII</b>	Qualitatively minor collagen , found in skin, intestine
<b>XIV</b>	Tissues containing collagen I, qualitatively minor component

#### 4.2.4 The basal lamina

Basal laminae, which are flexible, thin (140-120 nm) mats of specialised extracellular matrix, underlie epithelial cells. Most basal laminae contain type IV collagen, the large heparan sulphate proteoglycan perlecan and the glycoproteins laminin and nidogen. Perlecan (396-467 kDa) consists of a multi-domain core protein with two or three heparan sulphate/chondroitin sulphate side chains at its N-terminal end. It binds laminin and fibronectin. Nidogen (150 kDa) is a single polypeptide chain which has the major function of linking collagen IV and laminins. Laminins are a growing family of related proteins (400-1000 kDa) characterised by a heterotrimeric chain assembly ( $\alpha\beta\gamma$ ), a preferred localisation in basement membranes, and a multitude of biological activities (Ekblom and Timpl, 1996). So far 11 different assembly forms, laminins-1 to -11, have been identified, or suggested, based on five different  $\alpha$  chains, three  $\beta$  chains, and two  $\gamma$  chains.

The importance of cell-cell adhesion in differentiation and in the maintenance of the differentiated phenotype is well established for epithelial cells (Braga *et al.*, 1999). Epithelial cells are separated from the underlying connective tissue by a basement membrane that is composed of a variety of ECM molecules that control cell differentiation in many tissues through interactions with their cellular receptors, for example, with integrins (Boudreau and Bissell, 1998). The basement membrane is mostly composed of type IV collagen, different types of laminins, entactin and heparan sulfate proteoglycan (Beaulieu, 1997). ECM molecules, originating from both epithelial and underlying mesenchymal cells, create a framework that is essential for maintaining tissue integrity (Simon-Assmann and Keding, 1993). Thus, ECM proteins are involved in the control of adhesion, migration, proliferation, differentiation and gene expression of adjacent cells. This emphasizes the dynamic reciprocity between epithelial and mesenchymal cells (Bissell *et al.*, 1982). Additionally,

ECM is able to control the effects of trophic factors by sequestration outside of the cell (Simon-Assmann *et al.*, 1998) and by influencing their signalling pathways (Yamada and Geiger, 1997). It is known that cell adhesion to the ECM contributes to the apical-to-basal axis of polarity, *in vivo* as well as *in vitro*. Appearance of polarized cells coincides with the expression of laminin 1 in the developing kidney (Klein *et al.*, 1990). Similarly, the addition of laminin boosts the formation of polarized alveoles in various types of epithelial cells, including mouse mammary (Li *et al.*, 1987), human salivary (Hoffman *et al.*, 1996) and rat lung (Matter and Laurie, 1994) cells in culture. ECM-integrin interactions have either been demonstrated to be directly involved in ECM control of cell functions, or found to be aberrant in embryos or animals carrying mutations in integrin genes (Wang *et al.*, 1999).

Key features of a satisfactory model of the gastric epithelial cell surface are that the cells form a monolayer with tight junctions, that they remain differentiated and polarised. Regulation and maintenance of epithelial differentiation at the level of the gut mucosa is governed by extracellular signals present in the cell microenvironment. These are represented not only by growth factors but also by cell-to-cell interactions and interactions of cells with their underlying ECM (Montgomery *et al.*, 1999). Thus in this current study the effect of the matrix on tight junction formation, differentiation and polarity was assessed. Although collagen type I from different species and organs (e.g. rat, bovine, tendons, skin, placenta) is often used as an attachment matrix in epithelial cell culture, it is not a normal constituent of the basal lamina and this is the reason collagen IV and collagen IV plus laminin were also used to coat the membranes of Transwells.



## **4.3 Introduction- Methods for assessing differentiation and polarity**

### **4.3.1 Detection of mucin and mucous cells**

Gastric surface mucous cells originate from granule-free progenitor cells located at the isthmus of the gastric unit (Karam and Leblond, 1993). During upward migration, granule free pre-pit cell precursors acquire secretory granules that increase in number and size along with maturation into pit cells. The mature cells are characterised by their large granules, which consist of two major components: mucin positive for periodic acid Schiff and the galactose oxidase-Schiff (GOS) reactions, and surface-active phospholipids (Boland *et al.*, 1990). During the periodic acid Schiff reaction, the periodic acid, is employed as an oxidising agent that breaks the C-C bonds in mucin, converting them into dialdehyde groups, which then react with leuco fuschin (Schiff's reagent) to restore the colour of the fuschin (bright pink). The presence of mucin in the cytosol of the cells is therefore demonstrated by their staining bright pink. The above maturation process suggests that if mucous cells were to de-differentiate in culture they should lose their capacity to manufacture and release mucin.

#### **4.3.1.1 Gastric Mucin**

Gastric mucin is a high molecular weight glycoprotein. The MUC 5 gene is expressed by surface epithelial cells and MUC 6 by mucous neck cells (Ho *et al.*, 1995). Gastric mucus lines the epithelium and protects it from harmful constituents of the gastric lumen, including gastric acid, bacteria, and ingested toxins. The mucous coat provides an unstirred layer that helps segregate the luminal contents from the mucosal surface. Mucins have been difficult to study because of their complex, heterogenous molecular structure, and the difficulties encountered in attempting to separate the soluble pool in the gastric juice ("sol") from the functional poorly soluble pool adherent to the mucosa ("gel"). The gel is made up of water, mucin, other proteins, and lipids, into which a bicarbonate-rich fluid is secreted (Flemstrong,

1986). Moreover, the lack of an identifiable mucin activity, such as that of an enzyme, together with the polydispersity of the molecules, which makes assay by a single analytical procedure somewhat complex.

It has been shown (Neutra *et al.*, 1977) that the gastric surface mucous cells behave similarly to other mucus-secreting cells, such as intestinal goblet cells, in their uptake of labelled carbohydrate precursors into the cell. By measuring the incorporation of [<sup>3</sup>H] glucosamine hydrochloride by mucous cells into glycoprotein the presence of mucin can be indicated. The current study uses the above procedure to measure the synthesis and secretion of mucin by gastric epithelial cells so as to determine levels of differentiation and polarity. Mucin is an important marker because the apical release of mucin to form an extracellular layer of mucus is a major function of gastric surface cells which protects the epithelium (Allen *et al.*, 1993).

#### **4.3.2 Superoxide Release**

Phagocytes, especially neutrophils, undergo a respiratory burst in response to bacteria, resulting in robust production of reactive oxygen species (ROS) which include superoxide, hydrogen peroxide, hydroxyl radical and perchlorous acid (HOCL), which together function in bacterial killing. Circulatory phagocytic cells produce large amounts of ROS in response to infectious stimuli through the activation of the NADPH oxidase complex (phox system) (Leto, 1999). This multisubunit oxidase generates superoxide from oxygen, with secondary generation of hydrogen peroxide via superoxide dismutase. The membrane associated catalytic moiety, gp91phox, contains all the prosthetic groups needed to transfer electrons from NADPH to oxygen (flavin adenine dinucleotide [FAD], two heme moieties, and a binding site for NADPH) but is catalytically inactive in the absence of regulatory proteins. Another membrane protein, p22phox, complexes with gp91phox to form flavocytochrome b<sub>558</sub> and serves as a docking site for the cytosolic regulatory proteins p47phox, p67phox, p40phox,



and the small monomeric G-protein Rac. These proteins translocate to the membrane upon cell activation, and p67phox activates electron flow within gp91phox (Han *et al.*, 1998).

There is increasing evidence that distinct types of nonphagocytic cells, such as vascular smooth muscle cells (Patterson *et al.*, 1999), glomerular mesangial cells (Jones *et al.*, 1995), endothelial cells (Jones *et al.*, 1996), and fibroblasts (Meier *et al.*, 1993) express p22phox, p67phox and p47phox and can produce small amounts of O<sup>2-</sup> though a gene family encoding superoxide-producing NADPH oxidases, termed Nox (Table 4.3.2). Nox-1 in particular, an NADPH oxidase expressed predominantly in colon epithelium, shows a high degree of similarity to the phagocyte NADPH oxidase, generating superoxide when co-expressed with the p47phox and p67phox subunits of the phagocyte NADPH oxidase but not when expressed by itself (Bánfi *et al.*, 2002). Recently the production of superoxide by the NADPH oxidase component Nox-1 has also been shown to be a property of both guinea-pig (Teshima *et al.*, 1998) and human (Rokutan *et al.*, 1999) gastric surface cells. The enzyme is not present in other types of gastric cells (Rokutan *et al.*, 1999) and is therefore a useful marker of differentiated gastric surface cells. Furthermore, although the directionality of superoxide release has not been established, it could be a useful index of polarity.



**Table 4.3.2 Tissue expression of Nox/Duox family members (Lambeth, 2002).**

<b>Nox Homologue</b>	<b>Highest Expression</b>	<b>Some Expression</b>	<b>Established Function</b>
Nox 1	Colon	Stomach, vascular smooth muscle, prostate	
Nox 2 (Gp91 $phox$ )	Neutrophil Macrophage		Bacterial killing
Nox 3	Foetal kidney	Foetal : liver, lung, spleen	
Nox 4	Kidney	Osteoclasts, pancreas, Skeletal muscle, ovary, astrocytes	
Nox 5	Spleen, sperm, lymph node	Ovary, placenta, pancreas	
Duox 1	Thyroid, lung		Production of hydrogen peroxide for iodination of tyrosine
Duox 2	Thyroid, lung		

#### **4.4 Methods- Preparation of 4 % (w/v) paraformaldehyde in PBS**

In a 100 ml glass beaker 2.0 g of paraformaldehyde in 25 ml double distilled water was heated with stirring to 60 °C. At that exact temperature 1 M NaOH was added slowly in drops until the solution became clear (usually 7 drops). The resulting paraformaldehyde solution was transferred to another stirrer and was allowed to cool. 4% (w/v) paraformaldehyde solution was prepared by adding 25 ml of the paraformaldehyde solution to 25 ml of 2 x PBS.

#### **4.5 Methods- Proportion of mucous cells**

##### **4.5.1 Preparation of Schiff reagent**

Schiff reagent was prepared by dissolving 1 g of basic fuchsin in 100 ml boiling water to which 20 ml 1M HCl was added. The solution was then allowed to cool to 50 °C before addition of 300 mg activated charcoal. The solution was shaken for 5 min before removal of the charcoal by filtration through filter paper (No.1, Whatman, Maidstone). The charcoal addition and filtration was repeated once more and the resulting solution was stored in an amber glass bottle at room temperature until required. Immediately before use 30 ml Schiff reagent was incubated with 0.1 g sodium metabisulphite at 37 °C until a yellow color was obtained (usually 3 h).

##### **4.5.2 Fixation of cells**

Cells were detached from the wells by use of trypsin/EDTA (please refer to section 2.3) and centrifuged at 12,000 x g for 20 sec. The pellets were resuspended with 400µl of freshly prepared 4 % (w/v) paraformaldehyde in PBS and left at room temperature for 10 min after which the cells were once more centrifuged at 12,000 x g for 10 sec and the resulting pellets resuspended in 400 µl of ethanol: water (80:20). The microfuge tubes containing the fixed cells were stored at 4 °C.

#### **4.5.3 Preparation of Cytospins**

250  $\mu$ l aliquots of fixed cells (please refer to section 4.5.2) were transferred to the hopper of a cytocentrifuge (Shandon) and transferred to microscope slides by operating the centrifuge at 800 r.p.m. for 5 min.

#### **4.5.4 Staining of cells with periodic acid Schiff reagent**

Slides were immersed in periodic acid solution (1% w/v) for 20 min after which time they were washed under tap water for 5 min. Finally the slides were immersed in Schiff's reagent (please refer to section 4.5.1) for 20 min and rinsed in tap water for a further 10 min. Slides were then allowed to air dry, and mounted in DPX mounting agent.

#### **4.5.6 Counterstaining of cells with Hoechst 33258 ( bisbenzimidide)**

Following staining of cells with periodic acid Schiff reagent, the slides were then immersed in 8  $\mu$ l/ml Hoechst 33258 (bisbenzimidide) in PBS for 10 min after which they were washed by dipping once in a coplin jar containing tap water. The slides were then carefully drained onto an absorbent towel and were introduced 2 times into fresh 100 % ethanol. Slides were once again drained and briefly introduced once into a coplin jar containing xylene after which they were mounted in Fluomount. Alternatively sections were not dehydrated and mounted in aqueous agent comprised of glycerol (10 % v/v) in PBS and 2.5 % (w/v) 1,4-Diazabicyclo[2.2.2]octane (DABCO).

#### **4.5.7 Identification of mucous cells**

Following staining with periodic acid Schiff Reagent and counterstaining with Hoechst 33258, the proportion of mucous epithelial cells was determined. Mucous cells were identified with their positive staining (mucus appears bright pink): the total numbers of cells were



determined by counting nuclei stained with Hoechst 33258 using a fluorescent microscope under UV illumination.

## **4.6 Methods-Mucous synthesis and secretion**

### **4.6.1 Loading of cells with 2 $\mu$ Ci/ml of D-[6-<sup>3</sup>H]glucosamine hydrochloride**

Culture medium containing 2  $\mu$ Ci/ml of D-[6-<sup>3</sup>H] glucosamine hydrochloride was prepared under sterile conditions by adding 55  $\mu$ l of D-[6-<sup>3</sup>H] glucosamine hydrochloride (1 mCi/ml), to 27.5 ml of complete culture medium in the presence of 10 ng/ml EGF.

The medium change at 48 h was replaced with a medium change at 52 h of culture with complete medium containing 2  $\mu$ Ci/ml of D-[6-<sup>3</sup>H] glucosamine hydrochloride. At 52 h the plates were transferred to the culture hood and culture medium was removed from all plates. For both uncoated and collagen coated Transwells, medium was replaced with 1.5 ml per apical Transwell and 2.6ml receiving basal well of complete medium containing 2  $\mu$ Ci/ml of D-[6-<sup>3</sup>H] glucosamine hydrochloride. For 6-well TCP, medium was replaced with 1.5 ml per well of “labeled” culture medium. All the plates were returned to the incubator.

### **4.6.2 Glycoprotein synthesis and release**

Approximately 18 h later, the medium was removed and cells were rinsed with warm HBSS at the appropriate volume. HBSS was removed by gentle suction and the cells were then incubated with fresh HBSS for 1 h at 37 °C on a tilting platform. Apical and basal media were removed and centrifuged at 10,000 x g for 30 sec to remove any suspended cells. The supernatants, plus 15  $\mu$ g of bovine serum albumin, were mixed with trichloroacetic acid 10 % (w/v)/ phosphotungstic acid 1 % (w/v). Cells were obtained by treatment of wells with trypsin/EDTA. Cells were then centrifuged for 20 sec at 12,000 x g in an Eppendorf microfuge

and the pellets were resuspended in 1 ml of ice cold PBS per tube. The PBS was then removed and the pellets were resuspended in 750 µl of 10 % trichloroacetic acid/ 1 % phosphotungstic acid. All the tubes were then whirrmixed for 20 sec and were left overnight at 4 °C in the Radioisotope Fridge.

The following day all tubes were centrifuged at 12,000 x g for 30 min at 4 °C using a pre-cooled chilled Microfuge. The supernatants were removed and 1 ml 10 % trichloroacetic acid/ 1 % phosphotungstic acid was added to each of the tubes. The tubes were then inverted and centrifuged again as above. The supernatants were removed and replaced by 600 µl of 0.1 M NaOH/ 1 % SDS per tube. All of the tubes were then mixed and incubated for 4 h at 37 °C. All tubes were then left overnight at room temperature. 2 x 200 µl of samples were transferred to 2 counting vials. 5 ml optiphase HiSafe 3, were added to each of the vials. Radioactivity measurements were then taken using a scintillation counter.

In addition the protein content of the cell samples was measured by adding 2 x 10 µl samples to a 96-well plate for BCA protein assay (please refer to section 2.5).

#### **4.6.3 Electrophoresis and Immunoblotting**

Assessment of glycoprotein secretion was performed as above except that the cells were not preincubated with [<sup>3</sup>H] glucosamine. HBSS from three wells was pooled, dialysed against 2 x 2 l of water for 48 h at 4 °C and freeze-dried. Material was taken up into electrophoresis sample buffer without dithiothreitol and subjected to SDS polyacrylamide gel electrophoresis using a 4 % stacking gel and 8 % separating gel. A 1:1000 dilution of rabbit anti-rat gastric mucin antibody was employed for immunoblotting with detection by enhanced chemiluminescence (please refer to section 4.10 for details).

#### **4.7 Methods- Measurement of NADPH oxidase-derived superoxide anion producing activity**

Gastric epithelial cells were cultured for 72 h on 6-well TCP, on uncoated polycarbonate Transwells and on collagen coated polycarbonate Transwells. At 72 h TCP and Transwells were washed 3 times in non sterile conditions with warm PBS and incubated for 1 h with HBSS containing 80  $\mu$ M cytochrome C with 1.5 ml per well of TCP, and 1 ml per apical Transwell and 1.7 ml per basal well for 24 mm diameter Transwells. Superoxide dismutase (SOD, 100 u/ml) was added to reference cells. The amount of superoxide release was measured as the SOD-inhibitable reduction of cytochrome C at 550 nm with reference at 492 nm and was expressed at nanomoles per h per  $\text{cm}^2$ . In order to express results in nanomoles per milligram of protein per hour, immediately after incubation with cytochrome C, the culture media were removed to appropriate tubes and were replaced by trypsin /EDTA at the following volumes: 1 ml per well for TCP, and 1ml per apical well and 1.7 ml per basal well for the Transwells. All of the plates were then incubated for 20 min at 37  $^{\circ}$ C after which the trypsin /EDTA solutions containing the cells were removed and placed in tubes containing 1 ml of complete medium. All tubes containing the cells were then centrifuged at 12,000 x g for 30 sec. The supernatant was then removed and the pellets were rinsed with 1 ml per tube ice-cold PBS and were centrifuged again as above. The PBS was then removed and the pellets were resuspended with 800  $\mu$ l PBS/ 1 % (v/v) Triton X-100 per tube and were left at room temperature for 25 min after which they were stored at -20  $^{\circ}$ C. The following day all tubes were thawed, mixed, and centrifuged at 12,000 x g for 30 sec in order to be assayed for protein with the BCA assay.



## **4.8 Methods- Polyacrylamide Gel Electrophoresis and Immunoblotting**

The techniques of polyacrylamide gel electrophoresis (PAGE) and immunoblotting were used to separate proteins obtained from cells comprising the monolayers after 72 h in culture. An antibody was then used to probe for proteins immobilised on a filter to determine the presence or quantity of a particular protein of interest. There are a number of stages in this process which involves: solubilising the samples with detergents so that they can be separated by SDS-PAGE, transfer of proteins from the gel to a nitrocellulose filter, exposing the filter to antibody specific for the target protein and finally developing the blot so that the targeted proteins can be visualised.

### **4.8.1 Preparation for samples for PAGE**

Cells were cultured in duplicate conditions for 72 h on TCP, Transwells, Collagen I-Transwells and Collagen IV-Transwells. Cell samples were obtained by treatment with trypsin/EDTA, washed with 1 ml PBS containing 100 µg/ml of Soybean trypsin inhibitor, and dissolved into 200 µl hot electrophoresis sample buffer without dithiothreitol (DTT) (Table 4.8.1). All the above samples were then transferred to a heating block and heated for 3 min at 95 °C. The samples were then allowed to cool and duplicates were pooled into a single microfuge tube. A 20 µl sample from each tube was removed into microfuge tubes containing 60 µl of distilled water in order to determine protein concentration by the BCA protein assay (section 2.5). The buffer used to make the protein standards was made by mixing sample buffer without DTT with distilled water (1:3). A volume of 1 M DTT was then added to each tube to give a final concentration in the tube of 0.1 M. The tubes were then transferred to the heating block at 95 °C for a further 3 min. After cooling at room temperature the samples were either loaded onto

a gel or stored at  $-70^{\circ}\text{C}$ . The above procedure is necessary because DTT interferes with the BCA assay.

#### **4.8.2 Casting and running gel**

The polyacrylamide gel is composed of chains of polymerized acrylamide that are cross-linked with N, N'-methylenebisacrylamide. This forms pores in the gel which allow the SDS-protein complexes to pass through and creates tensile strength.

Two glass plates (16 cm x 16 cm), cleaned with ethanol and separated by two 0.75 mm spacers, were sandwiched together and clamped with cams vertically in the gel stand. The base of a yellow pipette tip was cut off and the end of the tip connected to a 10 ml syringe. The separating gel solution (Table 4.8.2.2) was introduced by the syringe between the glass plates to give a gel height of 11 cm from the base of the plates. The gel solution was then overlaid with a methanol/water (1:1) mixture, and allowed to set. When polymerization was completed, the overlay was removed by inserting a piece of filter paper into the gap between the plates. The top of the gel was rinsed with running buffer (0.025 M Tris, 0.2 M glycine, 0.15 (w/v) SDS, pH 8.3). A stacking gel (Tables 4.8.2.3, 4.8.2.4) (3 % for p67 phox and 4 % for glycoprotein secretion) was then prepared and the plates filled to the top. A clean gel comb was inserted into the stacking gel solution, without introduction of air bubbles, to create wells in which to load protein. As soon as the polymerization was complete, the cams and comb were removed and with a 10 ml syringe the wells were carefully washed and filled with running buffer. The length and width of the separating gel were measured.

**Table 4.8.2.1 1x sample buffer ( without DTT) composition**

Reagent	Volume	Final Concentration ( after addition of DTT)
0.5M TrisCl pH 6.8	1.0 ml	0.0625 M
Glycerol	0.8 ml	10 %
10% SDS	1.6 ml	2 %
0.5 mg/ml bromophenol blue	0.2 ml	0.0125 mg/ml 0.00125 %
Water	4.0 ml	
Total	7.6 ml	

**Table 4.8.2.2 Separating gel (8 %) components**

Reagent	Volume in ml	Final concentration
1.5M Tris.HCl (pH 8.8)	5	0.375 M
10% (w/v) SDS	0.2	0.1 %
Acrylamide/N, N'-bisacrylamide (30% /0.8% )	5.34	8 %
Distilled H <sub>2</sub> O	9.36	
Ammonium Persulphate (100mg/ml) Made up fresh	0.10	0.5 mg/ml
TOTAL volume	20.0 ml	

The above ingredients were added to a glass beaker and were gently mixed using a magnetic stirrer. 7.5  $\mu$ l N, N, N', N'- Tetramethylethylenediamine (TEMED) was then added and gently mixed.



**Table 4.8.2.3 Stacking gel (3 %) components**

Reagent	Volume in ml	Final concentration
0.5 M Tris.HCl (pH 6.8)	2.5	0.125 M
10 % (w/v) SDS	0.1	0.1 %
Acrylamide/N, N'-bisacrylamide (30%/0.8 %)	1.0	3 %
Distilled H <sub>2</sub> O	6.35	
Ammonium Persulphate (100 mg/ml) Made up fresh	0.05	0.5 mg/ml
TOTAL volume	10.0 ml	

The above ingredients were added to a glass beaker and were gently mixed using a magnetic stirrer. 5 µl TEMED was then added and gently mixed.

**Table 4.8.2.4 Stacking gel (4 %) components**

Reagent	Volume in ml	Final concentration
0.5M Tris (pH 6.8)	2.5	0.125 M
10 % (w/v) SDS	0.1	0.1 %
Acrylamide/Bis (30 %/ 0.8%)	1.33 ml	4 %
H <sub>2</sub> O	6.02	
Ammonium Pensulphate 100 mg/ml Made up fresh	0.05	0.5 mg/ml
TOTAL	10.0 ml	

The above ingredients were added to a glass beaker and were gently mixed using a magnetic stirrer. 5 µl TEMED was then added and gently mixed.

Before loading the protein samples (please refer to section 4.6.3 for glycoprotein secretion and 4.8.1 for p67 phox), they were resuspended 7 times through a 30 gauge needle to shear DNA.

The samples were then whirlmixed and centrifuged using an Eppendorf microcentrifuge (1 min at 12,000 x g). The supernatant was loaded to the appropriate wells in a volume to ensure equal protein loading in each well (25 µg protein per lane). One well was loaded with a sample of “rainbow markers”. Rainbow coloured protein molecular weight markers are markers of a known molecular mass so that it is possible to later estimate the molecular mass of the protein of interest. Where appropriate, 1 x sample buffer was added to top up the wells so that each well had an equal volume. An upper reservoir, containing running buffer was clamped to the top of the gel and the unit was immersed in running buffer (0.025 M Tris, 0.2M glycine, 0.1 % (w/v) SDS, pH 8.3). The electrophoresis unit was connected to a power supply and was set at 25 mA constant current with a maximum voltage at 500 V at a temperature of 10 °C. The electrophoresis unit was left to run for approximately 2 h or until the bromophenol blue dye reached the bottom of the separating gel.

#### **4.8.3 Transfer of proteins to nitrocellulose (Hybond P PDVF membrane)**

The resolved proteins in the gel were transferred to the nitrocellulose filter by direct, semi-dry electrophoretic transfer performed using LKB electrophoresis transfer unit (Multiphor II).

4 extra thick blotters and 6 sigma electrophoresis blotters were cut to the size of the gel using a scalpel. With scissors the Hybond P PDVF membrane was cut to the same size as well. All of the blotters were placed into transfer buffer (Table 4.8.3.1) and were allowed to soak.

**Table 4.8.3.1 Transfer buffer for PVDF membrane**

Quantity	Chemical	Final Concentration
3.03 g	Tris Base	25 mM
14.4 g	Glycine	190 mM
200 ml	Methanol	20 %
To make 1l of total volume	Double distilled H <sub>2</sub> O	

About 15 min before the electrophoresis run was to end the Hybond P PVDF membrane was treated as follows (Table 4.8.3.2) with gentle agitation.

**Table 4.8.3.2**

Reagent	Time
100 % methanol*	10 sec
Distilled H <sub>2</sub> O	5 min
Transfer Buffer	10 min

\*PVDF membrane is hydrophobic thus by pre-wetting it with methanol the PVDF becomes more hydrophilic and therefore can be wetted in distilled water.

The bottom of the graphite plate (anode) was saturated with distilled water and excess water was wiped off with paper towel. A gel sandwich was constructed. At the bottom of the electrode was laid 2 extra thick blotters followed by 3 sigma electrophoresis blotters. After completion of electrophoresis, the top glass was carefully pulled away, and the stacking gel was removed. The separating gel was flooded with transfer buffer and was overlaid with the prewetted PVDF membrane. The bottom glass was removed and the PVDF membrane/ gel was then transferred on the electrophoresis blotters. Another 3 sigma electrophoresis blotters were laid on the top of



the gel followed by two extra thick blotters. The top graphite plate was wetted with distilled water (cathode) and then placed on the top. Care was taken to exactly align each component of the sandwich during assembly and to ensure that no air bubbles were trapped, in order to optimise transfer. The electrodes were then connected to a maximum voltage of 50 V with a constant current of 118 mA ( 0.8 mA per cm<sup>2</sup>) for 90 min. After transfer the nitrocellulose filter was removed from the sandwich and was allowed to dry. The position of each molecular weight marker was recorded and the filter was stored at 4 °C between filter papers (No.1, Whatman, Maidstone) in a desiccated container.

#### **4.9 Methods- Immunoblotting with antibody**

Immunoblotting involves incubating proteins immobilized on the nitrocellulose filter with an antibody specific to the protein of interest, after first blocking non-specific binding sites. The nitrocellulose is then incubated with an anti-species IgG secondary antibody linked to horseradish peroxidase. A detection system can then be employed to visualise the targeted protein-antibody complexes.

The nitrocellulose filter is initially dipped for 15 sec in a dish containing methanol. The nitrocellulose filter was then transferred to a 25 ml roller bottle with the protein surface facing the inside the drum, and placed on a roller operating at a slow speed, for each of the incubation steps. The incubation steps were as follows:

**Table 4.9.1 Immunoblotting procedure** (Tween is Tween 20; milk is defatted powdered milk Marvel®).

Operation	Solution	Volume in ml	Time
Wash	PBS/Tween (0.1 %)	20 ml	10 min
Wash	PBS/Tween (0.1 %)	20 ml	10 min
Block	TBS Tween (0.1 %) 5 % milk	10 ml	60 min
Rinse	TBS Tween (0.1 %)	25 ml	15 sec
Primary Antibody p67 -phox	TBS Tween (0.1 %) 5 % milk Dilution 1:1000	2.5 ml	60 min
Rinse 1	TBS Tween (0.1 %)	25 ml	15 sec
Wash	TBS Tween (0.1 %)	25 ml	10 min
Wash	TBS Tween (0.1 %)	25 ml	10 min
Wash	TBS Tween (0.1 %)	25 ml	10 min
Secondary Antibody Anti rabbit IgG peroxidase *	TBS Tween (0.1 %) 5 µl in 5 ml	5 ml	60 min
Rinse 1	TBS Tween (0.1 %)	25 ml	15 sec
Wash	TBS Tween (0.1 %)	25 ml	10 min
Wash	TBS Tween (0.1 %)	25 ml	10 min
Wash	TBS Tween (0.1 %)	25 ml	10 min

The same procedure was followed for detection of glycoprotein but the primary antibody was replaced by a rabbit anti-rat gastric mucin antibody in a dilution of 1:1000.

\* From Amersham ECL kit

**Table 4.9.2 Preparation of TBS**

Quantity	Reagents	Final Conc
2.42 g	Tris Base	20 mM
8.0 g	NaCl	137 mM

The reagents were added to a 1 litre glass beaker and 900 ml distilled water was then added. Addition of HCl permitted adjustment to pH 7.6, and the volume was made up to 1l.

#### **4.10 Methods- Enhanced chemiluminescence detection**

The protein antibody complexes immobilized on the nitrocellulose filter were detected by the system of enhanced chemiluminescence (ECL) using a commercial kit (Amersham).

The steps undertaken in the following protocol were performed in a dark room using a red safety light. The nitrocellulose filter was placed on a sheet of Saranwrap (Dowchemical Co.) and covered with a solution containing equal volumes of detection solution 1 freshly mixed with detection solution 2 (containing luminol, hydrogen peroxide and a chemical enhancer at unknown concentrations, as provided by the Amersham kit). This was left for one minute. The blot was then lifted, drained briefly and placed onto an open photographic cassette where an acetate sheet had been previously placed protein side facing up. Another acetate sheet was then carefully placed on the top of the blot making sure no air pockets were formed. On the top of the acetate a piece of Kodak ECL film was placed and the lid of the cassette was closed. Exposure time was 15 sec after which the film was removed from the cassette and was placed for 1 min on a tray containing Kodak X-OMAT developer. Once the image appeared (usually 1-2 min, maybe quicker), the film was transferred to a tray containing water and then placed into a tray containing Kodak fixer for at least 1 min.

#### **4.11 Methods- Immunocytochemistry**

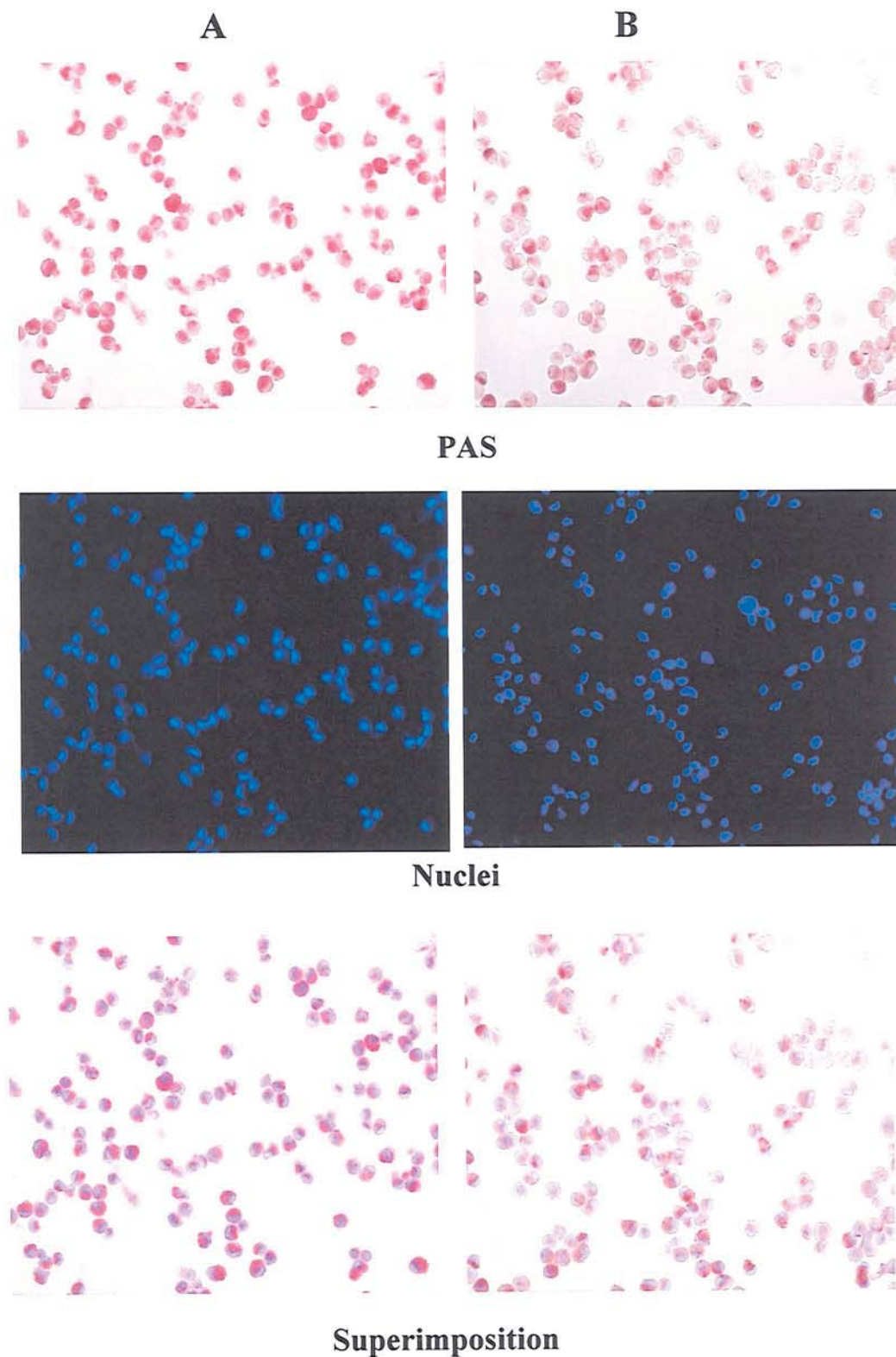
For immunocytochemistry filters were washed in PBS after fixation, permeabilised with 0.1 % (w/v) Triton X-100 in PBS for 10 min, blocked with 4 % (w/v) de-fatted milk in PBS for 30 min, incubated with a 1:25 dilution of rabbit anti-rat gastric mucin antibody for 1 h at room temperature, washed and finally exposed to 1:50 dilution of FITC-conjugated anti-rabbit IgG in PBS/milk for 30 min at room temperature. Filters were mounted in glycerol with 10 % PBS and 2.5 % (w/v) DABCO.



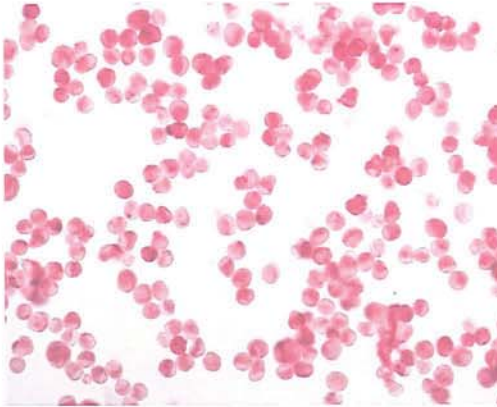
#### **4.12 Results- Proportion of mucous cells**

After culture for 72 h greater than 95 % of the cells removed from tissue culture plates, from uncoated Transwells or Transwells coated with collagen I or Collagen IV (Figure 4.12 A, B, C and D) stained positively for mucous glycoprotein.

**Figure 4.12** Effect of support matrix on proportion of mucous cells after 72h of culture on (A) tissue culture plastic (B) uncoated Transwells, (C) collagen I coated Transwells and (D) collagen IV coated transwells. Detached cells were transferred to microscope slides by using a cytocentrifuge (Shandon) and stained with periodic acid Schiff (PAS ) reagent. Nuclei were stained with Hoechst 33258.



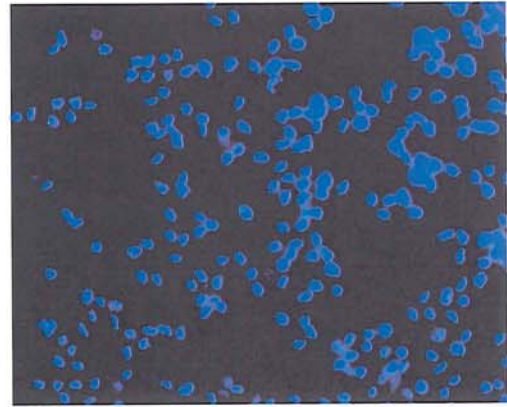
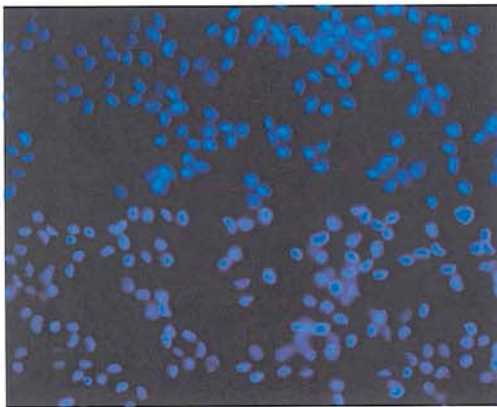
**C**



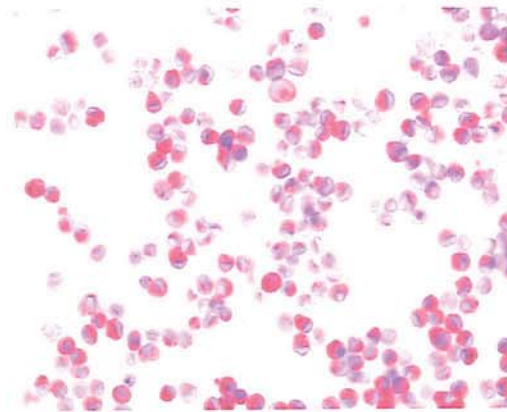
**D**



**PAS**



**Nuclei**



**Superimposition**



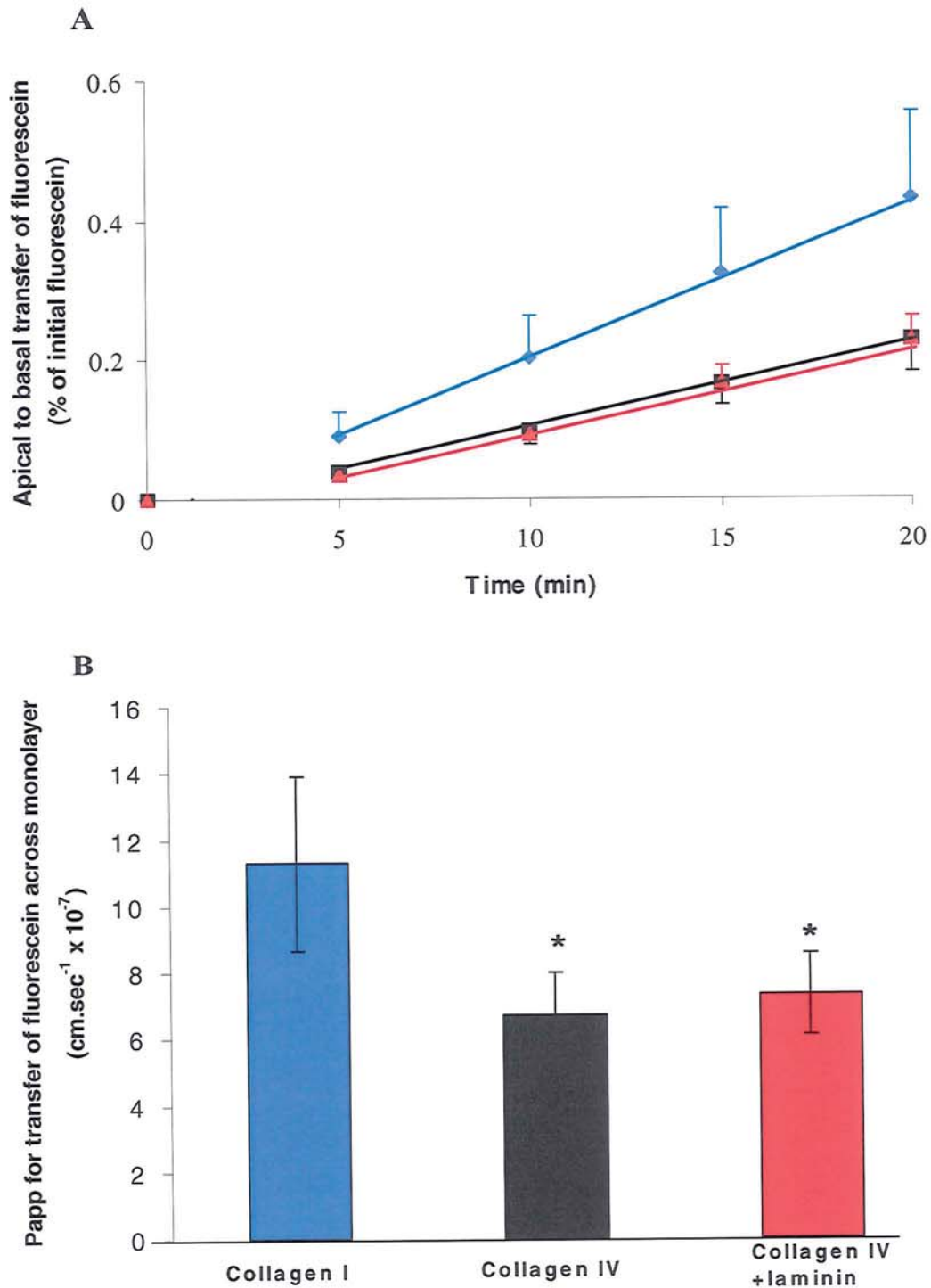
### **4.13 Results- Effect of matrix used to coat the Transwells on tight junction formation**

#### **4.13.1 Effect of collagen type I, collagen type IV, and collagen type IV plus laminin on permeability of fluorescein**

Transfer of fluorescein across the epithelial cell layer grown on Transwells coated with collagen type IV or collagen type IV plus laminin was lower ( $P < 0.05$ ) than for cell layers grown on membranes coated with collagen type I (Figure 4.13.1). No significant difference between collagen type IV and collagen type IV plus laminin was evident. There was some variation in  $P_{app}$  between experiments and it was necessary to use analysis of variance to remove this underlying effect. Nevertheless the effect of matrix was very consistent between experiments with the  $P_{app}$  values for transfer of fluorescein on Transwells coated with collagen IV and collagen IV plus laminin being respectively  $61 \pm 3 \%$  and  $67 \pm 6 \%$  of Transwells coated with collagen I.

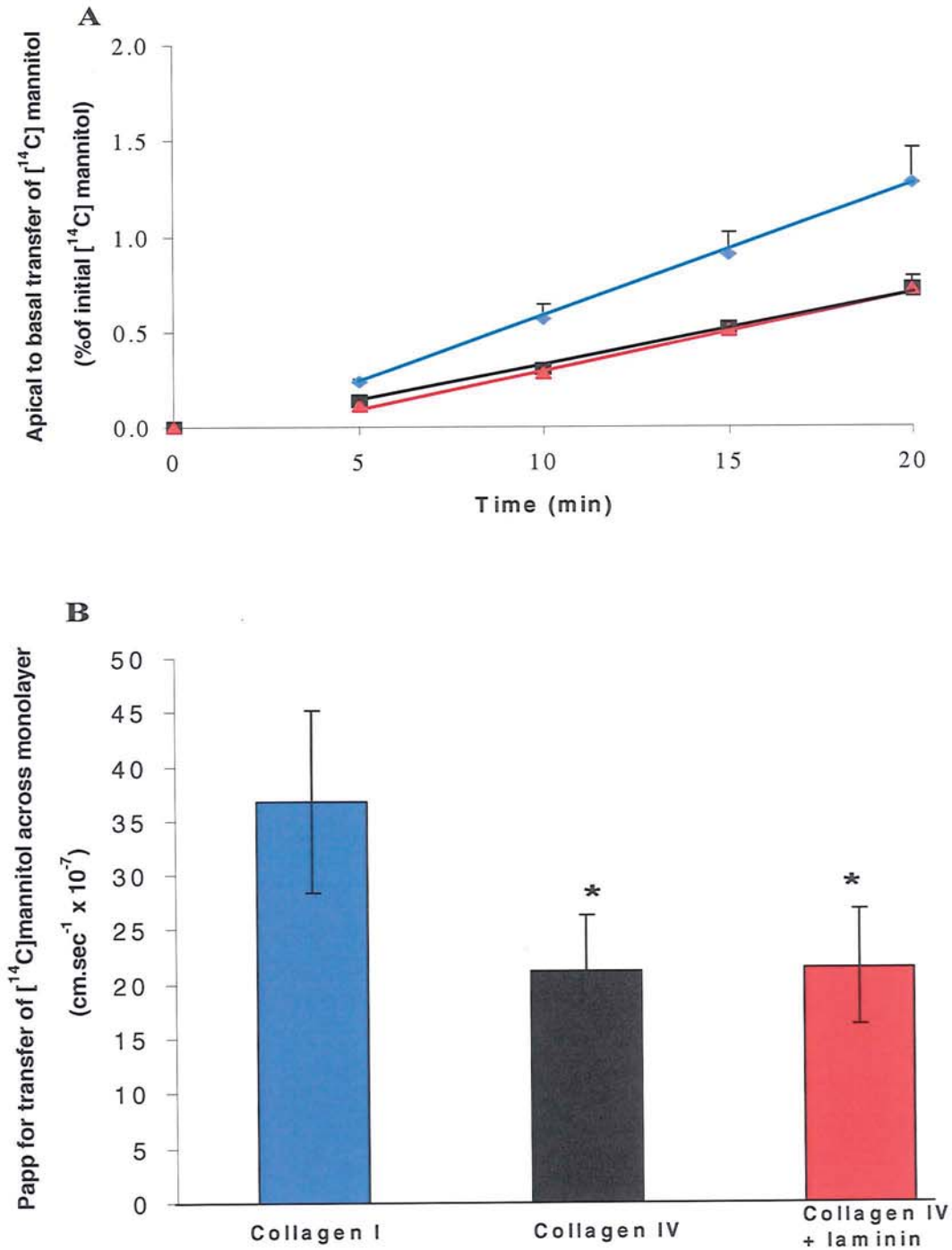
#### **4.13.2 Effect of collagen type I, collagen type IV, and collagen type IV plus laminin on permeability of [ $^{14}\text{C}$ ] mannitol**

Transfer of [ $^{14}\text{C}$ ] mannitol across the epithelial cell layer grown on membranes coated with collagen type IV or collagen type IV plus laminin was lower ( $P < 0.05$ ) than for cell layers grown on Transwells coated with collagen type I (Figure 4.13.2). No significant difference between collagen type IV and collagen type IV plus laminin was evident. The effect of matrix was very consistent between experiments with the  $P_{app}$  values for transfer of mannitol for monolayers on Transwells coated with collagen IV and collagen IV plus laminin being respectively  $58 \pm 4 \%$  and  $60 \pm 8.5 \%$  of preparations on Transwells coated with collagen I.



**Figure 4.13.1** Effect of collagen type I, collagen type IV, and collagen type IV and laminin on fluorescein paracellular permeability.

Effect of the matrix used to coat the Transwell on the transfer of (A) fluorescein in the apical to basal direction across the monolayer. Results are means  $\pm$  S.E.M. and are from three separate preparations with triplicate Transwells for each preparation. Blue line represents collagen I, black line represents collagen IV and red line represents collagen IV plus laminin. Apparent permeabilities ( $P_{app}$ ) (B) were calculated from the gradients of the lines over 5-20 minutes for fluorescein, and were compared by analysis of variance and a Newman-Keuls test. Results for collagen I were higher than for collagen IV or collagen IV plus laminin ( $P < 0.05$ ).



**Figure 4.13.2 Effect of collagen type I, collagen type IV, and collagen type IV plus laminin on mannitol permeability.**

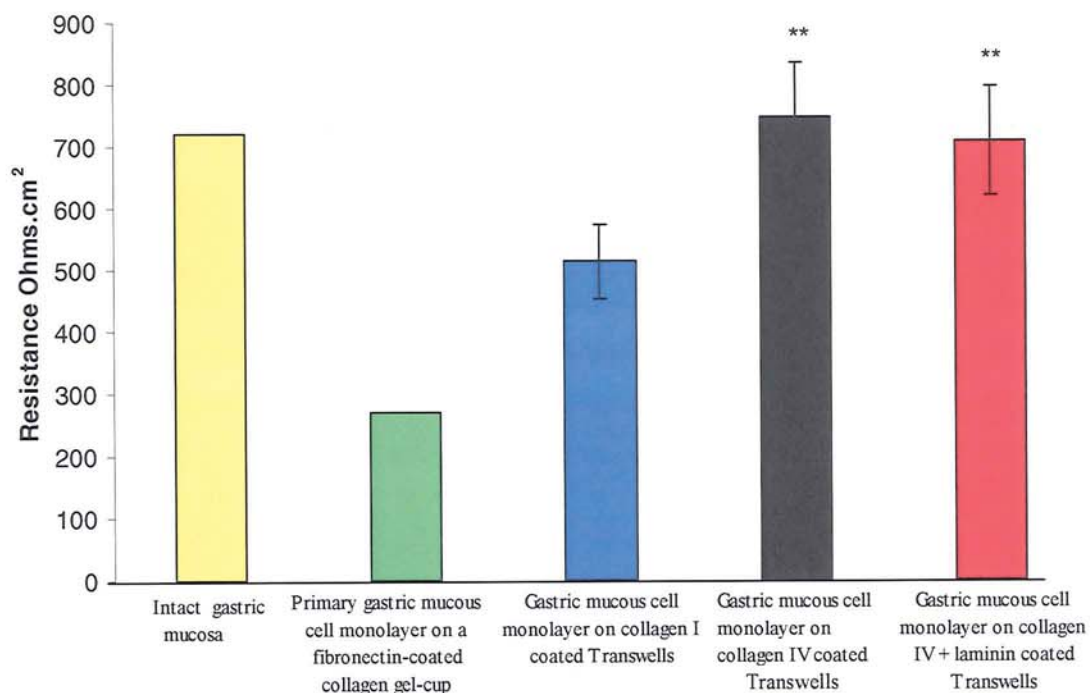
Effect of the matrix used to coat the Transwell on the transfer of (A) [<sup>14</sup>C]mannitol in the apical to basal direction across the monolayer. Results are means  $\pm$  S.E.M., and are from four separate preparations with triplicate Transwells for each preparation. Blue line represents collagen I, black line represents collagen IV and red line represents collagen IV plus laminin. Apparent permeabilities ( $P_{app}$ ) (B) were calculated from the gradients of the lines over 5-20 minutes for [<sup>14</sup>C]mannitol, and were compared by analysis of variance and a Newman-Keuls test. \*, results for collagen I were higher than for collagen IV or collagen IV plus laminin ( $P < 0.05$ ).



### 4.13.3 Effect of collagen type I, collagen type IV, and collagen type IV and laminin on TEER and comparison to other preparations

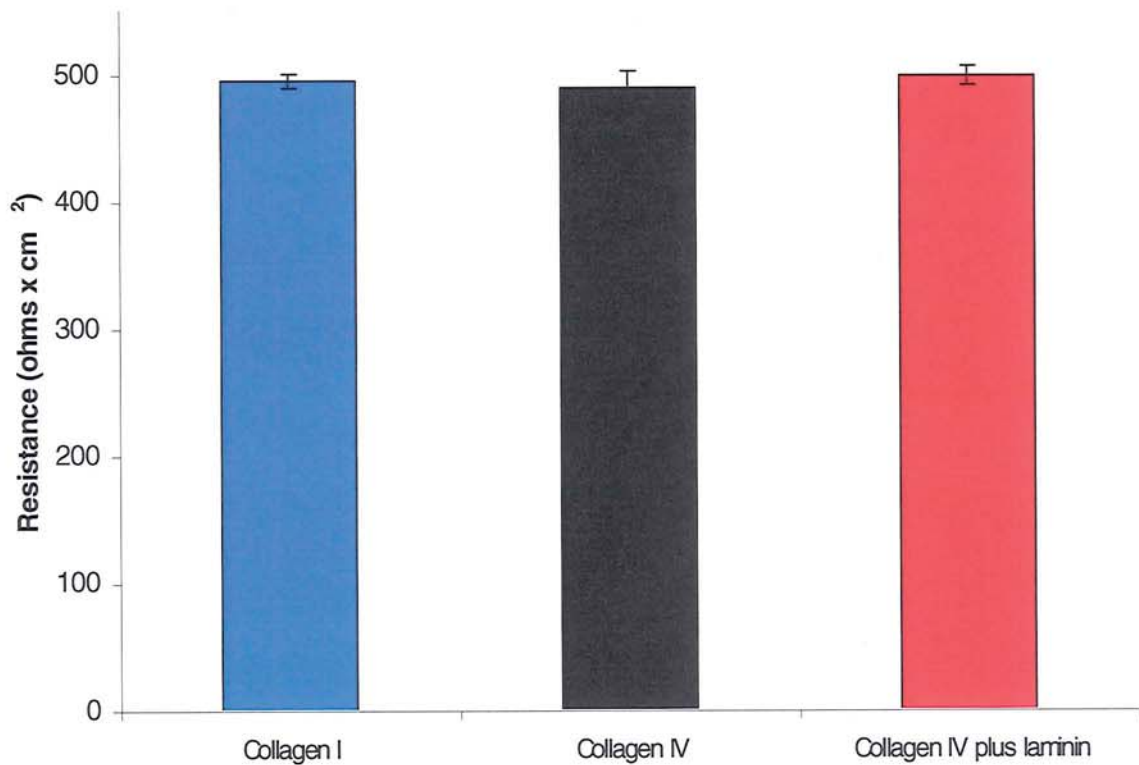
Cells from our preparation showed a significantly higher TEER when grown on membranes coated with collagen IV and collagen IV plus laminin, than with collagen I (Figure 4.13.3.1). However there was no difference between collagen IV and collagen IV plus laminin.

The TEER obtained with collagen type IV ( $748 \pm 88 \text{ ohms.cm}^2$ ) was similar to that of intact sheets of guinea-pig gastric mucosa *in vitro* ( $720 \text{ ohms.cm}^2$ ). There was no difference on TEER for Transwells coated with any matrix in the absence of cells (Figure 4.13.3.2).



**Figure 4.13.3.1 TEER of various guinea-pig preparations: intact sheets of gastric mucosa (Yanaka *et al.*, 1996) primary gastric mucous cell monolayers on a fibronectin-coated collagen gel-cup (Rattner *et al.*, 1985) and mucous cell monolayers grown on Transwells coated with collagen-I, collagen IV and collagen IV plus laminin respectively (present preparations).**

For the present preparations results are presented as means  $\pm$  S.E.M. of six separate preparations with triplicate determinations for each case. Data were analysed by a 2-factor ANOVA followed by Newman-Keuls multiple comparison test. \*\* $P < 0.02$  for comparison with collagen I. No difference between collagen IV and collagen IV plus laminin was found.



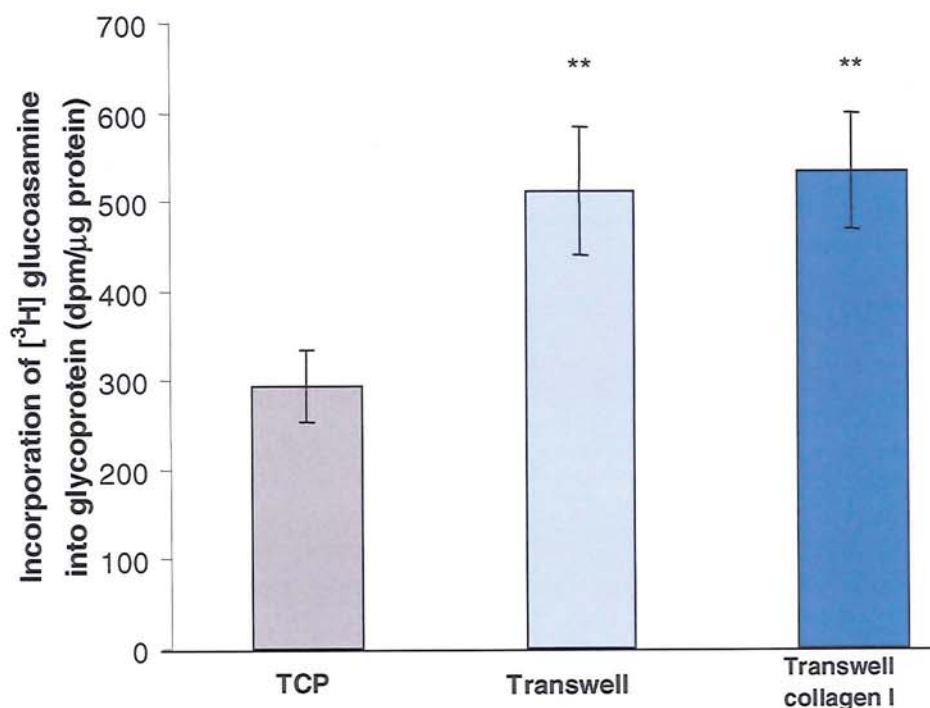
**Figure 4.13.3** TEER of Transwells coated with collagen I, collagen IV and collagen IV plus laminin in the absence of cells.

For the present preparations results are presented as means  $\pm$  S.E.M. of three separate preparations in each case with triplicate determinations for each case. No difference between matrixes by analysis of variance.

#### **4.14 Results- Effect of the matrix used to coat the Transwells on glycoprotein synthesis and secretion by gastric epithelial cells**

##### **4.14.1 Effect on glycoprotein synthesis of growing cells on TCP, uncoated Transwells and collagen I coated Transwells**

Incorporation of [<sup>3</sup>H]glucosamine into glycoprotein was increased, by comparison with TCP ( $P < 0.01$ ), when cells were grown on uncoated Transwells or Transwells coated with collagen I (Figure 4.14.1).



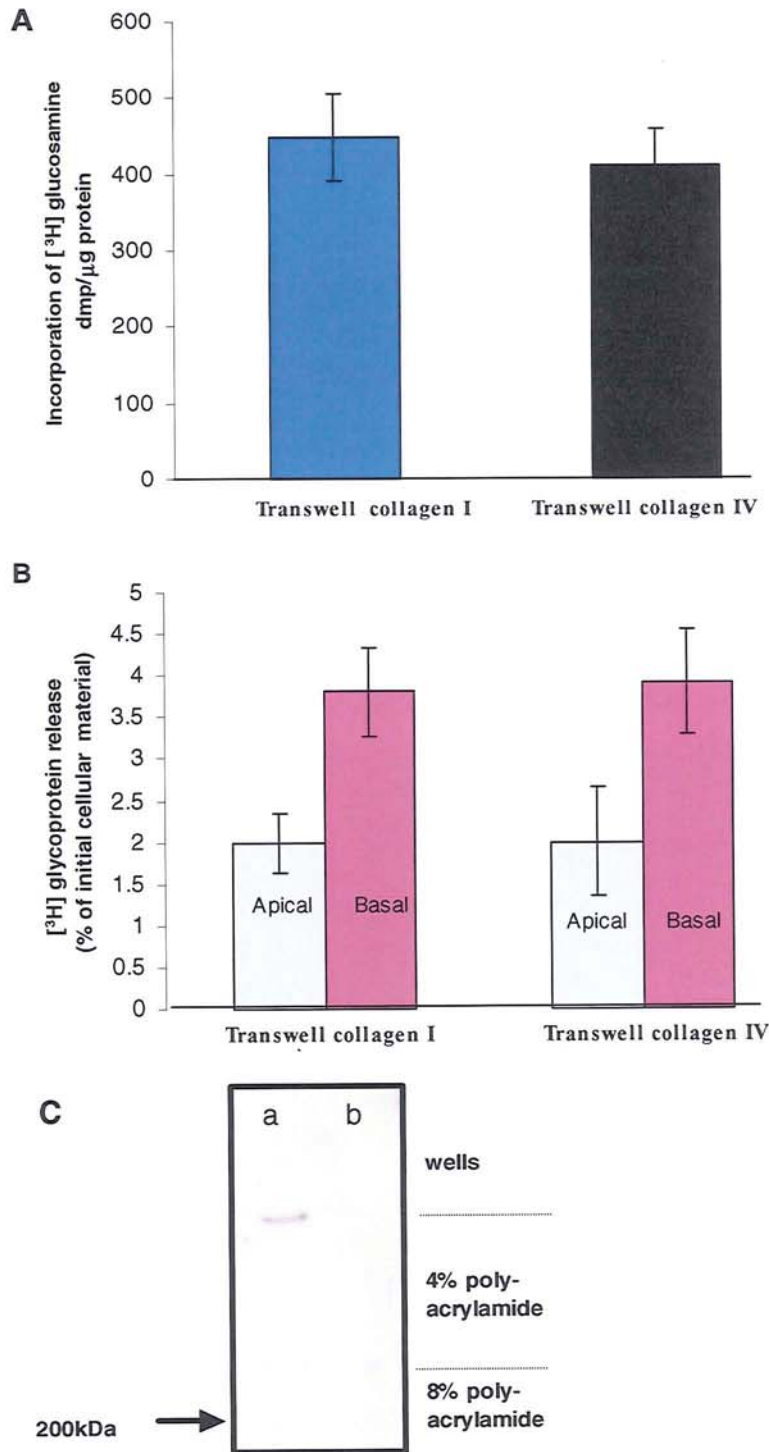
**Figure 4.14.1** Effect of tissue culture plastic, Transwell, and collagen I coated Transwell on glycoprotein synthesis of gastric epithelial cells as assessed by labelling of glycoprotein present within the cells with [<sup>3</sup>H]-glucosamine hydrochloride

Results are presented as means  $\pm$  S.E.M. of three separate preparations with triplicate determinations in each preparation. A medium change was performed at 48 h with the new culture medium containing 2  $\mu$ Ci/ml [<sup>3</sup>H]-glucosamine hydrochloride and label incorporation was assessed at 72 h without any challenge with mucus secretagogues. Means  $\pm$  S.E.M. of three separate preparations: \*\*P<0.01 for difference from TCP by ANOVAR and Newman-Keuls test. No difference between Transwell and collagen I coated Transwell was found.

#### 4.14.2 Effect of collagen I and collagen IV on glycoprotein incorporation and distribution

Incorporation of [<sup>3</sup>H]glucosamine was slightly, but significantly (P<0.05 paired t-test), reduced by growing cells on membranes coated with collagen IV rather than collagen I (Figure 4.14.2A). Cells grown on collagen IV released material labelled with [<sup>3</sup>H]glucosamine, which was precipitable by 10 % (w/v) trichloroacetic acid and 1 % (w/v) phosphotungstic acid, into both basal and apical media (Figure 4.14.2B). However, an antibody to native rat gastric mucin only recognised material released into the apical medium (Figure 4.14.2C). The position of this material, at the top of a 4 % polyacrylamide gel, was similar to that found previously for purified rat gastric mucin (Brown *et al.*, 1993).





**Figure 4.14.2 Effect of collagen I and collagen IV matrices on glycoprotein incorporation and distribution** (A) represents incorporation and (B) the distribution of secreted glycoprotein into the apical and basal compartments of Transwells coated with collagen I or collagen IV. In (A) and (B) glycoprotein release was assessed as material labelled with [<sup>3</sup>H] glucosamine precipitable by 10 % (w/v) trichloroacetic acid and 1 % (w/v) phosphotungstic acid, and is expressed relative to initial cellular material. Results are presented as means  $\pm$  S.E.M. from three separate preparations with triplicate Transwells for each preparation. In (C) material collected from apical (a) and basal (b) compartments of collagen IV coated Transwells was dialysed, freeze-dried and analysed by immunoblotting using an antibody against rat gastric mucin.

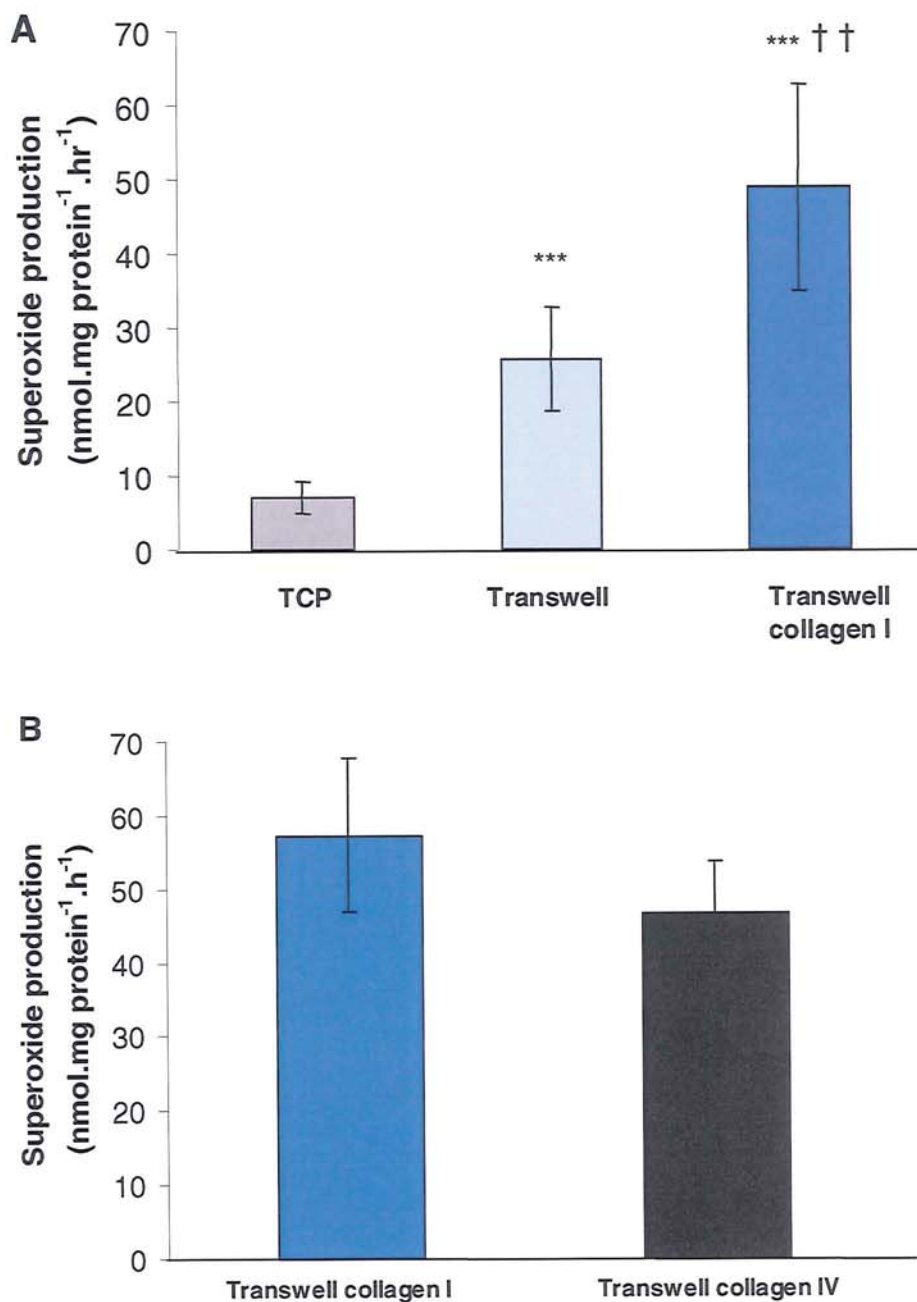
## **4.15 Results- Effect of the matrix used to coat the Transwells on NADPH oxidase activity in gastric epithelial cells**

### **4.15.1 Effect of matrix on the production of superoxide by gastric epithelial cells**

NADPH oxidase activity can be estimated by the reduction of cytochrome C by superoxide, which is inhibitable by superoxide dismutase. Activity was increased when cells were grown on Transwells or Transwells coated with collagen I by comparison with cells grown on tissue culture plastic (Figure 4.15.1A). There was also an increase, relative to Transwells alone, effected by coating Transwells with collagen I. However treating Transwells with collagen IV rather than collagen I did not further increase superoxide production (Figure 4.15.1B).

### **4.15.2 Effect of the matrix on the distribution of superoxide by gastric epithelial cells**

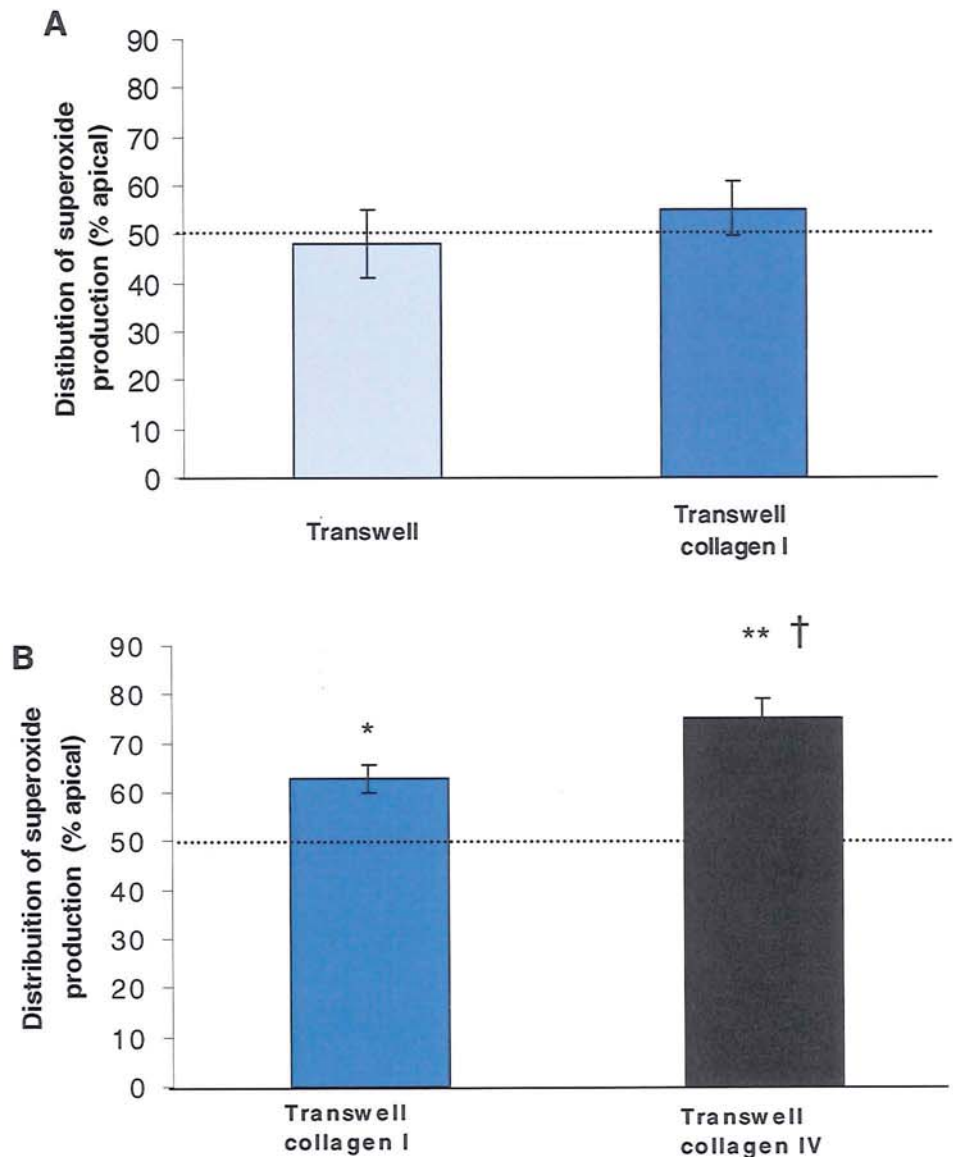
There was no difference between apical and basal release of superoxide for untreated Transwells and collagen I treated Transwells (Figure 4.15.2A). When Transwells were coated with collagen IV rather than collagen I the proportion of superoxide that was apically directed was increased such that the monolayer exhibited a significantly ( $P < 0.01$ , t-test) polarised distribution of this radical (Figure 4.15.2B).



**Figure 4.15.1** Effect of support matrix on NADPH oxidase-derived superoxide anion production by gastric mucous cells after 72h of culture on tissue culture plastic, uncoated Transwells and collagen I coated Transwells (A) and collagen I and collagen IV coated Transwells (B).

For (A) results are presented as means  $\pm$  S.E.M. of three separate preparations with triplicate determinations for each preparation. All results differ from each other by ANOVAR and Newman-Keuls test. \*\*\* $P < 0.001$  for difference from TCP, † †  $P < 0.01$  for difference from Transwell. For (B) results are presented as means  $\pm$  S.E.M. of four separate preparations with triplicate determinations for each preparation. No significant difference between the two matrices by a t-test.





**Figure 4.15.2 Effect of support matrix on the apical distribution of NADPH oxidase-derived superoxide anion release by gastric mucous cells on uncoated Transwells and collagen I coated Transwells (A) and on collagen I and collagen IV coated Transwells (B)**

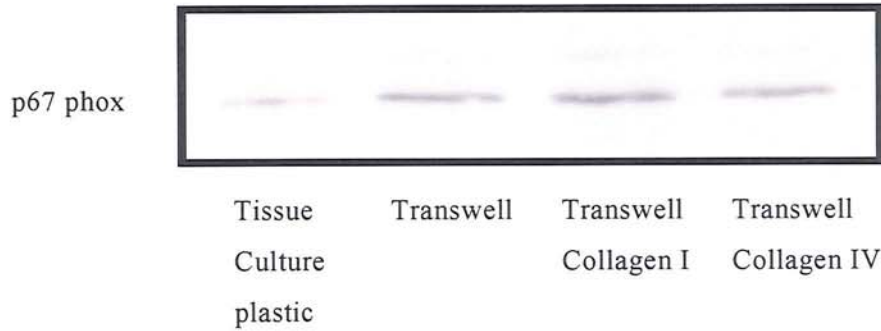
Results are presented for (A) as means  $\pm$  S.E.M. of three separate preparations with triplicate determinations for each preparation. There was no difference between apical/ basal distribution for uncoated Transwells and collagen I coated Transwells separately by t-test for apical release  $\neq$  50 %. No difference between % apical release between uncoated Transwells and collagen I coated Transwells by t-test.

Results are presented for (B) as means  $\pm$  S.E.M. of four separate preparations with triplicate determinations for each preparation. \* $P < 0.05$  between % apical/ basal distribution of collagen I coated Transwells and \*\*  $P < 0.01$  between % apical /basal distribution of collagen IV coated Transwells by t-test for apical release  $\neq$  50 %, and †  $P < 0.05$  for difference between % apical release between collagen I coated Transwells and collagen IV coated Transwells by t-test.

N.B. Apical represents the amount of superoxide anion release above the cell monolayer on the Transwell, and basal the amount of superoxide anion release below the cell monolayer (in the receiving well of the Transwell).

#### 4.15.3 Effect of matrix on the presence of NADPH oxidase component p67-phox

Immunoblotting demonstrated that a key component of the NADPH oxidase system, p67 phox was increased in cells grown on Transwells and Transwells coated with collagens I and IV relative to cells grown on tissue culture plastic.

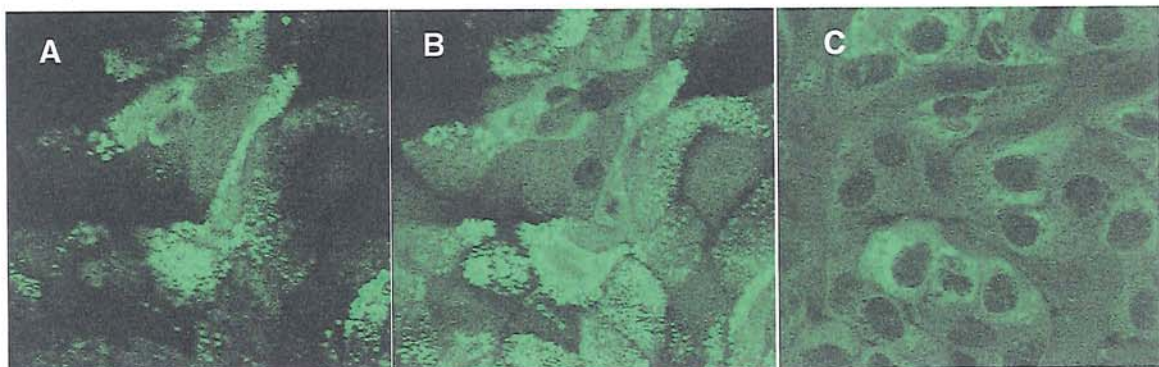


**Figure 4.15.3 Effect of matrix on the presence of NADPH oxidase component p67-phox.**

Cells were cultured for 72h on TCP, Transwell, collagen I and collagen IV coated Transwells. Cell samples, obtained by treatment with trypsin/EDTA, washed with PBS containing 100 µg/ml of trypsin inhibitor, and dissolved into hot electrophoresis sample buffer, were separated by electrophoresis (25 µg protein per lane) and analysed by immunoblotting using rabbit anti-human p67-phox antibody at a dilution of 1:1000.

#### 4.16 Results- Imaging of mucin granules

Optical sections (x, y plane) of monolayers, grown on collagen IV, and taken with a confocal microscope moving from the top of the monolayer down through the cells to the filter, demonstrated that the mucin granules were predominantly located above the nuclei. (Figure 4.16)



**Figure 4.16 Effect of collagen IV on mucin granule presence and location.**

A, B and C are confocal microscopic images of gastric epithelial cells grown on a Transwell coated with collagen IV and stained with an antibody to rat gastric mucin. The images (x,y plane) were taken 5, 8 and 13 µm from the top of the monolayer.



## 4.17 Discussion

The main finding of the work presented in this chapter was that collagen IV was required to provide an optimal monolayer of differentiated and polarised epithelial cells which acted as a permeability barrier. The mucosal cells used in this study were initially mixed mucosal cell preparations. However it was established that greater than 95% of the cells removed from the tissue culture plates, from uncoated Transwells or Transwells coated with collagen I or Collagen IV after 72 h of culture were mucous cells.

### 4.17.1 Assessment of monolayer formation

Collagen IV proved to be a superior matrix for the formation of tight monolayers as evidenced by a lower Papp for fluorescein and higher TEER than collagen I. These results were also supported by data on the transfer of [<sup>14</sup>C] mannitol across the monolayers. Permeability to mannitol and fluorescein and the value of the TEER largely reflect permeability through the tight junctions (paracellular permeability) (Tavelin *et al.*, 2002). The ECM could affect the formation of a “tight” monolayer by influencing the initial attachment of cells to the Transwell membrane, by influencing cell growth, or by activation of signal-transduction pathways which control tight junction formation and junction. The importance of ECM in cell attachment is illustrated by the higher numbers of cells from the conditionally immortalised rat intestinal epithelial cell line 2/4/A1 that attached to fibronectin, laminin and ECL (a mixture of entactin, collagen IV and laminin)-coated Transwells compared to the uncoated supports (Tavelin, 1999). Gastric epithelial tight junctions are subject to regulation by extracellular signalling molecules such as hepatocyte growth factor (Hollande *et al.*, 2001). It is therefore possible that signalling from the extracellular matrix via integrins could also modulate tight junctions. Indeed collagen, together with the Ca<sup>2+</sup> activation of E-cadherin, seems important in the early stages of tight junction formation between cells from the cultured canine renal epithelial cell line MDCK (Drubin, 1996).



The difference obtained here between cells grown on collagen I and collagen IV may relate to differences in the structure of these matrix proteins. Collagen IV forms a meshwork, is a normal constituent of basement membrane and is structurally different from the fibrillar collagen I. The structure of collagen IV differs in several ways from the fibrillar collagens. Unlike the procollagen precursors of the fibrillar collagens, collagen IV does not undergo proteolytic processing. The central triple helical region of collagen IV is about 25% longer than the fibrillar collagens, and unlike fibrillar collagens, it is interrupted into several positions by short non-triple helical sequences (Hulmes, 1992). Moreover, the two forms of collagen may differ in their interaction with cellular integrins (Kreis and Vale, 1999). Type IV collagen can interact with cells indirectly through laminin. Strong binding of collagen IV to laminin is mediated by nidogen/entactin (Timpl *et al.*, 1983; Carlin *et al.*, 1981), a glycoprotein of about 150 kDa which binds tightly to laminin (Poschl *et al.*, 1996) and has binding sites also for type IV collagen and cells (Timpl, 1996). In addition, direct low affinity interaction between laminin and collagen type IV is possible (Yurchenco and O'Rear, 1994). Type IV collagen also binds to heparin and heparan sulphate proteoglycan (Yurchenco and O'Rear, 1994). Many cells adhere directly to collagen type IV (Aumailley and Timpl, 1986). A major binding site in  $\alpha 1 (IV)_2\alpha 2(IV)_1$  heterotrimers is localised about 110nm from the N-terminus of the molecule and this triple binding site interacts with  $\alpha 1\beta 1$  and  $\alpha 1\beta 2$  integrins on cells. It has been shown that spreading of Caco-2 cells on collagen IV is mediated by  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrins (Schreider *et al.*, 2002). In Caco-2 cells integrin-dependent cell to ECM adhesion reinforced E-cadherin dependent cell-cell adhesion and a molecular crosstalk between the two adhesion systems maybe responsible for enterocyte differentiation (Schreider *et al.*, 2002). Thus it could be possible that spreading and epithelial differentiation of the current preparation may also be mediated by the same family of integrins through interaction with E-cadherin.

However, no improvement in permeability characteristics of the monolayer was found if laminin was added to the collagen IV solution used to coat the Transwells. Although laminin is a normal component of the basement membrane it may not be an essential component for monolayer formation in this system. These results agree well with an investigation (Rhodes *et al.*, 1994) that showed that laminin compared to other ECM constituents was the least effective in promoting epithelial cell adhesion of primary guinea-pig gastric epithelial cells. However the importance of laminin in gastric healing has been demonstrated both in rat and rabbit since the expression of the laminin receptor was increased during gastric healing in rat (Piotrowski *et al.*, 1993) and addition of laminin to wounded cultured gastric epithelial cells significantly accelerated repair (Fujiwara *et al.*, 1995). The same effect was observed for collagen I, collagen IV but not fibronectin.

Another possibility for a matrix could have been Matrigel, which is harvested from basement membrane-secreting tumour cells, and which consists of laminin, collagen IV and fibronectin, as well as cytokines and other molecules which influence cell adhesion (Kleinman *et al.*, 1986). The availability of binding sites for the guinea-pig gastric cells may have been greater on the Matrigel. (Rhodes *et al.*, 1994). When guinea pig gastric epithelial cells were seeded on matrigel coated TCP, they extended lamellipodia and started to migrate faster than did the cells on collagen IV and laminin, which remained round and with a smaller contact area to the substrate. The rapid formation of lamellipodia, is typical of mucous cells *in vivo* during restitution. The use of matrigel in our preparation however was rejected since it has a rather gel-like appearance which might retard drug diffusion, it is difficult to mix completely to allow even spreading on the filter support and it is not cost-effective.

An additional method for assessing monolayer integrity was by measuring the resistance across the monolayer. The TEER for gastric epithelial monolayers grown on collagen IV-coated transwells was  $748 \Omega \cdot \text{cm}^2$  which is higher than corresponding values for



the intestinal cell lines: Caco-2 cells (Tavelin *et al.*, 1999),  $234 \Omega \cdot \text{cm}^2$ ; 2/4/A1 cells (Tavelin *et al.*, 1999),  $25 \Omega \cdot \text{cm}^2$  and IEC 18 cells (Versantvoort *et al.*, 2002),  $50 \Omega \cdot \text{cm}^2$ , and most importantly is close to that obtained with intact guinea-pig gastric epithelium (Yanaka *et al.*, 1995). Mannitol permeability (Papp) across the gastric monolayer was  $1.6 \times 10^{-6} \text{ cm} \cdot \text{sec}^{-1}$  which is lower than that for 2/4/A1 cells,  $15.6 \times 10^{-6} \text{ cm} \cdot \text{sec}^{-1}$  and IEC-18 cells,  $12 \times 10^{-6} \text{ cm} \cdot \text{sec}^{-1}$ , but higher than that for Caco-2 cells,  $0.24 \times 10^{-6} \text{ cm} \cdot \text{sec}^{-1}$ , the lower paracellular permeability of which may be explained by their colonic origin (Tavelin, *et al.*, 1999). These comparisons suggest the formation of a reasonably tight monolayer of gastric epithelial cells. There was some variation in permeability to mannitol between monolayers grown on collagen IV, which seemed related to TEER. Measurement of TEER should enable the determination whether a particular preparation was suitable for screening purposes, thus any preparation cultured on collagen IV coated Transwells with a TEER above  $650 \text{ Ohms} \cdot \text{cm}^2$  was accepted for permeability studies.

Cell adhesion is also an important regulator of apoptosis. The induction of a programmed cell death pathway through abrogation of anchorage to the substratum occurs most prominently in epithelial and endothelial cells, and has been termed “anoikis”. A key set of early observations (Frisch and Francis, 1994) showed that when untransformed epithelial cells are deprived of anchorage to an ECM substratum, the cells die by an apoptotic mechanism. By contrast, fibroblasts respond to loss of anchorage by a G1 arrest. The role of anoikis in normal physiological function is unclear but one possibility is that it may serve as a tumour-suppressive function, because cells that lose contact with their normal supporting ECM tend to die rather than survive to invade or metastasise elsewhere. The mechanistic basis of anoikis is the subject of current investigation. Because anchorage to ECM is mediated largely by integrins, one would presume that integrins provide the initial signals governing anoikis. Apoptotic activity was not assessed as a function of the matrix in this work but it is



conceivable that by reducing apoptosis the collagen matrices could have promoted monolayer formation.

#### **4.17.2 Polarity and Differentiation**

##### **4.17.2.1 Glycoprotein synthesis and secretion**

Incorporation of glucosamine into glycoprotein was considerably enhanced by growing cells on transwells, but a major influence of collagen I or collagen IV was absent. Labelled glycoproteins were released into both apical and basal compartments, but immunoblotting experiments showed that only the material released into the apical compartment was mucin. The basally secreted material could represent proteoglycan. The increased incorporation of labelled glucosamine into cells grown on transwells relative to culture plates may simply represent an enhanced access of label to the cells, however it should be noted that another marker of differentiation, NADPH oxidase activity (see below) was also enhanced by growing cells on Transwells. Although the incorporation of labelled glucosamine into material precipitable by trichloroacetic and phosphotungstic acids has been employed as an index of mucin synthesis in human (Kelly and Hunter, 1990), canine (Boland *et al.*, 1990), rabbit (Yoshida *et al.*, 1987), rat (Hiraishi *et al.*, 1991) and guinea-pig (Takao, 1993) gastric mucosa, the present data suggest that this is an oversimplification, and other glycoproteins may be labelled and secreted.

The demonstration by immunoblotting of the apical secretion of mucin served to demonstrate a polarised organisation of the cells grown on Transwells coated with collagen IV.

##### **4.17.2.2 Superoxide Release**

Production of superoxide was enhanced by growing cells on transwells, and by using collagen I to coat the Transwells, relative to tissue culture plastic. These changes were not due to differences between the preparations in the proportion of mucous cells, and seem at least in part to be due to differences in protein expression, as evidenced by immunoblotting for p67-

*phox*, which is an important component of the NADPH oxidase (Chanock *et al.*, 1994). If it is accepted that NADPH oxidase activity is an important differentiated function of gastric surface cells then it appears that differentiation is enhanced by growing cells on transwells and coating them with collagen (I and IV). A possible explanation could be that when these cells are cultured on Transwells they have the opportunity to establish polarity which is an essential feature of all epithelial cells (Chambard *et al.*, 1983). Unless the cell is cultured in such a way as to allow this polarity to develop, complete differentiation is not expressed. One of the best examples is thyroid epithelium, where cells grown on plastic become only partially polarised (Chambard *et al.*, 1983) but when grown on collagen coated filter wells, a complete functional polarity is evident. Another example is a study on colonic cells cultured on Transwells. These cells were able to transport amino acids from the apical to the basal surface (Hidalgo and Borchardt, 1990), that is from the filter well to the underlying medium, and transport toxins from the basal to the apical surface by virtue of the localisation of the efflux protein P-glycoprotein on the apical surface (Meyers *et al.*, 1991). Although there was no major difference in NADPH oxidase activity between cells grown on collagen I and Collagen IV, in the latter case there was a clear apical direction of superoxide release. This appears to be the first demonstration that superoxide production by gastric cells is polarised towards the gastric lumen and it may reflect an interaction between tight junction formation and apical/basal direction of cellular proteins (Drubin, 1996). Collagen IV which is a normal constituent of the basal lamina might have provided the spatial cue for the establishment of cell polarity.

A clear role for superoxide production by gastric epithelial cells has not yet been established. The evidence that gastric mucosal cells possessed a phagocyte NADPH oxidase-like system may provide a previously unrealised pathway for initiating an anti-pathogen response in gastric mucosa. A study by Teshima *et al.* (1998), has shown that the presence of

cells. However the  $O_2^-$  alone appeared not to be enough for killing *H. pylori in vitro*. The gastric epithelial cells produced  $O_2^-$  for several days but did not exhibit a respiratory burst-like production. The predominantly apical release superoxide by gastric cells could however suggest a host defence role for  $O_2^-$  release. Induction of Nox-1 in colon cells by  $\gamma$ -interferon could suggest a role in host defence role since,  $\gamma$ -interferon induces immune response proteins (Lambeth, 2002).

Alternatively NADPH-oxidase in gastric cells may be involved in regulation of proliferation and apoptosis (Teshima *et al.*, 2000). When production of ROS by Nox-1 which is normally expressed in vascular smooth muscle was inhibited, cell growth was suppressed, suggesting a possible role of Nox-1 in normal cell growth. Thus, fibroblasts contain a respiratory burst oxidase-like enzyme that manufactures  $O_2^-$  in response to growth factors (Meier *et al.*, 1993) and endothelial cells manufacture  $O_2^-$  in response to sheer stress (Laurindo *et al.*, 1994). The cells in the current culture (Table 4.9.2.2) release similar amounts of superoxide by Nox-1 as with human colonic epithelial cells in primary culture (Perner *et al.*, 2003).



**Table 4.17.2.2 Comparison of superoxide production with other tissues (note the change in units between shaded and unshaded panels)**

Guinea pig gastric cells	47 nmol/mg protein/h
Human colonic epithelial cells in primary culture <i>Perner et al., 2003</i>	40 nmol/mg protein/h
Guinea pig gastric cells	0.26 nmol/10 <sup>6</sup> cells/min
Human aortic smooth muscle cells + thrombin <i>Patterson et al., 1999</i>	0.48 nmol/10 <sup>6</sup> cells/min
Mouse macrophages activated with phorbol myristate acetate <i>Teshima et al., 1998</i>	0.07 nmol/10 <sup>6</sup> cells/min
Guinea-pig neutrophils activated with phorbol myristate acetate <i>Teshima et al., 1998</i>	9.15 nmol/10 <sup>6</sup> cells/min

#### 4.17.2.3 Imaging

The nucleus of the surface mucous cell is not distinctive because it was not stained prior to confocal microscopy. It is located in the basal cytoplasm, may be extensively infolded, and contains a centrally located nucleolus. In the current study, localisation of mucin granules by confocal microscopy showed that most were supra-nuclear, which again supports a polarised organisation of the cells. Mucin granules in some cells appeared to be higher above the Transwell than in others. Although this result could reflect irregularity in the height of cells in the monolayer it might also be an artefact of fixation or because of slight ‘ruffling’ of the Transwell.

Studies of electron micrographs (Rhodes, D. *et al.*, 1994) of a typical guinea-pig mucous cell prior to plating have shown that membrane bound mucous granules predominated in the

cytoplasm, which also had abundant Golgi, endoplasmic reticulum and mitochondria. The cells had lost membrane polarity in the isolation process and thus extended filopodia at various sites along the cell periphery. However, cytoplasmic polarity remained since mucous granules were congregated together near one side of the cell while the nucleus was at the opposite side of the cell. When cells were plated on collagen IV coated TCP, cells not only adhered to the plates but concomitant with attachment the cells became polarised and changed shape. This effect can be seen in some of the cells grown on TCP in figure 4.3.A but of course is dependent upon the orientation in which the cells is viewed as so no comment can be made as to its prevalence. By contrast all cells grown on collagen IV coated Transwells were shown by confocal microscopy to have a polarised distribution of mucin granules.

#### **4.18 Conclusions**

- 1) Greater than 95 % of the cells removed from tissue culture plates, from uncoated Transwells or Transwells coated with collagen I or Collagen IV were mucous cells.
- 2) Since the TEER, and fluorescein and [<sup>14</sup>C]mannitol transfer across monolayers grown on Collagen Type IV was respectively higher and lower, than for Collagen Type I, it was concluded that Collagen IV is a superior matrix for the achievement of a monolayer with fully developed tight junctions. There was no evidence that addition of laminin to Collagen IV resulted in any further improvement.
- 3) The monolayers exhibited apically polarised secretion of mucin and superoxide when cultured on collagen IV coated Transwells.
- 4) Monolayers cultured on collagen IV coated Transwells exhibited mucin granules with a supra-nuclear location, supporting a polarised organisation of the cells.

## **Chapter 5**

### **Effect of pH on viability of gastric epithelial cells**



## 5.1 Aims

1) To examine the effect of the pH of the culture medium on cell viability after 24 h of exposure for cells grown on TCP. 2) To investigate the effect of apical acidification for 30 min on [<sup>14</sup>C] mannitol flux and TEER for monolayers cultured on collagen IV coated Transwells.

## 5.2 Introduction

The gastric mucosa produces potentially damaging agents such as gastric acid and the proteolytic enzyme pepsin. To maintain mucosal integrity, an effective defence mechanism is required. The concept of a “gastric mucosal barrier” was originally introduced by Code *et al.* (1955) to account for the relative impermeability of gastric epithelium to passive movement of ions. These studies showed that flux of H<sup>+</sup> from the lumen of the stomach (acid back-diffusion) and that of Na<sup>+</sup> in the opposite direction are normally very small. However, a marked increase in ionic permeability, a precipitous fall in transmucosal potential difference, and an outpouring of alkaline interstitial fluid were found to accompany acute gastric mucosal damage induced by exposure to bile salts, ethanol, or salicylates. In most of these experiments, the injurious agent (“barrier breaker”) was only present for a limited period of 30 minutes, and normal ionic permeability was restored over the ensuing few hours, presumably by a process of restitution. Restitution is the migration of undamaged cells from the gastric pits to reform a continuous epithelium.

The gastric barrier consists of three major components: the mucous-bicarbonate layer which overlies the epithelium; the epithelial cells themselves and the mucosal blood flow which removes noxious agents. The gastric mucosal barrier to luminal acid thus consists of pre-epithelial, epithelial, and post-epithelial elements. The pre-epithelial element involves mucus and bicarbonate secretion, the epithelial element involves epithelial cell mechanisms,

and the post-epithelial element involves the removal of the  $H^+$  and supply of substrate via mucosal blood flow.

The first evidence to suggest that electrolyte secretion by the stomach contains bicarbonate ( $HCO_3^-$ ), in addition to acid, was presented as early as 1892 by the Danish physiologist Schierbeck. His results also suggested that in dogs, gastric secretion of  $HCO_3^-$ , like that of acid, is a stimulatable process. A few years later, Pavlov proposed that “alkaline mucus lining the gastric mucosa” neutralised luminal acid but his suggestion gained little support at that time (see, Flemström, 1994). Heatly (1959), hypothesised that adherent mucus could act as an unstirred layer that would allow a pH gradient to develop at the surface of the stomach. Although alkaline secretion by the stomach has been studied in all common laboratory animals and in humans, most of the current information on the origin and cellular mechanism of  $HCO_3^-$  transport has been obtained using mucosal sheets isolated from amphibians (Allen *et al.*, 1993). Such studies have demonstrated that the secretion is a metabolism-dependent process and that it originates from the surface epithelial cells of the gastric mucosa (Flemström, 1977). Secretion is stimulated by physiological stimuli such as the presence of acid. The  $HCO_3^-$  secretion alkalinises the viscoelastic mucus gel adherent to the intact mucosa and provides the first line defence against luminal acid.

Kiviluoto *et al.* (1993) were able to demonstrate that the mucus-bicarbonate layer preserved intracellular pH in *Necturus* gastric antral mucosa exposed to acid. They were able to simultaneously measure luminal pH, surface pH, and intracellular pH, using intracellular microelectrodes. In three experimental series, the investigators inhibited luminal bicarbonate secretion by removing serosal bicarbonate, digested the mucus gel with pepsin, and altered the structure of the mucus gel with N-acetyl-cysteine, a mucolytic agent. A clear-cut intracellular acidification occurred in all three cases, demonstrating the need for the integrity of the mucus-bicarbonate layer in the protection against acidification of the antral surface cell.

The presence of a pH gradient from an acidic lumen to a near neutral pH at the mucosal surface of gastric mucosa has been measured using pH-sensitive microelectrodes. The existence of these pH gradients provides direct evidence for effective surface neutralisation of acid by  $\text{HCO}_3^-$  secreted from the mucosa. Conversely the gradient breaks down at luminal  $\text{H}^+$  concentration of 1.1-1.7 *in vitro* and *in vivo* (Bahari *et al.*, 1982; Ross *et al.*, 1981; and Takeuchi *et al.*, 1983). Consequently, in this series of experiments, the effect of juxtamucosal pH seemed pivotal for the use of this model as a screening tool that would mimic *in vivo* physiological conditions. Thus, experiments were conducted on the effect of pH on the gastric epithelial cells. Firstly the effect of pH, for a period of 24 h on cells grown on TCP was investigated. Next monolayers with culture medium at a pH of 7.4 on the basolateral side were exposed on the apical side for 30 minutes to media with pH values ranging from 7.4 to 1.5. The long-term experiments, with cells grown on TCP, were intended to establish that the cells had an intrinsic sensitivity to pH. For reasons which will become clear from the data it was necessary to limit the experiments using cells grown on Transwells to 30 minutes duration.



### 5.3 Methods- pH dependence of viability of gastrointestinal cells on TCP

After 48 h of culture in 12-well plates, medium was removed by gentle suction and 2 ml of fresh medium was added followed by the appropriate amount of 6M HCl in order to achieve the desired pH. The amounts of 6M HCl per well were as follows:

**Table 5.3.1**

<b>Amount of 6M HCl (<math>\mu</math>l) added in 2 ml of medium into well at 48 h of initial cell culture</b>	<b>pH of medium + 6M HCl</b>
1.33	6.96
2.33	6.5
3.33	6.0
7.3	4.9
8.0	4.0

The plates were returned to the incubator and 24 h later, the medium was removed from the wells into 7 ml tubes, and the tubes capped. 500  $\mu$ l of (0.4 % (wt./vol.) crystal violet in 30 % methanol (v/v)) was added to each well of the plate for assay of cell number. Immediately afterwards, the pH of medium in the tubes was measured. In cell free conditions the same procedure was followed but no cells were present in the wells.

## 5.4 Methods- Acidification of the apical medium

In transfer experiments HBSS (pH 7.4) is used as the apical medium. Acidification of HBSS to pH 3.0, 2.0, and 1.5 was achieved by adding 5 M HCl until the correct pH was achieved. A solution of pH 4.5, buffered with 25 mM acetate, was achieved by adding acetic acid to a concentration of 25 mM and then adjusting the pH to 4.5 with NaOH. A pH of 5.5 was obtained by adding solid MES to give a concentration of 25 mM and then adjusting the pH with 1 M HCl. The pH values of the above adjusted solutions were stable in the absence of cells.

**Table 5.4**

<b>pH</b>	<b>HBSS volume in ml</b>	<b>Amount of 5 M HCl</b>	<b>Amount of 1 M HCl</b>	<b>Amount of 1 M NaOH</b>
5.5 after addition of MES	25 ml	N/A	30 $\mu$ l	N/A
4.5 after addition of 25 mM acetate	25 ml	N/A	N/A	130 $\mu$ l
3.0	20 ml	19 $\mu$ l	5 $\mu$ l	N/A
2.0	20 ml	30 $\mu$ l	70 $\mu$ l	N/A
1.5	20 ml	100 $\mu$ l	260 $\mu$ l	N/A

## **5.5 Methods- Effect of gastric cells on initial pH of medium**

Transfer experiments were performed as described in section 2.8.2 but apical HBSS was substituted with acidified HBSS (please refer to section 5.4) containing [<sup>14</sup>C] mannitol (0.45 μCi/ml, 7.75 μM). Basal HBSS was substituted with complete medium (pH 7.4) in order to maximize buffering of the basal aspect of the cells.

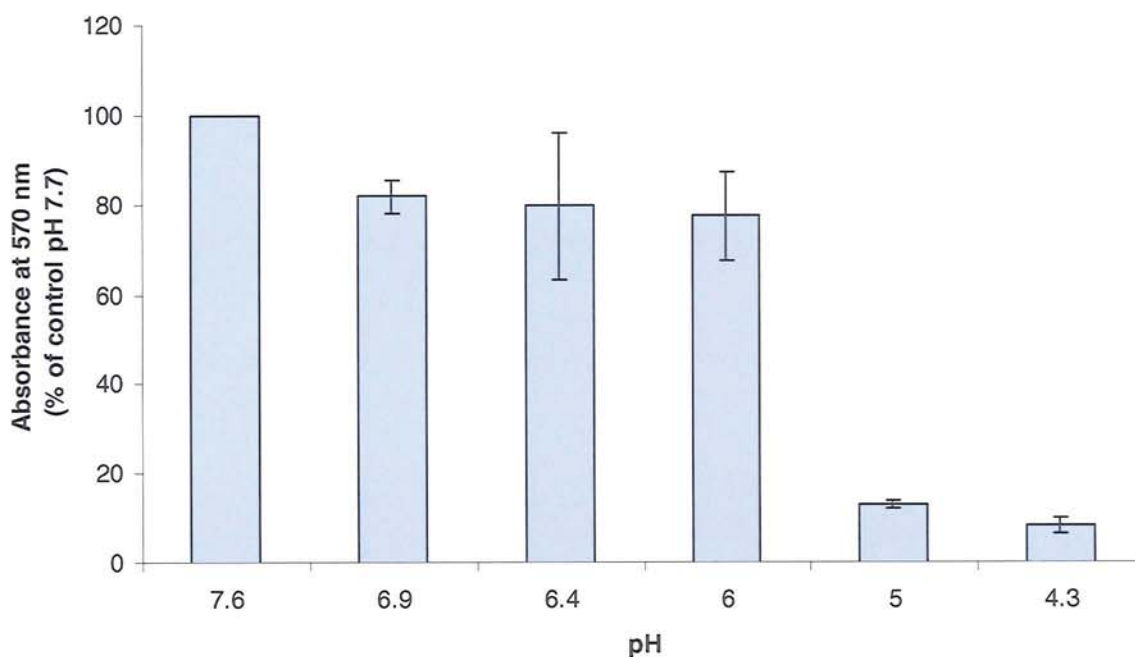


## 5.6 Results- Effect of pH of the culture medium on cell attachment and growth for cells cultured on TCP

The drop in pH obtained after acidification of the culturing medium (Table 5.6) recovered after 24 h when the initial pH value was above 6.0, both in the presence or absence of cells, which indicates that this recovery was due not to a significant buffering effect of the cells but rather the culture medium. Assessment of cell number by the crystal violet assay (Figure 5.6.1) showed the viability of the cells to be pH dependent. Cell number dramatically drops when pH is around 5.

**Table 5.6 Change in pH after acidification in the presence and absence of cells**

Well	Initial pH of medium no cells	pH of medium after 72 h in the absence of cells	pH of medium after 72 h in the presence of cells
1	7.6	7.57	7.73
2	7.6	7.57	7.85
3	6.9	7.68	7.67
4	6.9	7.58	7.67
5	6.4	7.36	7.54
6	6.4	7.34	7.56
7	6	7.46	7.54
8	6	7.36	7.47
9	5	5.2	4.58
10	5	4.75	4.78
11	4.3	4.03	3.71
12	4.3	2.78	4.25



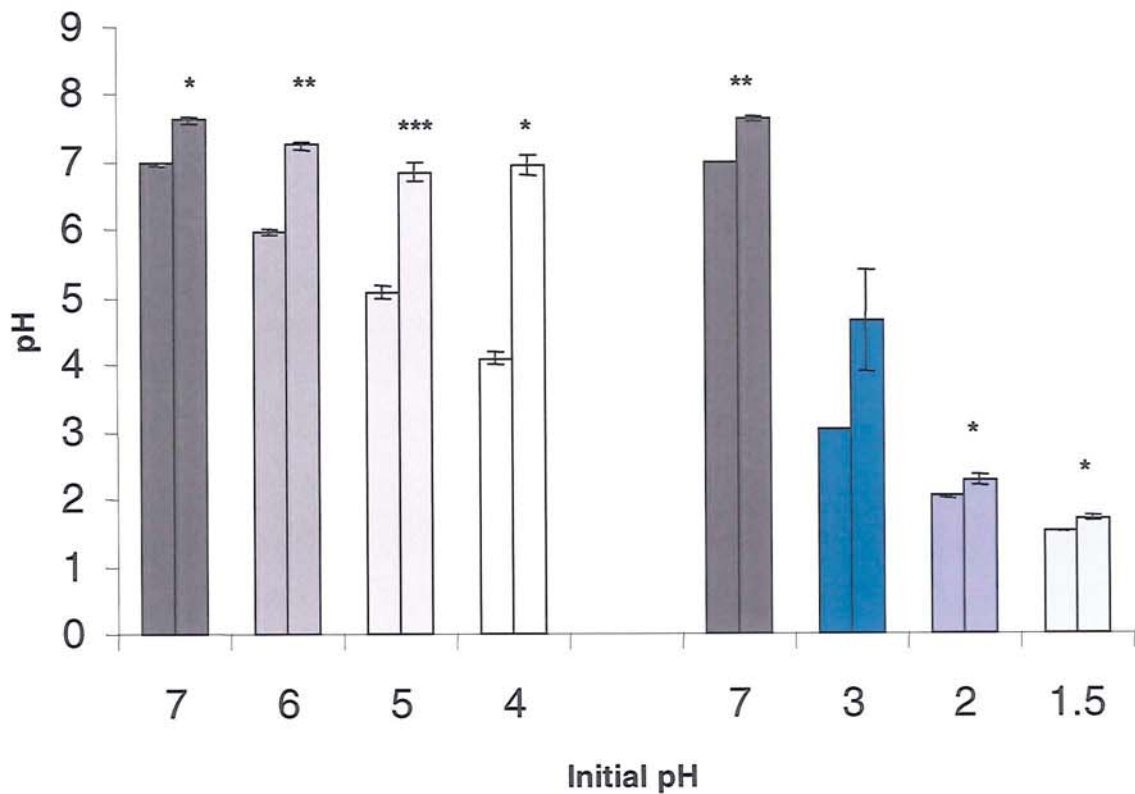
**Figure 5.6.1** Effect of exposure to altered pH for 24 h on number of gastric cells assessed by the crystal violet assay.

Results for absorbance values are expressed as percentage of control cells i.e. no acid added. Cells were plated at  $12 \times 10^5$  cells / well. Results are means of two wells in each case.

## 5.7 Results- Response of gastric surface cells grown on collagen IV to apical acidification

### 5.7.1 Effect of gastric cells on initial pH of medium

There was a significant increase in apical pH, relative to initial pH values of 7, 6, 5, 4, 2, and 1.5, over the 30 min incubation (Figure 5.7.1.1). When the initial pH was 4 or above the final pH was close to neutrality and therefore apical acidification was only transient. However, acidification to pH 3.0 or below produced a sustained reduction in apical pH (Figure 5.7.1.1). These changes in medium pH did not take place in the absence of cells, so changes in pH from initial pH values of 7, 6, 5, 4 and 2 were due to cellular activity (Figure 5.7.1.1)



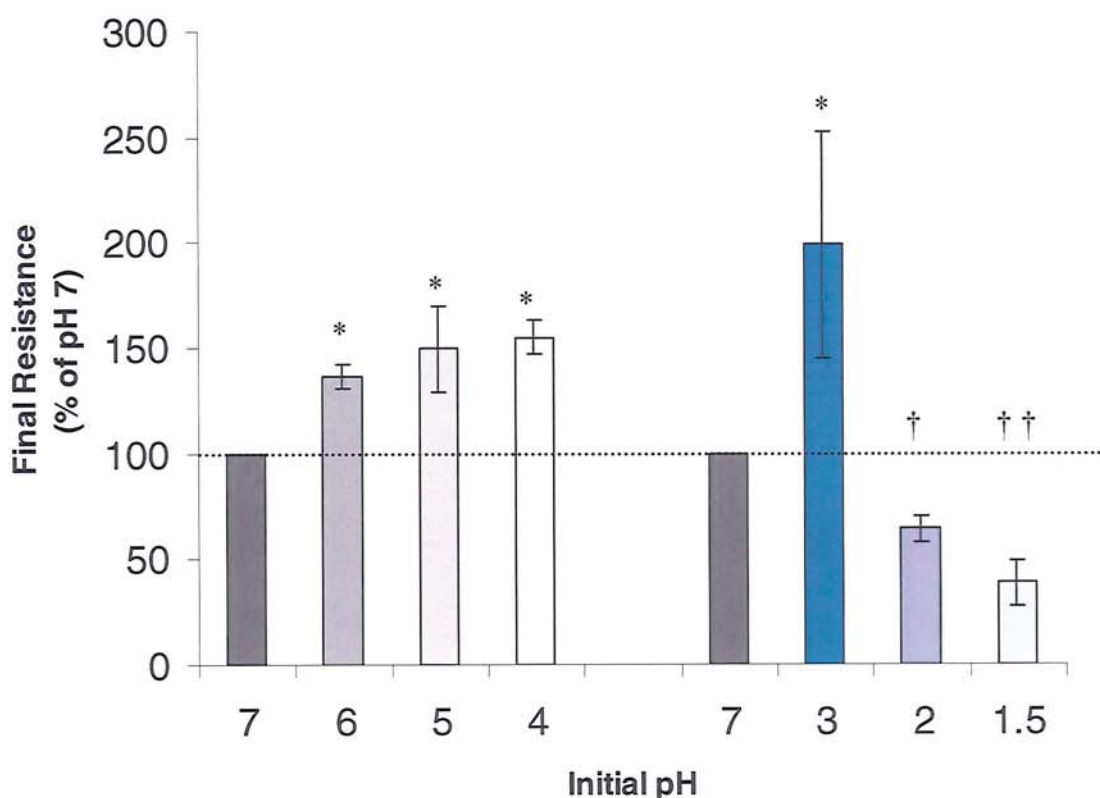
**Figure 5.7.1.1 Final pH the apical medium relative to initial apical pH for monolayers grown on collagen IV coated Transwells.**

Results are presented as means  $\pm$  S.E.M. from three separate cell preparations with triplicate determination in each experiment. Comparison of final pH (columns on the right) with initial pH (columns on the left). \* $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\* $P < 0.001$  for comparison between initial and final pH by paired t-test.

### 5.7.2 Effect of pH on TEER

An initial apical pH of 6, 5, 4, or 3 produced a significant elevation in final resistance relative to that at pH 7. In particular, when initial apical pH was 3, the TEER increased 100 % in relation to a neutral pH. By contrast, a further decrease in initial pH, to 2 or 1.5 produced a TEER which was significantly lower than that at pH 3. (Figure 5.7.2).



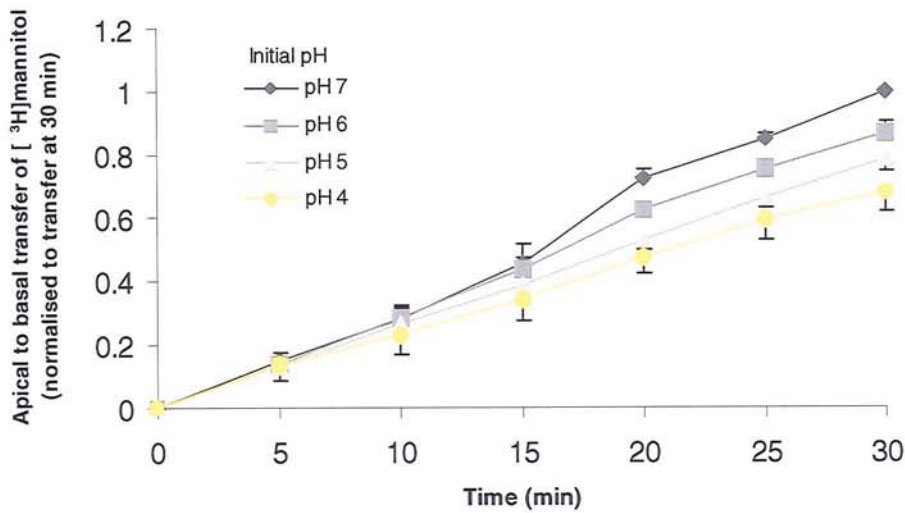


### 5.7.2 Effect of pH on TEER of monolayers grown on collagen IV coated Transwells

Results are presented as means  $\pm$  S.E.M. from three separate cell preparations for each data set with triplicate determination in each experiment. Effect of initial pH on final resistance expressed as a percentage of that at pH 7. \* $P < 0.05$  for comparison with pH 7 by analysis of variance and a Newman-Keuls test. Right-hand data set: †  $P < 0.05$ ; ††  $P < 0.01$  for comparison with pH 3.

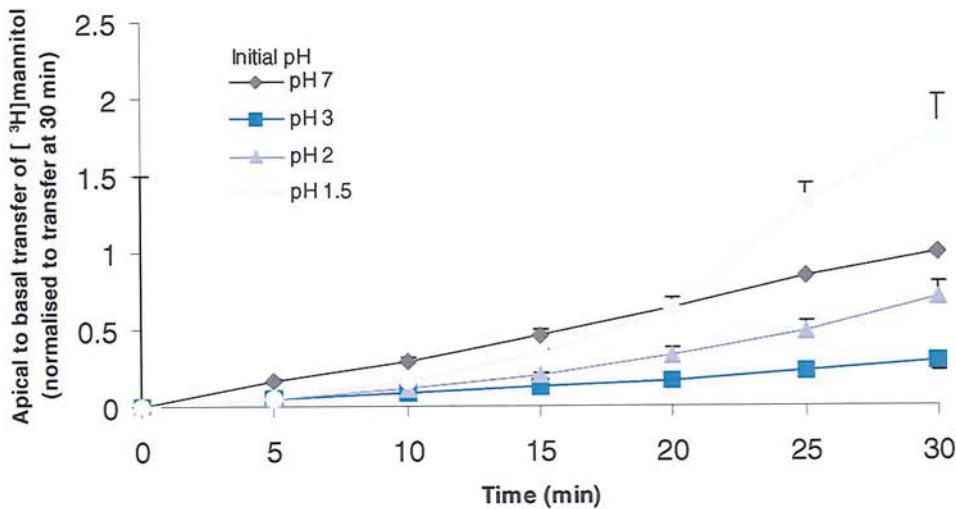
### 5.7.3 Effect of pH on [ $^3\text{H}$ ] mannitol transfer across epithelial cell monolayers

Mannitol transfer showed a graded reduction with decreasing initial pH over the range 7 to 4 (Figure 5.7.3.1) with analysis of variance followed by a Dunnett's test showing the  $P_{\text{app}}$  at pH 4,  $1.1 \times 10^{-6} \pm 0.13 \times 10^{-6} \text{ cm}\cdot\text{sec}^{-1}$ , to be significantly lower ( $P < 0.05$ ) than that at pH 7,  $1.8 \times 10^{-6} \pm 0.46 \times 10^{-6} \text{ cm}\cdot\text{sec}^{-1}$  ( $n=3$ ). The sustained reduction in pH caused by acidification to pH 3 gave a  $P_{\text{app}}$  of  $0.61 \times 10^{-6} \pm 0.2 \times 10^{-6} \text{ cm}\cdot\text{sec}^{-1}$ , which was significantly lower ( $P < 0.05$ , paired t-test) than that at pH 7,  $1.8 \times 10^{-6} \pm 0.43 \times 10^{-6} \text{ cm}\cdot\text{sec}^{-1}$  ( $n=3$ ). Although mannitol transfer at an initial pH of 2 and 1.5 started out low relative to pH 7, it increased during the incubation, this effect being particularly noticeable at pH 1.5 (Figure 5.7.3.2).



**Figure 5.7.3.1** Effect of acidification of the apical medium on apical to basal transfer of mannitol for monolayers grown on collagen IV coated Transwells.

Results are presented as means  $\pm$  S.E.M. from three separate cell preparations with triplicate determination in each experiment. Results have been normalised to transfer at 30 min at pH 7 which was made equal to unity to facilitate comparison between the data sets from Figure 5.7.3.1 and Figure 5.7.3.2 (please refer to Figure below).



**Figure 5.7.3.2** Effect of acidification of the apical medium on apical to basal transfer of mannitol for monolayers grown on collagen IV coated Transwells.

Results are presented as means  $\pm$  S.E.M. from three separate cell preparations with triplicate determination in each experiment. Results have been normalised to transfer at 30 min at pH 7 which was made equal to unity to facilitate comparison between the data sets from Figure 5.4.3.1 (please refer to Figure above) and Figure 5.4.3.2

## 5. 8 Discussion

The main finding of this chapter is that gastric epithelial monolayers grown on Transwells coated with collagen type IV are able to withstand transient apical acidification to pH 3, which is associated with a decrease in [<sup>3</sup>H] mannitol flux and an increase in TEER, a probable reflection of a tighter monolayer relative to pH 7.4.

When cells were cultured on TCP, and after 48 h of culture the medium was replaced by culture medium acidified to lower pH values the cells which had to tolerate for 24 h pH values ranging from 5.2 to 2.78, lost viability. When the pH after initial acidification was above 6, the culture medium had the ability to recover its pH and no major loss of viability occurred. A physiological property of gastric surface cells is their ability to secrete HCO<sub>3</sub><sup>-</sup> (Allen *et al.*, 1983). However the change in pH of the medium in the absence of the cells did not suggest that the cells produced substantial amounts of HCO<sub>3</sub><sup>-</sup> in order to buffer the medium. Furthermore, in a single compartment system it is difficult to envisage how separation of H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> could occur.

Although a direct comparison with monolayers cultured on collagen IV coated transwells cannot be made, it is interesting to note that when the monolayers cultured on collagen IV Transwells were exposed to stepwise acidification down to pH 4.0, within less than 30 minutes, the pH reached close to neutrality. This increase in pH of the apical medium during the incubation was probably caused by bicarbonate secretion. In frogs, *in vitro* experiments have revealed that gastric bicarbonate secretion is dependent on luminal chloride ions, indicating the presence of an apical Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger (Flemström, 1980). When, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), an inhibitor of the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger as well as Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> cotransport, was applied luminally to rat gastric mucosa, bicarbonate secretion seemed to be inhibited suggesting at least the presence of an apical Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger in the surface epithelial cells (Phillipson *et al.*, 2002). In the current study,



it seems that the rate of bicarbonate secretion was such that only when the initial pH was 4 or above was the pH brought close to 7 during the incubation. Under physiological conditions gastric epithelial  $\text{HCO}_3^-$  neutralises luminal acid within the matrix of the adherent mucus gel. A pH gradient is established from an acid pH in the lumen which has a value of pH 2 or above, to a near-neutral pH at the mucus-epithelium interface. The adherent mucus gel creates a stable unstirred layer at the mucosal surface that acts as a mixing barrier, restricting the movement of newly secreted  $\text{HCO}_3^-$  and preventing it from being overwhelmed by the vast excess of acid in the lumen. In an *in vivo* study in the rat corpus, an inverse relation between mucous gel thickness and intracellular acidification in the surface epithelium was found (Engel *et al.*, 1995). Therefore, indicating, that a thicker mucus layer would protect the underlying cells from back-diffused acid. A study by Atuma *et al.* (2001) has demonstrated the presence of two types of mucus gel secretion, one that is firmly adherent to the mucosal surface and one above it that is relatively loosely adhered and can be removed by suction. Clearly in the present system bicarbonate moving from the cell layer is mixing, at least at some extent with the medium. Further experiments are required to establish for certain whether a layer of secreted mucus overlies the cell monolayer. However pilot experiments using a transversely mounted monolayer grown on a Transwell and viewed in an inverted phase contrast microscope did not give clear evidence of a layer of mucus.

A key property of the gastric mucosal barrier, is that the apical surface of the epithelium displays low permeability to  $\text{H}^+$ , thus being able to maintain a  $\text{H}^+$  concentration gradient  $>10^6$  across the epithelial lining. In the present preparation the transient decrease in pH did cause a reduction in paracellular permeability through tight junctions with an accompanying increase in TEER. Such effects were greater with a sustained reduction in apical pH achieved by acidification to pH 3 with an increase in TEER of 100 %. In intact gastric epithelia, increasing luminal acidity is associated with an increase in TEER and

therefore a tightening of the barrier to  $H^+$  (Spencer *et al.*, 1992). Further reduction in pH below 3 caused disruption of the monolayer as evidenced by a reduced TEER and increased mannitol flux relative to pH 7.0. Similar findings have been obtained with intact isolated rabbit gastric mucosa where acidification to pH 2.8 produced an increase in TEER by 43 %, which however declined at pH values less than 2.8 (Spencer *et al.*, 1992). Examination by scanning electron microscopy to of rabbit gastric mucosa exposed to a mucosal pH of 2.8 showed little difference compared with control tissues with a pH of 7.4. In comparison, in tissue exposed to pH 1.8, necrotic cells together with cellular debris were clearly evident, and the apical plasma membranes of numbers of surface epithelial cells were disrupted. Spencer *et al.* (1992) suggested that the increase in TEER upon a moderate acidification (pH 2.8) in intact sheets of gastric mucosa is a phenomenon of the mucosal surface, as identical acidification of the serosal surface did not result in an increase in TEER. In contrast, the serosal surface was more sensitive to damage by acidification. In canine gastric and gastric chief cell monolayers, basolateral acidification below a pH value of 5.5 produced rapid loss of tight junctional and monolayer integrity (Sanders *et al.*, 1985; Chen *et al.*, 1994). There was insufficient time to establish if this was the case for the present monolayer grown in Transwells.

In canine gastric mucosal monolayers composed of chief and parietal cells increasing apical  $H^+$  produced effects which were interpreted in three *phases*. In *phase 1*, as the apical pH decreased from 7 to about 2.5, TEER increased, in *phase 2*, an increased paracellular permeability developed at pH below 2.5, which was evidenced by an increase in [ $^3H$ ] mannitol flux. Finally in *phase 3*, after sustained exposure to an apical pH below 2, monolayer integrity was lost, accompanied by decreased TEER and increased in [ $^3H$ ] mannitol flux (Chen *et al.*, 1994). The authors suggest that involvement of the tight junction in the above events is twofold. Firstly, apical  $H^+$  induced an increase in TEER due to



decreased paracellular permeability (*phase 1*); and secondly, the earliest injury in response to sustained apical acidification is a decrease in TEER associated with a selective increase in paracellular permeability which is not accompanied by loss of monolayer integrity. These results resemble those obtained here in that the acidification down to pH 3 decreases mannitol permeability and increases TEER relative to pH 7; acidification down to pH 2 causes some disruption as evidenced by TEER not significantly different from pH 7 but still with reduced mannitol permeability; while acidification to pH 1.5 clearly disrupts the monolayer with a large increase in mannitol permeability relative to pH 7.

*In vivo* gastric epithelial cells can withstand exposure to pH values of 1.5. The difference from *in vitro* preparations may reflect involvement of other defence mechanisms *in vivo*. Thus, removal of backdiffused  $H^+$  by the mucosal circulation, buffering of  $H^+$  with systemic  $HCO_3^-$ , and the influence of mesenchymal cell types.

## 5.9 Conclusions

1. When cells were grown on TCP, the viability of the cells was reduced when the pH of the culture medium dropped between 6 and 4.
2. The present preparation seems to exhibit a sensitivity and resistance to acid conditions similar to that of intact gastric mucosa *in vitro* (Spencer *et al.*, 1992).



## **Chapter 6**

### **Accumulation of indomethacin in gastric epithelial cells**

## 6.1 Aims

To examine the effect of the pH of the apical incubation medium on the transfer of the acidic drug indomethacin (pKa 4.5) across a gastric mucous epithelial cell monolayer, and to determine whether indomethacin accumulates in cells exposed to a low apical pH.

## 6.2 Introduction- Martin's Hypothesis

Theoretical considerations (Martin, 1963) suggest that after the oral administration of acidic drugs, appreciable quantities of drug anion ( $\text{RCOO}^-$ ) may accumulate in the gastric mucosal cells. The arguments presented were as follows. A pH gradient exists between the gastric fluid and the gastric mucosal cells. The pH within the mucosal cells is assumed to be at pH 7, but the surface of the cell which borders the gastric lumen is in contact with gastric fluid of pH 2.

Moderately acidic drugs (pKa 3-5) are mainly in their unionised ( $\text{RCOOH}$ ) form at the pH of the gastric fluid and thus will readily diffuse into the mucosal cells, but at a pH of about 7.4, they will be mainly ionised and will hardly be able to diffuse out of the cells again (Table 6.2, Figure 6.2.1).

**Table 6.2 Effect of pH on the dissociation of weak acids**

Percentage of undissociated acid		
pKa	pH 2	pH 7
3.5	97%	0.03%
4.5	~100%	0.3%
5.5	~100%	3%
7.0	~100%	50%

According to Martin, if the cell membrane is selectively permeable to the undissociated acid molecule then:

$$\text{Rate of entry into the mucosal cell} = k ([\text{RCOOH}]_A - [\text{RCOOH}]_B) \quad (1)$$

Where  $[\text{RCOOH}]_A$  and  $[\text{RCOOH}]_B$  represent, respectively the concentration of the undissociated acid in solution at the mucosal surface of the stomach, and in the mucosal cell, and  $k$  is the specific rate constant for a particular acid crossing the cell membranes. On entering a mucosal cell, an acid will dissociate according to its  $\text{pK}_a$ , and only that fraction remaining undissociated will contribute effective potential to its rate of removal (Figure 6.2.).

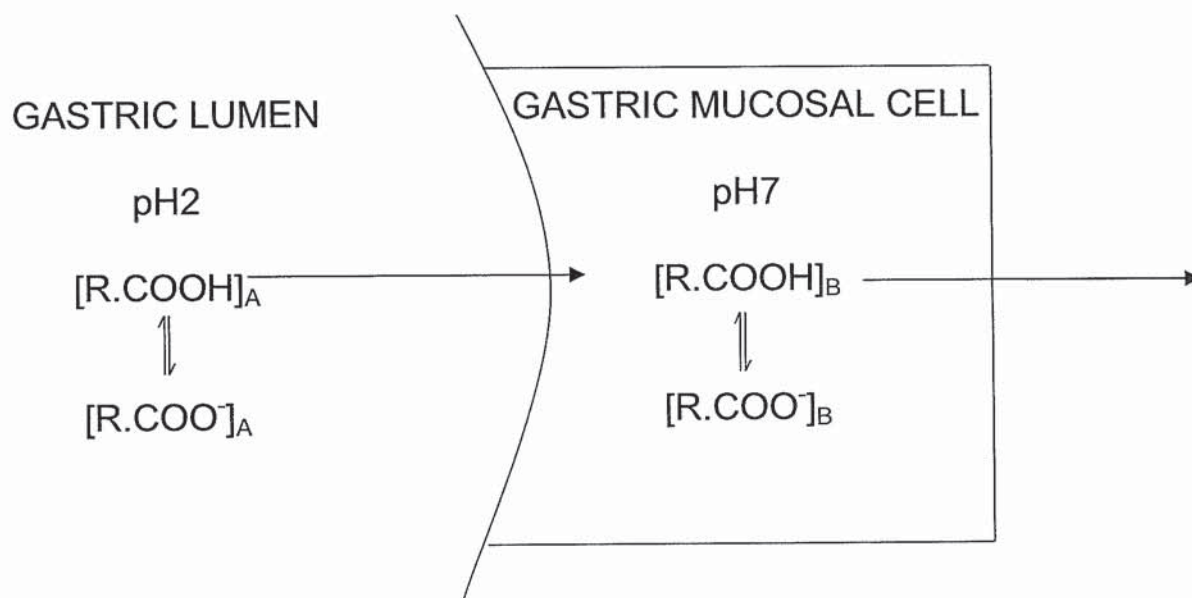


Figure 6.2.1 Diagram illustrating the pH gradient which exists in the stomach between the gastric fluid and a mucosal cell, and the equilibrium between the dissociated and undissociated forms of an acid  $\text{RCOOH}$ .



The total concentration of acid in the cell,  $[\text{RCOOH}]_B + [\text{RCOO}^-]_B$ , will rise until the rate of diffusion of  $\text{RCOOH}$  out of the cell is equal to the rate of entry. If it is assumed that the acid diffuses from the cell into a medium where it remains at negligible concentration, then:

Rate of removal from mucosal cell =  $k[\text{RCOOH}]_B$ . (2) Equating the above two rates (equation 1 and 2), a steady state will result when  $[\text{RCOOH}]_B = 1/2[\text{RCOOH}]_A$

An acid of  $\text{pK}_a$  7 is 50 per cent undissociated at  $\text{pH}$  7 and a steady state will therefore be attained when the total concentration of acid in the cell equals the concentration in the gastric lumen. But for stronger acids, the total concentration of the acid in the cell must rise appreciably above the lumen concentration before this steady-state can be attained. The net effect is the accumulation of the drug anion,  $\text{RCOO}^-$ , in the mucosal cell. The accumulation will be considerably greater with stronger acids. It is suggested that if a stronger acid ( $\text{pK}_a$  3) is rapidly absorbed, the mucosal cell may suffer damage due to the accumulation of anion.

Martin's hypothesis may be significant in two respects:

- 1) The oral administration of certain acids which are well absorbed from the stomach, for example, aspirin, frequently give rise to gastric mucosal damage. The fact that the intestine is seldom involved may be related to the much smaller  $\text{pH}$  gradients existing between the intestinal contents and the mucosal cells.
- 2) Any attempt to relate the rate of absorption of acids to the  $\text{pK}_a$  of the acid, in so far as the  $\text{pK}_a$  controls the concentration of undissociated molecules in the stomach, is not readily acceptable, for most acids are largely in the undissociated form at the  $\text{pH}$  of the stomach. If the  $\text{pK}_a$  does influence the rate of absorption of an acid, this effect may be exerted within the mucosal cell rather than in the gastric lumen.

Martin's hypothesis arises essentially as a consequence of the theory that the transfer of acids across the membranes of the mucosal cells is restricted largely, if not entirely, to the undissociated acid and therefore an intracellular accumulation of over 400-fold relative to the apical concentration could also be hypothesised with a drug of  $pK_a$  4.5 at a luminal pH of 3. This hypothesis requires that there is no mucus-bicarbonate layer to bring the apical surface pH up to 7 (Allen *et al.*, 1993), but is worthy of investigation because the mucus-bicarbonate barrier collapses at a luminal pH below 1.5 (Ross *et al.*, 1981) and NSAIDs also inhibit bicarbonate secretion (Wallace, 1997).

Martin's hypothesis which is referred to as 'ion-trapping', is often mentioned in reviews (e.g. Wallace, 1997, Hawkey, 2000), and also appears as a statement of fact in textbooks (Ghosh and Kinnear, 2003) however it lacks experimental proof since it is based on biophysical considerations which to this date have not satisfactorily been tested.

In 1970, Morris *et al.* (1970) studied the absorption and distribution of radiolabelled aspirin by placing x-ray plates in contact with mucosal and serosal surfaces of the stomach. This, approach, provided only limited information on mucosal distribution which requires study in the opposite (transverse) plane of the stomach wall. However, the first experimental evidence that the trapping of salicylate anions occurs in stomach cells after ingestion, as proposed by Martin, was obtained by Brune *et al.* (1976). This group used autoradiography to determine the distribution of [ $^{14}C$ ] salicylic acid in sections of rat mucosa and showed that there was a gradient of concentration of salicylate between the gastric surface cells and the base of the mucosa immediately after intragastric administration *in vivo*, thereafter, the concentration declined rapidly and had fallen to almost blood levels after about 45 minutes. These results agree well with those of Garner (1977), who studied the distribution and concentration of radiolabelled [ $^{14}C$ ] aspirin in the stomach wall of the guinea pig using autoradiography and showed that immediately after intragastric instillation of 20 mM aspirin



for 10 min, grain density in the upper region of the glandular mucosa was 35-fold greater than in the submucosa. In addition, Garner (1977) showed that at that time, the concentration in the stomach wall was about 40 times greater than in the blood but within 30 min the drug was evenly distributed throughout the stomach wall and that the concentration was similar to that in the blood. However, neither of these studies addressed the key prediction of Martin's hypothesis that the concentration in the apical cells will be much higher than that in the gastric lumen. Frey and El-Sayed (1977) attempted to selectively scrape off gastric surface cells after exposure to a variety of NSAIDs including aspirin and indomethacin *in vivo*, but with the exception of flufenamic acid were unable to show significant accumulation relative to the concentration of NSAID in the gastric lumen. This group suggested that NSAIDs caused disruption of the cell membranes through which the diffusion of the uncharged molecules is supposed to occur, such that a free diffusion of both ionised and the unionised drug will be possible and no accumulation in the mucosa in comparison to the lumen will consequently occur.

### **6.3 Introduction- Cell damage**

It is suggested that if an acid (pKa 3) is rapidly absorbed, the mucosal cell may suffer damage due to the accumulation of the anion. The dissociation of the acid may disturb the buffer system of the cell, while the accumulation of anion may interfere with metabolism (Garner, 1977). Trapping of large amounts of salicylate anions together with protons would occur in cells of the stomach mucosa during absorption. These molecules would then disturb the buffer system of the cell, interfere with mitochondrial functions and eventually lead to cell death. Others reported cellular effects of NSAIDs include disturbances in oxidative phosphorylation, active transport, transmembrane ion fluxes, and cell-to-cell adhesion (Brooks and Day, 1991).



## 6.4 Introduction- Non-steroidal anti-inflammatory drugs (NSAIDs)

The impact of NSAIDs on public health and the sensitivity that surrounds this issue have driven a search for safer but equally effective analgesic/anti-inflammatory drugs. NSAIDs exhibit a well-established action to induce gastrointestinal mucosal lesions, perforations and bleeding, which result in significant morbidity and even mortality in NSAID users (Allison *et al.*, 1992). Strategies to reduce the injurious effects of these drugs to the gastroduodenal region by means of enteric coatings have had limited success. Although it is clear that the GI side effects of NSAIDs are in part attributable to their ability to inhibit the biosynthesis of gastroprotective prostaglandins, a significant amount of evidence exists that NSAIDs act locally on the mucosa to induce GI ulcers and bleeding by a prostaglandin-independent mechanism (Wallace, 1997).

A chemical feature common to most NSAIDs is that they are functionally acidic, i.e. they have a carboxylic acid, ketoenolic or amide moiety, giving them a  $pK_a$  of 3 to 6. This is important for their propensity to accumulate in inflamed sites, where the pH (about 6 to 6.5) is often lower than that of normal tissues. Moreover, with the exception of salicylates (other than diflunisal) the NSAIDs have two or more aromatic groups, conferring upon them relatively marked lipophilic properties, which assist them to cross lipid membranes and accumulate at inflamed sites. This also accounts for their unique pharmacokinetics, of high protein binding and low volume of distribution (Rainsford, 1994). The main action of NSAIDs is the inhibition of arachidonate cyclooxygenases, as described originally by Vane (1971).

### 6.4.1 Cyclooxygenase (COX) enzymes

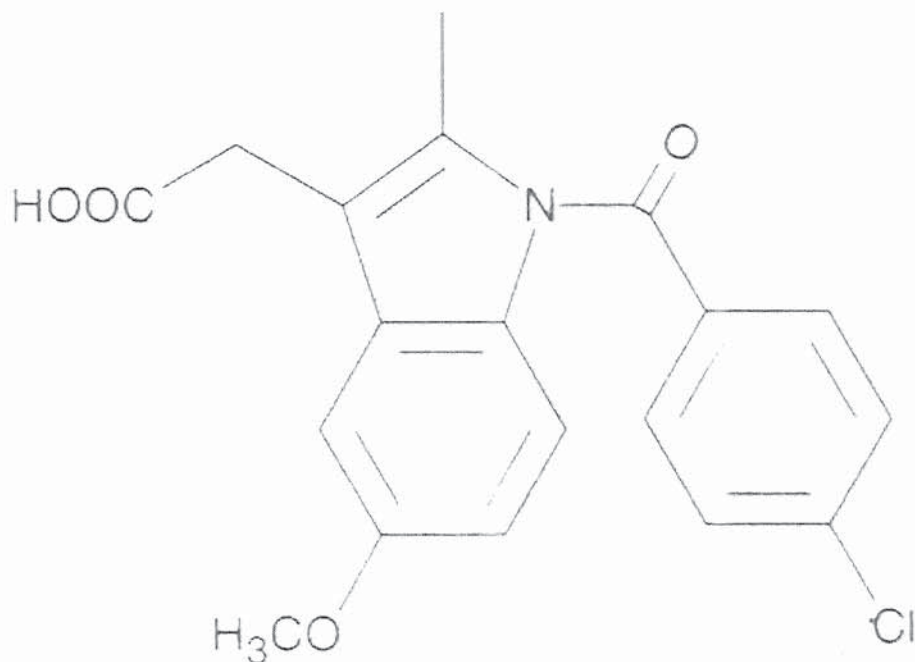
COX, an enzyme responsible for the synthesis of physiologically important prostaglandins, is known to play a key role in maintaining the integrity of gastric mucosa (Szabo, 1991). There are three forms of cyclooxygenase, COX-1 which is constitutively

expressed in most cell types including platelets, prostate and stomach cells, COX-2 which is induced by various stimuli (eg. growth factors, cytokines, lipopolysaccharides) (Labenz *et al.*, 1996), and COX-3 which is a splice variant of the COX-1 gene and which may be the target for paracetamol (acetaminophen) (Chandrasekharan *et al.*, 2002). Expression of the mitogen-inducible isoform COX-2 is enhanced in gastric epithelial cells after growth stimulation *in vitro* and in gastric epithelium after acid induced damage *in vitro* (Sawaoka *et al.*, 1997; Sun *et al.*, 2000). Although COX-2 can be induced during inflammation in the stomach, COX-1 seems to be the dominant functional source of prostaglandins in both *Helicobacter pylori*-negative and *Helicobacter pylori*-positive humans (Jackson *et al.*, 2000). COX-2 is very similar to COX-1 but it differs in certain key characteristics. Particularly it is a valine/leucine substitution at position 523 that creates a defect in the inner shell of the COX-2 enzyme channel to give a side pocket that can be assessed by drugs selective for COX-2. COX-2 inhibition is not instantaneous but requires a time-dependent conformational change in the molecule (Hawkey, 2000).

#### **6.4.2 Indomethacin**

Indomethacin 1-(p-chlorobenzoyl)-5-methoxy-2methylindole-3-acetic acid (Figure 6.4.2), is an NSAID with anti-pyretic and analgesic properties discovered and developed by the Merck Sharp and Dohme Research Laboratories in the 1960's (O'Brien, 1984). It is slightly more toxic but in some circumstances more effective than aspirin. In the laboratory, it is among the most potent of the inhibitors of prostaglandin synthesis. Indomethacin is not suggested for general use as an analgesic. It has been effectively used however, in the management of patients with moderate to severe rheumatoid arthritis, ankylosing spondylitis, pericarditis, osteoarthritis, tendonitis and acute gouty arthritis (Katzung and Furst, 1998). Indomethacin is also extensively used in obstetrics to delay uterine contractions and in the neonatal unit to facilitate patent ductus arteriosus closure in premature infants.





**Figure 6.4.2 Structure of Indomethacin**

Indomethacin was the NSAID chosen to test the hypothesis proposed by Martin (1963) because with a pKa of 4.5 (O'Brien, 1984), it would be largely unionised at pH 3.0 and essentially fully ionised at pH 7.0, and it is available as a [<sup>14</sup>C]-labelled compound. The choice of a suitable concentration is difficult because gastric luminal concentrations depend on dissolution, gastric emptying and whether the agent is taken with food or water. Furthermore, *in vivo* concentrations may also be significantly less than those attainable under *in vitro* conditions at pH 7.4 because NSAIDs, in acidic environments show low solubility. (Minta and Williams, 1985). Assuming that indomethacin is passively transferred, the concentration within the gastrointestinal tract must at some time be higher than the peak plasma concentration of 25  $\mu$ M (Alvan *et al.*, 1975; obtained after a standard dose of 100 mg). We therefore chose to expose gastric cells to half this concentration in apical media of different pH, but to also investigate lower concentrations at pH 3.0. Previous investigators



have reported that 100  $\mu\text{M}$  indomethacin is non injurious under *in vitro* conditions (Durbin *et al.*, 1991). Data from both human and rat experimentations suggest that serum indomethacin concentrations are frequently greater than 20  $\mu\text{M}$  (Daggan, 1972; Alvan, 1975).

## **6.5 Methods- Effect on gastric cells of the initial pH of the medium in the presence of indomethacin- Specific methodology**

HBSS containing [<sup>14</sup>C] indomethacin (12.25 μM, 0.225 μCi/ml), and [<sup>3</sup>H] mannitol (26 μM, 0.45 μCi/ml) was added to the apical compartment in experiments to examine the effect of the pH of the apical medium. In experiments involving variation in indomethacin concentration in an apical medium of pH 3.0, a solution of 7 μM [<sup>14</sup>C] indomethacin was serially diluted into HBSS to give concentrations of 0.7 and 0.07 μM. Unlabelled indomethacin was added to the 7 μM solution in an attempt to achieve a 70 μM concentration, but at pH 3.0 the solubility of indomethacin was exceeded with the medium becoming very slightly hazy. The basal medium in all experiments was complete culture medium in order to maximise buffering of the basal aspect of the cells. The Transwells were incubated on a gently tilting (1.5 cycles/min) platform in a hybridisation oven at 37 °C. Rapid agitation to minimise the potential impact of an aqueous boundary layer was not employed since the aim was to model conditions in the stomach *in vivo*. Transwells were moved to fresh basal medium at 5 min intervals for 30 min, which ensured that the transfer of indomethacin and mannitol from apical to basal was always under 'sink' conditions (ratio of basal to apical concentration <0.1). A sample of the apical medium was taken at 30 min to assess the apical concentration of indomethacin at this time. [<sup>3</sup>H] mannitol and [<sup>14</sup>C] indomethacin were assessed by scintillation counting (see section 2.9).

The equation:

$$\text{Transfer (pmol/min)} = \left( P_{\text{app AH}} \left( \frac{\text{Total conc of indomethacin ( } \mu\text{M)}}{10^{(\text{pH} - \text{pKa})} + 1} \right) + P_{\text{app A}^-} \left( \frac{\text{Total conc of indomethacin ( } \mu\text{M)} \times 10^{(\text{pH} - \text{pKa})}}{10^{(\text{pH} - \text{pKa})} + 1} \right) \right) \times 60 \times \text{Surface area} \times 1000$$

was used to calculate an apparent permeability for the ionised form of indomethacin at pH 7.4 , with the assumption that transfer at pH 3.0, when 97% of indomethacin (pKa =4.5) will be unionised, was due wholly to the unionised species. In this equation the rate of apical to basal transfer of indomethacin is related to the apparent permeability of indomethacin [ $P_{\text{app}}$ ], the area of the monolayer (A) and the initial concentration in the apical chamber ( $C_0$ ) by the equation:

$$\text{Transfer} = P_{\text{app}} \times A \times C_0$$

Total transfer is presumed to be made up of contributions from the unionised species, with apparent permeability  $P_{\text{app AH}}$ , and the anion, with apparent permeability  $P_{\text{app A}^-}$  and the Henderson-Hasselbalch equation is used to estimate concentrations of unionised species and anion as a function of pH.

### 6.5.1 Intracellular concentration of indomethacin

After 30 min of incubation the membrane was removed and rapidly washed in 2 x 50 ml of phosphate-buffered saline (0.01 M Phosphate, 0.0027 M KCl, 0.137 M NaCl) at 4°C. Membranes were cut from Transwells, incubated with 1.0 M NaOH for 30 min and then scintillant was added for assay of radiolabelled material. Tissue water was determined in parallel



incubations involving exposure of both surfaces of the Transwell to [<sup>14</sup>C] urea (17 μM, 1 μCi/ml) for 30 min. A correction was made for urea associated with the polycarbonate membrane in the absence of cells.

Non-specific binding of indomethacin to the membrane was measured by incubating Transwells for 30 min in complete culture medium (pH 7.4) using indomethacin concentrations from 1 to 100 μM. The justification for using complete culture medium at pH 7.4 in such binding experiments is that the monolayer will shield the Transwell from contact with HBSS and from the low apical pH when present. The linear relationship that was found between indomethacin concentration and non-specific binding to the membrane (see section 6.9) could be used to make a correction to experimental data, if the indomethacin concentration in the region of the membrane was estimated. There is likely to be a gradient of indomethacin concentration across the membrane from highest on the cell side to lowest on the side facing the basal medium. The concentration, which best described that to which the polycarbonate membrane was exposed in the presence of cells, was therefore taken to be the average of the intracellular concentration and that present in the basal medium at the end of the 25-30 min collection.

## 6.6 Results- Effect of reduction in the apical medium pH on the monolayer in the presence of indomethacin (12 $\mu$ M)

The pH of the apical media increased slightly, but nevertheless significantly, (statistical analysis by paired t test, n=5) during the 30 min of incubation from:  $7.4 \pm 0.03$  to  $7.6 \pm 0.02$ ,  $P < 0.05$ ;  $5.5 \pm 0.002$  to  $5.7 \pm 0.05$ ,  $P < 0.05$ ;  $4.5 \pm 0.001$  to  $4.8 \pm 0.02$   $P < 0.01$ ; and  $3.01 \pm 0.02$  to  $3.43 \pm 0.07$ ,  $P < 0.01$ . Trans-epithelial electrical resistance (TEER), after 30 min exposure of the apical surface of the monolayer to media of pH 5.5, 4.5 and 3, was significantly elevated relative to the final TEER of monolayers exposed to pH 7.4 (Figure 6.6.1).

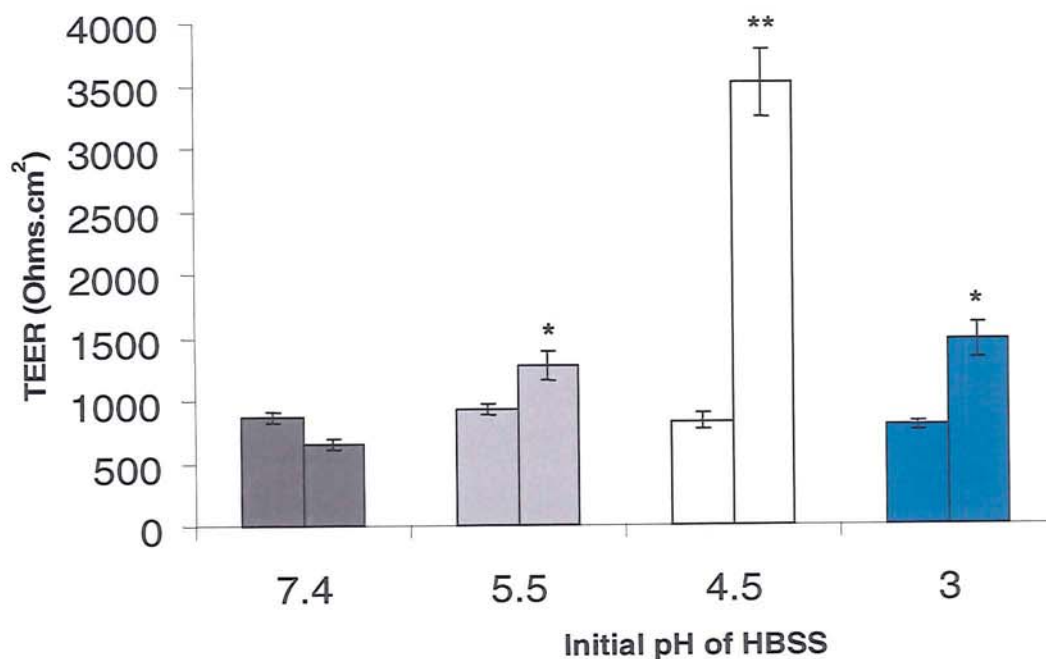
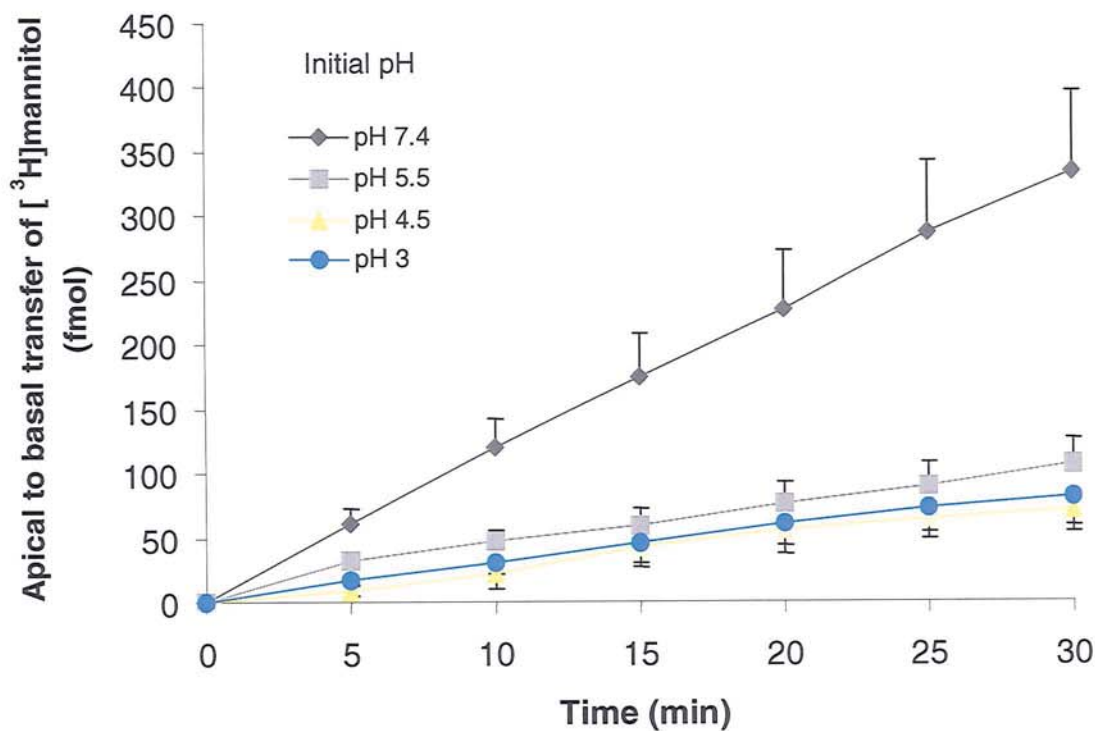


Figure 6.6.1 Effect of incubation for 30 min in the presence of apical media of different pH on the trans-epithelial electrical resistance (TEER) of monolayers.

Results are means  $\pm$  S.E.M. from five separate cultures with triplicate determinations within each experiment. Left-hand bars represent initial TEER and right-hand bars final TEER in culture medium before the change to HBSS of different pH. Results have been subjected to analysis of variance and Dunnett's test, \*  $P < 0.05$ , \*\*  $P < 0.01$  for difference of final TEER from final TEER at pH 7.4. The initial concentration of indomethacin in the apical medium was 12  $\mu$ M.

Transfer of mannitol from the apical to basal compartment was linearly related to time from 5-30 min whatever the initial pH of the apical medium (Figure 6.6.2). Apparent permeabilities ( $P_{app}$ ,  $\text{cm}\cdot\text{sec}^{-1}$ ) for mannitol transfer at pH 5.5, 4.5 and 3.0 were respectively ( $n=5$ , means  $\pm$  S.E.M.):  $4.17 \times 10^{-7} \pm 7.86 \times 10^{-8}$ ,  $3.26 \times 10^{-7} \pm 9.59 \times 10^{-8}$ ,  $3.25 \times 10^{-7} \pm 1.16 \times 10^{-7}$  and were lower ( $P<0.001$ ) than that at pH 7.4 which was  $1.59 \times 10^{-6} \pm 1.87 \times 10^{-7}$ .



**Figure 6.6.2** Effect of apical media pH on transfer of [ $^3\text{H}$ ] mannitol transfer over time in the presence of indomethacin from the apical to the basal compartment with apical media at different initial pH.

Results are means  $\pm$  S.E.M. of five different cultures with triplicate determinations within each culture.  $12 \mu\text{M}$  indomethacin was present in the apical medium in all incubations.



## 6.7 Results- Effect of pH on the transfer of indomethacin across the monolayer

When the initial apical medium pH was 5.5 or below, transfer of indomethacin into the basal compartment was non-linear with respect to time (Figure 6.7.1.1). This result probably reflected the greater reduction in the apical concentration of indomethacin over the period 0-30 min at low pH. Thus the percentage of apical indomethacin transferred into the basal compartment by 30 min was respectively (means  $\pm$  S.E.M., n=5)  $10.3 \pm 0.58$ ,  $35.1 \pm 1.9$ ,  $40.8 \pm 0.73$  and  $41.7 \pm 1.3\%$  at pH 7.4, 5.5, 4.5 and 3.0. The rate of transfer of indomethacin was consequently calculated between 5 and 15 min when an approximate linear relationship with time obtained (Figure 6.7.1.1).

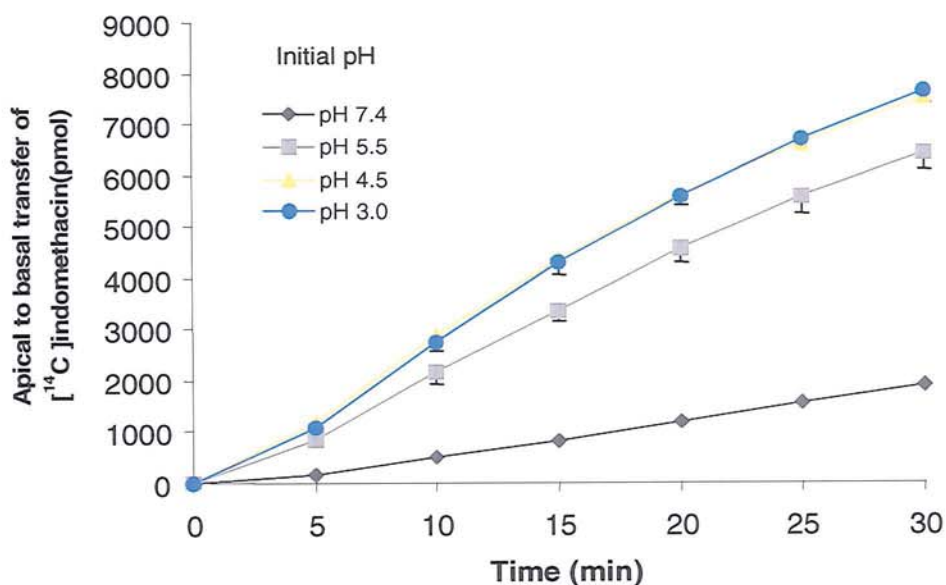
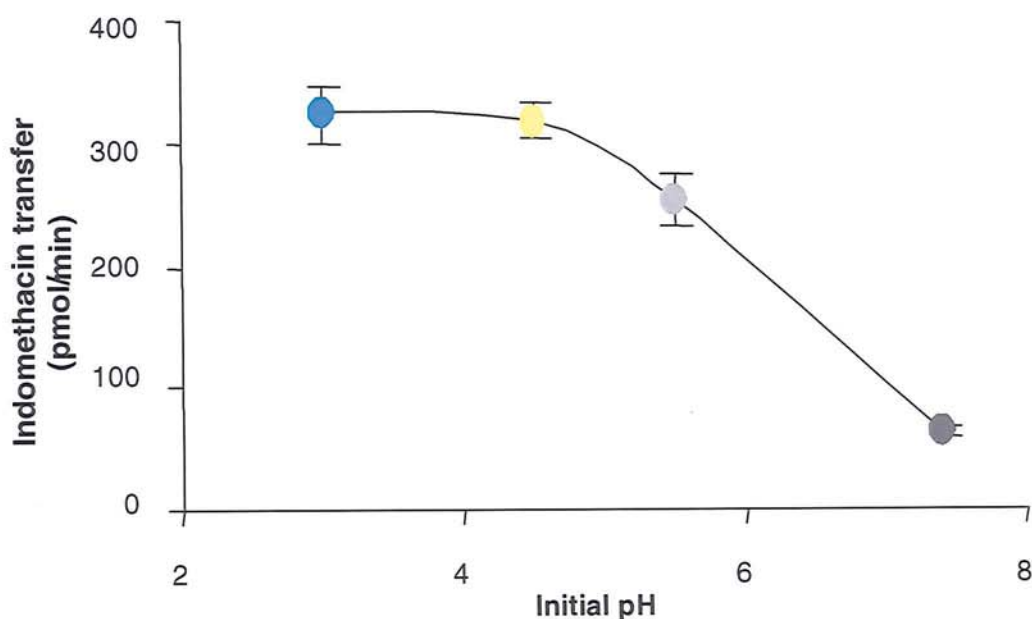


Figure 6.7.1.1 Effect of apical pH on [ $^{14}\text{C}$ ] indomethacin transfer over time from the apical to the basal compartment with apical media of different initial pH.

Results are means  $\pm$  S.E.M. of five different cultures with triplicate determinations within each culture. The initial concentration of indomethacin in the apical medium was  $12 \mu\text{M}$ .

The rate of transfer of indomethacin increased with decreasing pH (Figure 6.7.1.2). At pH 3.0, 96.9% of indomethacin ( $pK_a = 4.5$ ) will be unionised and the experimental permeability at pH 3.0 can be assumed to derive virtually entirely from movement of the undissociated acid ( $P_{app} = 9.62 \times 10^{-5} \text{ cm}\cdot\text{sec}^{-1}$ ) (Appendix 6.12). If the ionised form of indomethacin is assumed not to permeate the monolayer, then transfer at pH 7.4 can be calculated from the proportion of the unionised species at pH 7.4 (0.126%) to be 0.42 pmol/min. However, the actual experimental value at pH 7.4 was  $64 \pm 4$  pmol/min, which is significantly ( $P < 0.01$ , t-test) greater than that predicted. The simplest explanation is that the ionised form of indomethacin can cross cell membranes and in order to explain the experimental result at pH 7.4 it is necessary to postulate a  $P_{app}$  for the ionised species of  $1.85 \times 10^{-5} \text{ cm}\cdot\text{sec}^{-1}$  (Appendix 6.12).



**Figure 6.7.1.2** Effect of initial pH on the rate of transfer of indomethacin (12  $\mu\text{M}$ ) from the apical to the basal compartment.

Results are means  $\pm$  S.E.M. of five different cultures with triplicate determinations within each culture.

## 6.8 Results- Effect of concentration of indomethacin on apical to basal transfer

A log/ log plot indicated that indomethacin transfer was linearly related to concentration over two orders of magnitude.

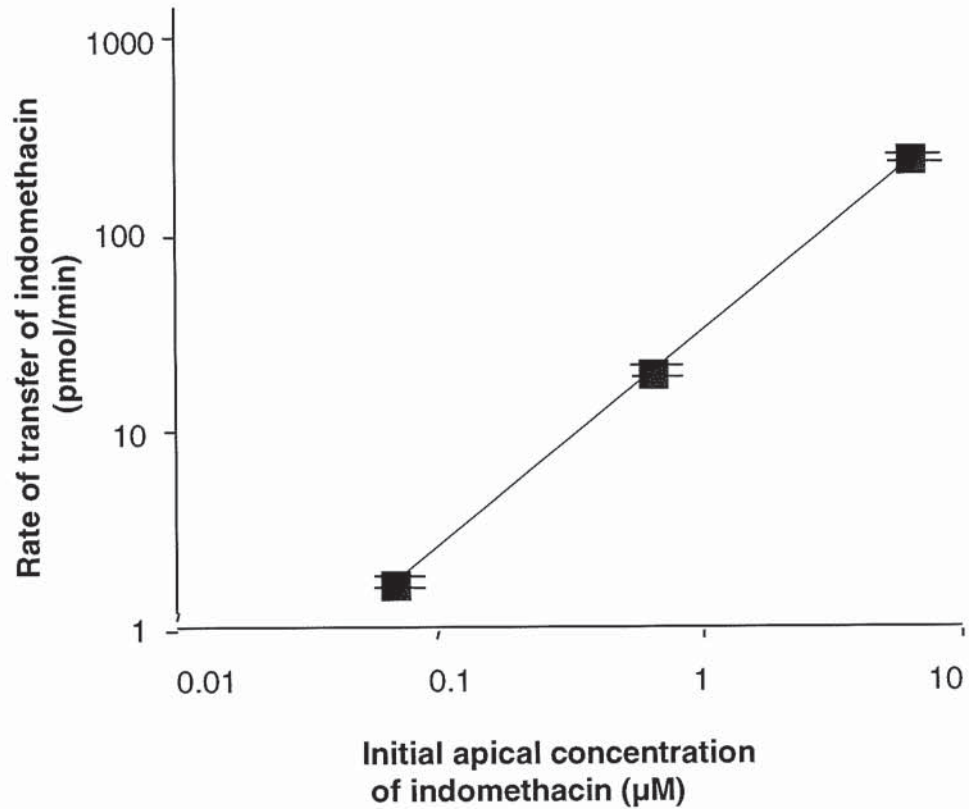


Figure 6.8 Effect of the concentration of indomethacin on the rate of transfer (5-15 min) of indomethacin from apical to basal with an initial pH of 3.0 in the apical medium.

Results are means  $\pm$  S.E.M. of five experiments with duplicate determinations within each experiment.



## 6.9 Results- Accumulation of indomethacin in gastric mucous epithelial cells

Indomethacin bound to collagen IV coated polycarbonate membranes. Binding of indomethacin was directly proportional to concentration over the range 1-100  $\mu\text{M}$  and was described by the equation:  $\text{pmol bound} = 3.1122 \times \text{concentration of indomethacin } (\mu\text{M})$  ( $r^2 = 0.9958$ ) (Figure 6.9.1)

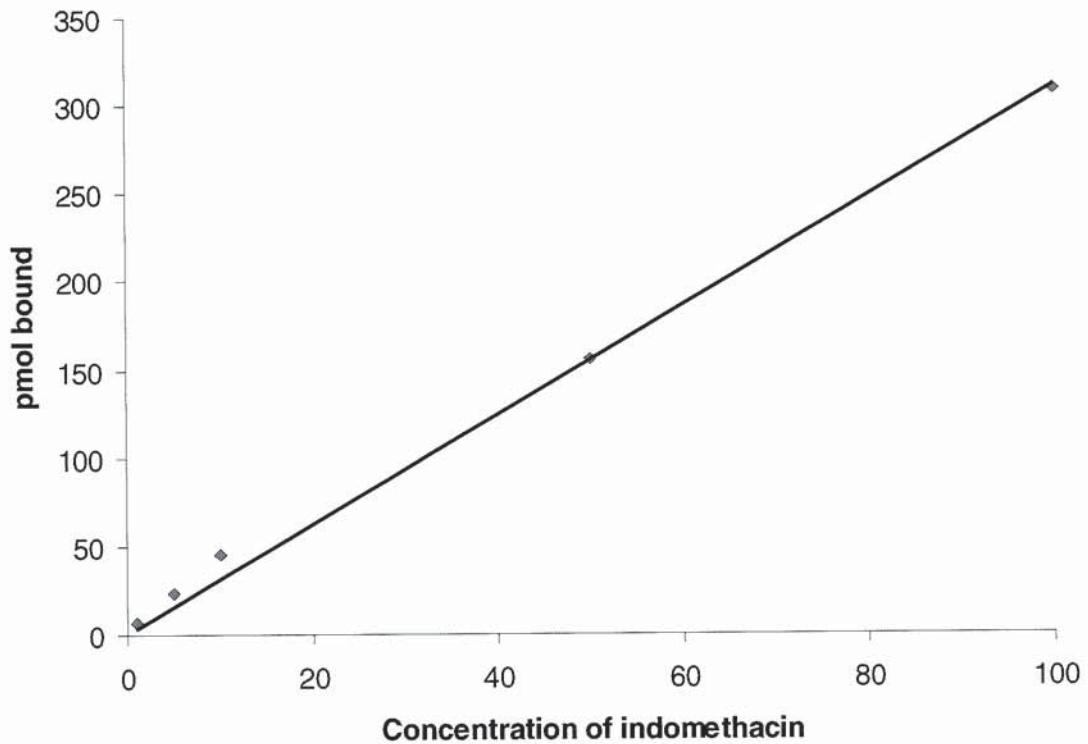
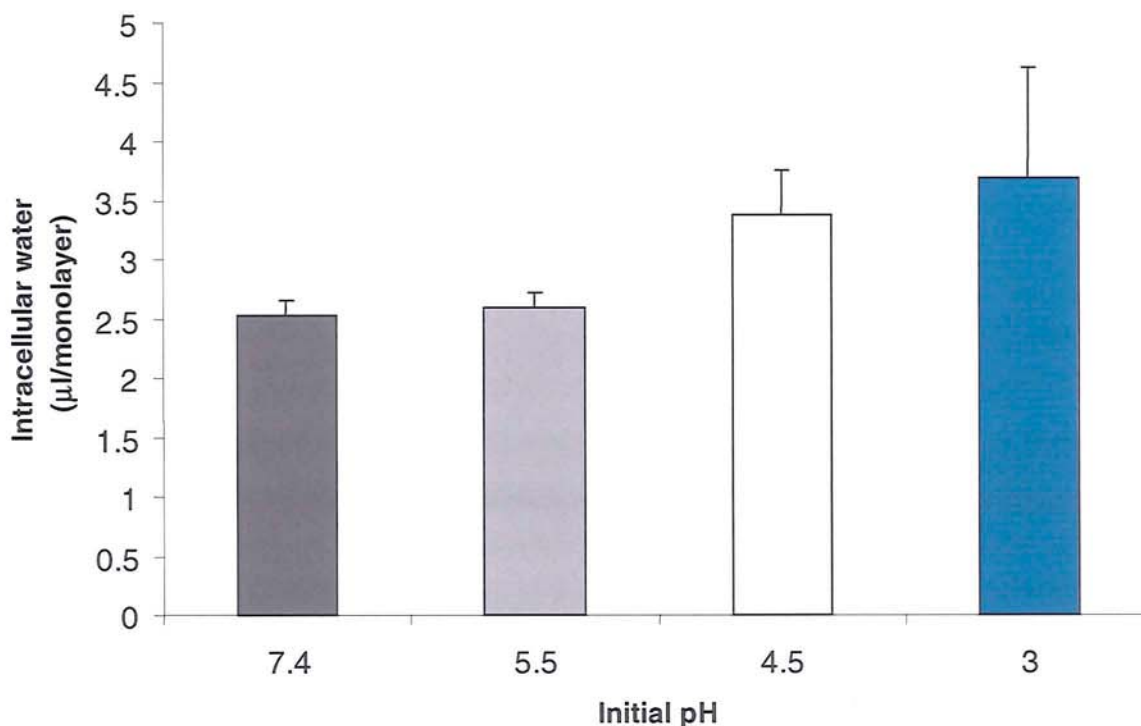


Figure 6.9.1 Effect of indomethacin concentration on binding to collagen IV coated polycarbonate membranes.

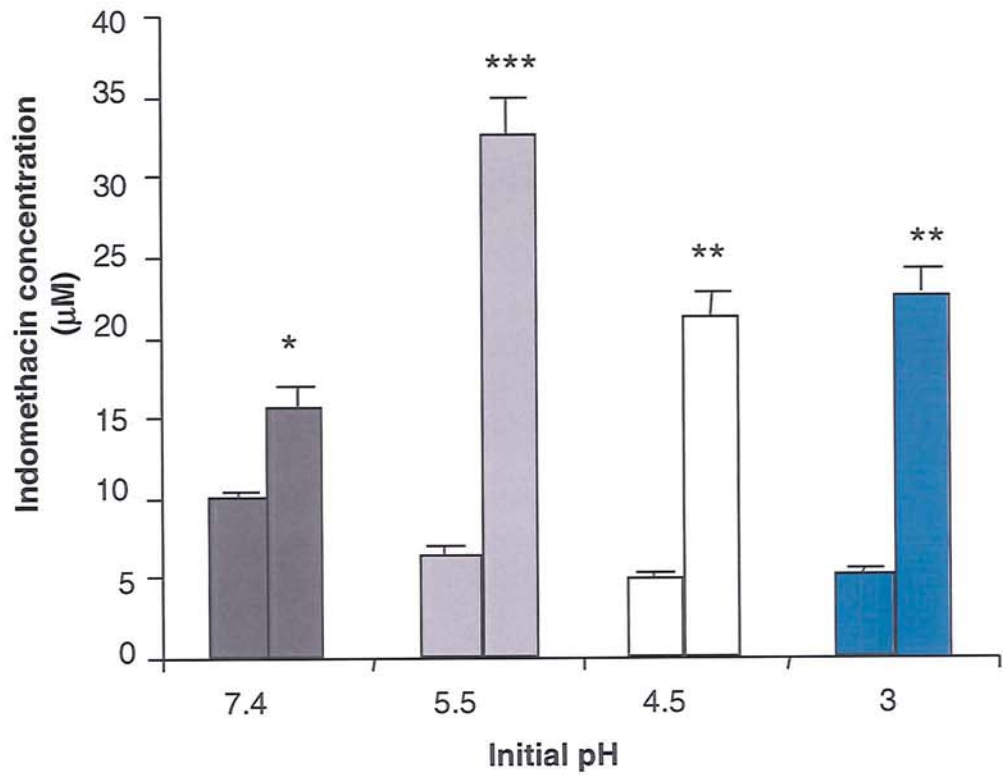
The data in Figures 6.9.2 and 6.9.3 were corrected for indomethacin binding to the membrane as described in the Methods. Cell water was estimated from the distribution of urea to be ( $\mu\text{l}$  per monolayer, means  $\pm$  S.E.M.,  $n=5$ ):  $2.53 \pm 0.13$ ,  $2.59 \pm 0.13$ ,  $3.37 \pm 0.39$  and  $3.69 \pm$

0.94 at pH 7.4, 5.5, 4.5 and 3.0 respectively (no effect of pH by analysis of variance) (Figure 6.9.2).



**Figure 6.9.2** Effect of the pH of the apical medium on intracellular water of gastric epithelial cell monolayers. Results are means  $\pm$  S.E.M. from five separate cultures. The indomethacin concentration in the apical medium was 12  $\mu$ M.

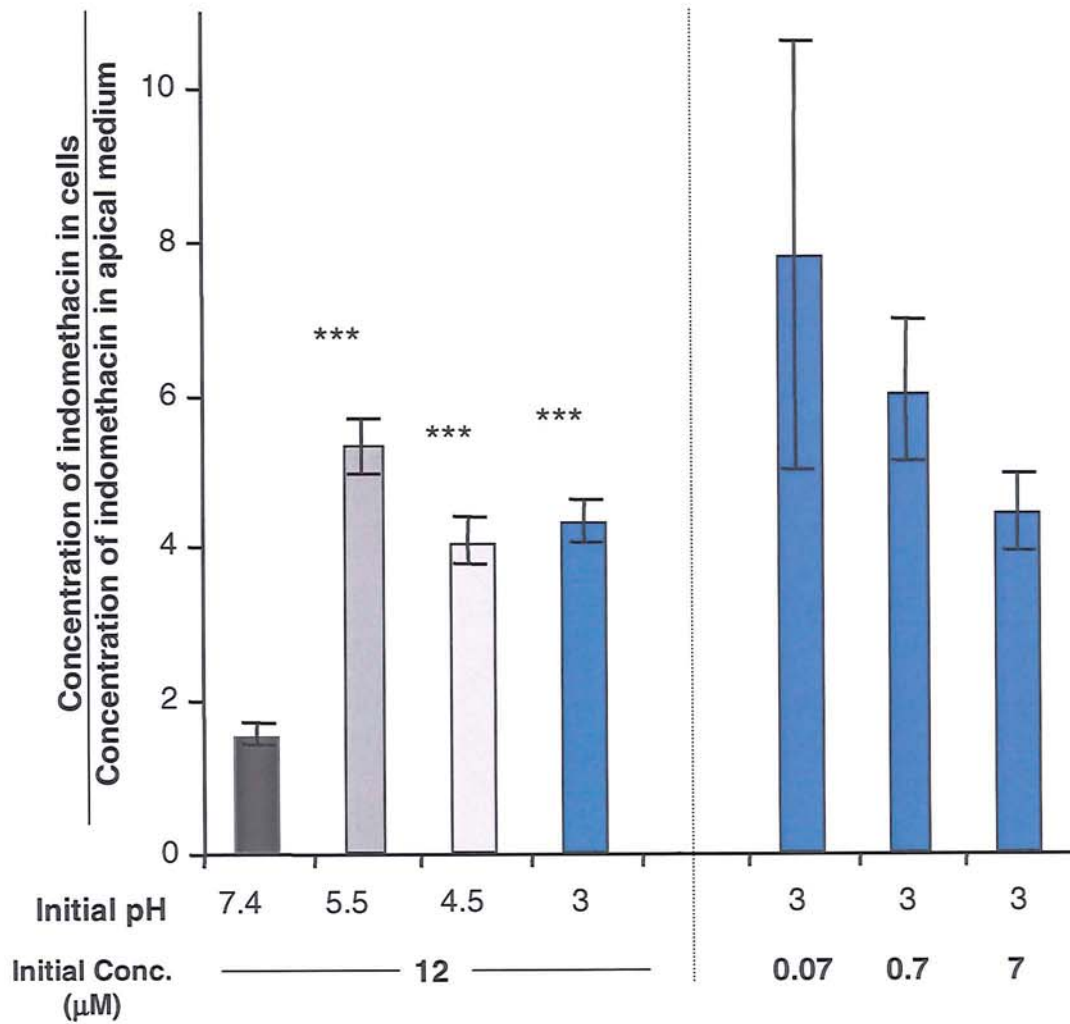
At pH 7.4 cell water was  $6.91 \pm 0.36$   $\mu$ l per mg of protein which is reasonably close to the 7.8  $\mu$ l per mg of protein of human tracheal epithelial cells (Liedtke and Cole, 2002). Intracellular indomethacin (pmol) was:  $40 \pm 2.9$ ,  $85 \pm 5.6$ ,  $72 \pm 5.5$  and  $84 \pm 5.7$  at pH 7.4, 5.5, 4.5 and 3.0 respectively. Indomethacin concentration in the cells was greater than that in the apical medium at pH 7.4, 5.5, 4.5 and 3.0 (Figure 6.9.3), with the ratio of indomethacin concentration in the cell to that in the medium (accumulation ratio) at pH 5.5, 4.5 and 3.0 being much higher than at pH 7.4 (Figure 6.9.4). There was no effect of indomethacin concentration on the accumulation of indomethacin at pH 3.0 (Figure 6.9.4).



**Figure 6.9.3** Effect of pH of the apical medium on the initial concentration of indomethacin inside the gastric epithelial cells, and in the apical medium, after 30 min of incubation.

Right-hand columns represent indomethacin concentration inside the gastric epithelial cells and left-hand columns represent indomethacin concentration in the apical medium. Results are means  $\pm$  S.E.M. from five separate cultures. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  and \*\*\*,  $P < 0.001$  for the comparison between intracellular and apical indomethacin concentration by paired t test.





**Figure 6.9.4** Effect of the pH of the apical medium, and the concentration of indomethacin in the apical medium, on the accumulation of indomethacin in gastric mucous epithelial cells.

Accumulation is expressed as the ratio of the intracellular concentration to that in the medium at the end of the 30 min incubation period. Results are means  $\pm$  S.E.M. of five separate cultures for both the effects of pH and the effects of concentration. \*\*\*,  $P < 0.001$  for a difference for results in the left-hand panel from the result at pH 7.4 by analysis of variance and a Newman-Keuls test. There was no significant effect of indomethacin concentration on the accumulation of indomethacin at pH 3.0.

## 6.10 Discussion

This study showed clearly for the first time that indomethacin accumulated in gastric epithelial cells at low apical medium pH. Furthermore it demonstrated that both undissociated and ionised forms of indomethacin could permeate through the apical membrane of gastric surface epithelial cells.

This interpretation requires that the monolayer was not disrupted by acidification of the apical medium or by the indomethacin. Indeed, as demonstrated in chapter 5, gastric epithelial monolayers grown on Transwells coated with collagen type IV are able to withstand transient apical acidification to pH 3, with a decrease in [<sup>3</sup>H] mannitol flux and an increase in TEER. Such changes are a reflection of a tighter monolayer relative to pH 7.4. By contrast Caco-2 cell monolayers exhibit increased permeability of mannitol when the pH is reduced to 4.5 (Palm *et al.*, 1999). Furthermore, the decrease in [<sup>3</sup>H] mannitol transfer with decreasing pH also occurred in the presence of indomethacin (12 µM).

Most importantly it is not possible to ascribe the increased transfer of indomethacin with reduced apical pH to an opening of tight junctions consequent on damage to the monolayer. The increase in final TEER relative to pH 7.4 with apical acidification also supports an increased resistance of tight junctions on exposure to acid, although the large response at pH 4.5 does not correlate with mannitol movement and might involve an additional effect of the acetate buffer on current flow (Rutten *et al.*, 1985).

By contrast with skin (Watkinson *et al.*, 1993) the plot of NSAID transfer against pH shows significant deviations from a theoretical relationship based on changes in the concentration of a permeant, undissociated acid with pH, and with negligible permeation of the anion. If transfer at pH 3.0 is ascribed entirely to movement of the undissociated acid then an apparent

permeability can be calculated for the anion at pH 7.4 which is five times lower than that of the acid. (see Appendix 6.12).

Whether or not a significant permeability of the monolayer to the anion is assumed the plot of indomethacin movement against pH should 'pivot' around pH 4.5 (the pKa for indomethacin). However the experimental data are shifted to the right. An aqueous boundary layer might produce such a shift (Wohnsland and Faller, 2001). The unstirred water layer is an aqueous film at the membrane interface where solute movement is purely diffusion controlled. The unstirred water layer makes up a considerable proportion of the total resistance to transfer of highly permeable compounds through membranes (Wohnsland and Faller, 2001), so that effects of changes in the proportion of ionised species are not seen until they become more extreme. However bicarbonate moving from basal to apical would be expected to accumulate in such a layer and by elevating apical pH actually shift the experimental curve to the left of the theoretical one. Furthermore we have been unable to detect a layer of mucus overlying the monolayer when Transwell membranes are mounted transversely and viewed through an inverted microscope under phase contrast. Addition of  $10^{-5}$  M indomethacin on guinea pig gastric epithelial cell monolayers had no effect on mucin synthesis in comparison to control (Takao *et al.*, 1993) although it might have affected mucin secretion. An alternative explanation is in the environment of the membrane the pKa of indomethacin is higher than 4.5. There was no evidence that transfer of indomethacin was carrier-mediated since movement from apical to basal was linearly related to concentration over two orders of magnitude.

Calculation of the intracellular concentration of indomethacin required a correction for binding of indomethacin to the polycarbonate membrane. Since the basal medium is changed every 5 min there will almost certainly be a gradient of indomethacin concentration across the membrane with a higher concentration on the side next to the cells. In the present study the



assumption was made that indomethacin binding to the membrane will be best estimated by taking a concentration which is the average of the cell concentration and that in the basal medium at 30 min. This may be an over-correction because the presence of cells may reduce the availability of non-specific binding sites for indomethacin on the polycarbonate membranes. The correction does not however substantially alter the level of accumulation. Thus if no correction at all is made for binding of indomethacin to the membrane the accumulation ratio at pH 5.5 increases from a corrected result of  $5.3 \pm 0.4$  to  $8.6 \pm 0.6$ . The lack of higher accumulation at pH of 4.5 or 3.0 in the apical medium, relative to 5.5, seems to reflect the small changes in transfer of indomethacin over this pH range, and the tendency, albeit not significant, for tissue water to increase with reducing apical pH. It is important to note that the decreasing solubility of indomethacin at pH 3.0 and below will limit the intracellular concentrations obtainable *in vivo*.

In human subjects a 50 mg dose of indomethacin caused acute gastroduodenal damage in 100% cases, with maximal damage 24 hours after administration. The damage includes ulceration, bleeding, perforation, and stricture formation, complications that may be life threatening. However mucosal damage was more marked in the stomach than in the duodenum. It has been shown that with continued intake the damage resolves through mucosal adaptation. The mechanisms though which the mucosa adapts remain unknown (Shorrock *et al.*, 1990). Despite the accumulation of indomethacin within the cells, even at pH 3.0 there was no evidence from permeability of mannitol, or from TEER, that indomethacin caused cell damage over the time-scale of the experiment. This is fortunate since it enabled an accurate assessment of routes of transfer of indomethacin across the gastric epithelial cell layer. However, longer exposure to indomethacin might well have caused damage. For example rabbit gastric mucosal epithelial cells grown on plastic, and exposed to indomethacin concentrations of 50  $\mu\text{M}$  and above for 4 h,

showed reduced viability in the 3-(4,5-dimethyl-2-thiazoyl)-diphenyl-2H-tetrazolium bromide (MTT) assay (Takahashi and Okabe, 1996). Furthermore, it has been shown that indomethacin inhibited PGE<sub>2</sub> production by monolayers of primary rat gastric mucous cells (Ota *et al.*, 1988). Previous studies have demonstrated that PGE<sub>2</sub> stimulates increases in intracellular Ca<sup>2+</sup> and cyclic AMP and also promotes sealing of tight junctions (Palant *et al.*, 1983), but no such effects of PGE<sub>2</sub> could be inferred here as indomethacin did not affect indicators of tight-junctional status. Kokoska *et al.* (1998) suggest that indomethacin predisposes human gastric cells to injury by disturbing intracellular Ca<sup>2+</sup> homeostasis through mechanisms independent to prostaglandin synthesis. Such effects might have been seen with the present preparation had exposure been for a longer period. As mentioned in previous chapter it has been proposed that Ca<sup>2+</sup> homeostasis is critical in maintaining mucosal integrity (Tepperman *et al.*, 1991) and that this cation plays a major role in promoting mucosal injury induced by a variety of noxious agents (Tripp and Tepperman, 1996).

The inhibition of prostaglandin synthesis and the breaking of the mucosal barrier are not synonymous. Salicylic acid breaks the mucosal barrier however without inhibiting prostaglandin synthesis. The mechanism behind this prostaglandin-independent process has not been fully elucidated and triggers among other mechanisms the dissipation of the mucus bicarbonate barrier, an effect on paracellular pathways, epithelial cell toxicity and metabolic changes occurring because of high mucosal concentrations. Furthermore, in order for the mucosal barrier to be maintained, sufficient mucosal perfusion must be supplied, a process that can be compromised by the inhibition of prostaglandin synthesis *in vivo*. Thus, although it has been suggested that there are two routes to mucosal injury, topical toxicity and inhibition of prostaglandin synthesis, it has not been proven whether all NSAIDs possess topical toxicity which is independent to prostaglandin synthesis (Hawkey, 2000). High mucosal concentrations

might not only enhance the ability of NSAIDs to inhibit prostaglandin synthesis but could also have a direct effect on enzyme activity, uncoupling of oxidative phosphorylation, and inhibition of fatty acid metabolism-properties that have been demonstrated at high NSAID concentrations. (Hawkey, 2000). However, within the short period of the present experiment evidence for topical or other damage by NSAIDs was absent.

### **6.11 Conclusion**

In conclusion, this study represents the first direct demonstration that an NSAID accumulates in gastric epithelial cells exposed to low apical pH. The results strongly suggested that both undissociated and anionic forms of indomethacin were able to pass across membranes of gastric surface epithelial cells.



**Chapter 7**  
**General Discussion**

## 7.1 General discussion and conclusions

The purpose of this study was to produce a model of the surface gastric epithelium suitable for screening of new chemical entities and dosage forms. If this model were to represent fully the gastric epithelial surface it should function as a permeability barrier resistant to apical acidification, and it should retain the differentiated functions of surface epithelial cells. Furthermore, the cells should show evidence of polarity with a clear distinction between apical and basal surfaces.

In order to assess the functioning of the model as a permeability barrier it was necessary to grow cells on filters. The most successful approach was to use Transwell inserts with polycarbonate filters of 0.4  $\mu\text{m}$  pore size coated with collagen IV. The current preparation is to our knowledge the first primary culture of surface gastric epithelial cells, that have been fully characterised with respect to permeability, differentiation and polarity. The proportion of mucous cells, which stained positively for mucin with periodic acid reagent, was greater than 95 %. Tight junction formation of cells grown on Transwells for three days, was assessed by measurement of trans-epithelial electrical resistance (TEER) and permeability of mannitol and fluorescein. TEER for cells grown on Transwells with collagen IV was close to that obtained with intact guinea-pig gastric epithelium *in vitro*, and apparent permeabilities for mannitol and fluorescein were only slightly higher than those for Caco-2 cell monolayers, which are known to represent an intestinal-like epithelium.

The monolayers grown on Transwells coated with collagen IV, exhibited many mucin granules with a predominantly supra-nuclear location, and mucin was secreted only across the apical membrane. Nox-1 associated production of superoxide has been established in gastric surface cells (Teshima *et al.*, 1998; Rokutan *et al.*, 1999). This feature of differentiation was retained in the present preparation and we showed for the first time more superoxide was released in the apical than the basal direction.

A key feature of the present preparation is that it is the first primary culture of gastric surface epithelial cells where a sensitivity and resistance to acid conditions similar to that of intact gastric mucosa *in vitro* (Spencer *et al.*, 1992), has been established.

The present preparation represents a major step towards modeling the gastric epithelial surface in a manner suitable for screening drugs and dosage forms for their effects on the integrity of the gastric barrier. It should prove useful to examine transfer into and across the gastric epithelium, and to screen for the effects of drugs and dosage forms on cellular viability and on gastric paracellular permeability. Furthermore its resistance to acid will enable examination of the effect of pH on drug uptake as evidenced by the study of indomethacin transfer and accumulation undertaken in this thesis. Additional applications would be the examination of the effects and transfer of antibiotics or omeprazole in the presence of *Helicobacter pylori*, or to use it as restitution model.

With the exception of the relatively poorly characterised human cell preparation of Rutten *et al.* (1999), this is the first primary culture of surface gastric epithelium from any species that has been grown on Transwells. The advantage of using guinea pigs is the easy digestibility and ready availability of the tissue, and the potential to scale up the



preparation to perform large-scale screens. Although there was insufficient time during this thesis to perform a pilot screen of a group of compounds, this has subsequently been successfully performed for 10 non-steroidal anti inflammatory drugs. The major disadvantage of using guinea pigs is the possibility of species differences from man influencing and/ or reducing the relevance of the data obtained with the model.

In conclusion the major goals of this project have been successfully met, creating a very promising fully characterised gastric epithelium model with numerous future potential applications.

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## 2.10 Appendix

Abbreviation	Meaning
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CaCl <sub>2</sub>	Calcium chloride
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
FCS	Foetal calf serum
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HCO <sub>3</sub> <sup>-</sup>	Bicarbonate
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NSAID	Non steroidal anti-inflammatory drugs
P <sub>app</sub>	Apparent permeability
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor
PAGE	Polyacrylamide gel electrophoresis
PLA	Poly (L-lactide)
PLGA	Poly (L-lactide co-glycolide)

SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TCP	Tissue culture plastic
TEER	Trans-epithelial electrical resistance
TEMED	1 N, N, N', N'- Tetramethylethylenediamine

<b>Reagent</b>	<b>Supplier</b>
[2- <sup>14</sup> C] indomethacin	Amersham Pharmacia
Acrylamide/N, N'-bisacrylamide	BDH
Activated charcoal	Sigma
Ammonium Persulphate	BDH
Amphotericin B	Gibco
Anti rabbit IgG-peroxidase	Amersham Pharmacia
BCA protein assay reagent kit	Pierce
Bromophenol blue	Biorad
BSA-fraction V	ICN
Calcium chloride (CaCl <sub>2</sub> )	Sigma
Chloroform	Fisher
Collagen Type I from Rat Tail	Sigma



Collagen IV from human placenta	Sigma
Collagenase	Sigma
Crystal Violet	Sigma
Cytochrome C	Sigma
D-[6- <sup>3</sup> H] glucosamine hydrochloride	Amersham Pharmacia
D-[1- <sup>3</sup> H(N)] mannitol	Amersham Pharmacia
D-[1- <sup>14</sup> C]mannitol	Perkin Elmer
DABCO	Sigma
Defatted powdered milk	Marvel®
Developer (Kodak)	Sigma
Diazepam	May and Baker
Dimethylchloride	Fisher
DMSO	Sigma
DTT	Sigma
DPX mounting agent	Sigma
ECL Detection Reagents	Amersham Pharmacia
EGF	Sigma
FITC-conjugated anti-rabbit IgG	Amersham Pharmacia
Fixer (Kodak)	Sigma
Fluomount	Sigma
Fluorescein	Sigma
FCS (F2442)	Sigma
Glacial acetic acid	BDH

Glutaraldehyde	BDH
Glycerol	Sigma
Glycine	BDH
HBSS	Sigma
HCl	BDH
Hoechst 33258 (bisbenzimidide)	Amersham Pharmacia
Indomethacin	Sigma
Mouse laminin	Sigma
NaOH	Sigma
Optiphase HiSafe 3 scintillation fluid	Wallac
Rabbit anti-p67 phox	gift from Dr. B.M. Babior, Scripps Research Institute, La Jolla
Paraformaldehyde	Sigma
PBS	Sigma
Penicillin	Gibco
Percoll	Sigma
Phosphotungstic acid	Sigma
Pronase E	Merck
Rabbit anti-rat gastric mucin antibody	Peter Hanson
Rainbow markers	Amersham Pharmacia
RPMI 1640	Gibco
SDS	Sigma
Sodium metabisulphite	Sigma

Sodium pentobarbitone (hypnorm)	May and Baker
Soybean trypsin inhibitor	Sigma
Streptomycin	Gibco
Superoxide dismutase	Sigma
TCA	Sigma
TEMED	BDH
Transwells	Costar
Tris	BDH
Triton X-100	Sigma
Trypsin/EDTA	Sigma
Tween 20	Sigma



## 6.12 Appendix (with acknowledgement to Dr. P. J. Hanson)

$$\begin{aligned} \text{Transfer (pmol/min)} &= \\ &(\text{Papp}_{\text{AH}} \left( \frac{\text{Total conc of indomethacin (}\mu\text{M)}}{10^{(\text{pH}-\text{pKa})} + 1} \right) \\ &+ \text{Papp}_{\text{A}^-} \left( \frac{\text{Total conc of indomethacin (}\mu\text{M)} \times 10^{(\text{pH}-\text{pKa})}}{10^{(\text{pH}-\text{pKa})} + 1} \right)) \times 60 \times \text{Surface area} \times 1000 \end{aligned}$$

(i) At pH 3.0 if we assume that transfer 322.38 pmol/min is entirely due to the undissociated acid (pKa indomethacin = 4.5):

$$322.38 = \text{Papp}_{\text{AH}} \frac{12.25}{(10^{-1.5} + 1)} \times 60 \times 4.7 \times 1000$$

which gives  $\text{Papp}_{\text{AH}} = 9.62 \times 10^{-5} \text{ cm}\cdot\text{sec}^{-1}$

(ii) At pH 7.0 we get a calculated transfer of AH as follows:

$$\text{Transfer} = 9.62 \times 10^{-5} \times \left( \frac{12.25}{(10^{2.9} + 1)} \right) \times 60 \times 4.7 \times 1000$$

Transfer = 0.42 pmol/min

(iii) Actual transfer is 64.29 pmol/min

If a significant permeability of  $\text{A}^-$  is postulated  $\text{Papp}_{\text{A}^-}$  can be calculated as follows:

$$64.29 = 0.42 + (\text{Papp}_{\text{A}^-} \times \left( \frac{12.25 \times 10^{2.9}}{(10^{2.9} + 1)} \right) \times 60 \times 4.7 \times 1000)$$

Which gives  $\text{Papp}_{\text{A}^-} = 1.85 \times 10^{-5} \text{ cm}\cdot\text{sec}^{-1}$

## 2.10 Appendix

<b>Abbreviation</b>	<b>Meaning</b>
BCA	Bicinchoninic acid
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## Awards

- **Best Poster Award**  
**8<sup>th</sup> Society of Biomolecular Screening (SBS) Conference, The Hague, The Netherlands.** September 2002 (please refer below).
- **Best Oral Presentation Award**  
**Postgraduate Research symposium, School of Life & Health Sciences, Aston University, UK.** October 2002 (please refer below).

## Publications

The authors of all the listed publications are as follows:

Kavvada, K., Coombes, A., Murray, J., Moore, V. and Hanson, P.

- **Annual PhD Review Day for students sponsored by AstraZeneca R&D Charnwood.** October 2001, Poster Presentation.

Modelling the gastric epithelium using a primary culture of guinea-pig gastric mucous cells

- **8<sup>th</sup> United Kingdom and Ireland Controlled Release Society (UKICRS) Conference, AstraZeneca R&D Charnwood, UK.** January 2002, Poster Presentation.

Modelling the gastric epithelium using a primary culture of guinea-pig gastric mucous cells.

- **British Pharmaceutical Conference (BPC), Manchester International Convention Center, UK.** September 2002, Poster Presentation

Monolayer formation by guinea-pig gastric epithelial cells grown on polycarbonate membranes.

- **8<sup>th</sup> Society of Biomolecular Screening (SBS) Conference, The Hague, The Netherlands.** September 2002, Poster Presentation

Guinea-pig gastric epithelial cells grown on polycarbonate membranes as a model for the gastric epithelium.

- **Postgraduate Research symposium, School of Life Sciences & Health Sciences, Aston University, UK.** October 2002, Oral Presentation.

Modelling the gastric epithelium for testing new entities.

- **Annual PhD Review Day for students sponsored by AstraZeneca R&D Charnwood.** November 2002, Poster Presentation.

Modelling the gastric epithelium for biopharmaceutical screening: collagen IV is superior to collagen I in promoting tight junction formation.

- **American Association of Pharmaceutical Sciences (AAPS) Conference, Salt Lake City, USA.** October 2003, Poster Presentation.

Guinea-pig gastric epithelial cell monolayers grown on polycarbonate membranes are a good model of the stomach surface for biopharmaceutical screening.



- **Society of Biomolecular Screening (SBS) and Sebiot, Madrid, Spain.** November 2003, Poster Presentation.

Characterization of a primary culture model of the stomach surface suitable for pharmaceutical screening.

- **Annual PhD Review Day for students sponsored by AstraZeneca R&D Charnwood.** November 2003, Oral Presentation

Guinea-pig gastric epithelial cell monolayers grown on polycarbonate membranes are a good model of the stomach surface for biopharmaceutical screening.

- **In press (Journal of Biomolecular Screening)**

A collagen IV matrix is required for guinea-pig gastric epithelial cell monolayers to provide an optimal model of the stomach surface for biopharmaceutical screening.

- **In press**

Accumulation of indomethacin in cultured gastric surface epithelial cells exposed to low apical pH.

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