THE ISOLATION, PURIFICATION, TISSUE LOCALIZATION AND IDENTIFICATION OF A GLYCOPROTEIN FOUND IN THE CRUDE MUCUS GEL OF PATIENTS WITH CARCINOMA OF THE STOMACH

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THIS THESIS IS DEDICATED WITH LOVE TO THE MEMORY OF MY LATE MOTHER – HILDA CHIRWA

PUBLICATIONS

Published peer reviewed abstracts related to this work:

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AWARDS

This study won two major awards:

1. The Bunny Angorn Prize for the best presentation at the South African Surgical Research Society (SRS), held at Bloemfontein, South Africa, 27 to 28 July 2007. The candidate was then able to present his work at the Society of Academic and Research Surgery (SARS), Birmingham, UK, 9 to 11 January 2008.
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2. The Roc Kaschula Award for the best presentation by a young researcher at the International Academy of Pathology (South African Division), 19 to 21 July 2008, Cape Town, South Africa.
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PREFACE

This study was carried out from February 2006 to September 2008 under the supervision of Professor Anwar Suleman Mall, in the Department of Surgery, Faculty of Health Sciences, University of Cape Town, South Africa.

These studies represent the original work by the author and have not been submitted in any other form to another University. Where use has been made of the work of others, it has been duly acknowledged in the text.

Nthato Chirwa	Prof. Anwar Suleman Mall
September 2008	September 2008

ABSTRACT

The thin layer of crude mucus lining the human gastric mucosa protects the delicate gastric epithelium from the high shear forces associated with digestion. Gastric mucus is composed largely of water (>90%) and a complex mixture of organic components such as enzymes, various serum and cellular macromolecules, sloughed cells, bactericidal proteins, plasma proteins, inorganic ions and very importantly the mucins, that impart to it its gel-forming properties. Mucins are large heterogenous polymers that are difficult to characterize by traditional biochemical methods. However mucins from different regions of the body do share common features such as a low protein content of approximately 20% by weight and a carbohydrate content of 70 to 80% by weight. Mucins are characterized by a variable number of tandem repeat regions rich in serine, threonine and proline with the serine and threonine being potential sites for O-glycosylation. In contrast to the glycosylated region is the 'naked' region rich in cysteine and susceptible to proteolysis. The cysteine residues enable mucin monomers to form polymers by disulphide bridges.

Alterations of mucin expression take place in gastric carcinomas. Our laboratory previously reported the presence of a 40-50 kDa glycoprotein in the mucus of patients with gastric cancer that associated with albumin. The primary aims of our study were to develop an antibody to this 40-50 kDa glycoprotein, and to determine the location of its expression in gastric tissue by the immunohistochemical method, ranging from normal, to premalignant to cancer. The final aim was to identify this unknown glycoprotein by proteomic analysis. The reactivity of the antibody to the mucin and its 40-50 kDa component was determined by Western Blotting.

We collected gastric mucus scrapings by gentle scraping of the mucosa with a sterile glass slide from 27 gastrectomy specimens resected for carcinoma of the stomach. Mucus samples from each patient were collected within 20 min after surgical resection, on ice. Mucins were extracted from the crude mucus gel scrapings in 6 M guanidinium chloride in a cocktail of proteinase inhibitors and purified by density gradient ultra-centrifugation in CsCl/4 M guanidinium chloride, for 48 h at 105 000 x g, twice. Purified mucin was dialysed against distilled water and its purity assessed by

sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE). The 40-50 kDa glycoprotein was separated from the mucins by SDS-PAGE, stained with periodic acid Schiff (PAS) base. Its association with albumin was confirmed by staining with Coomassie Brilliant Blue. A Ponceau S stain of this gel indicated the position of albumin, allowing the gel to be sliced in exactly the position of the 40-50 kDa glycoprotein, which was then prepared for immunization of a rabbit. The antibody developed was then tested for homogeneity and cross reactivity by dot blot and western blotting methods, and further used to determine the localization of this 40-50 kDa glycoprotein in human gastric tissue.

In the normal gastric mucosa, the 40-50 kDa glycoprotein was expressed in parietal cells and not in the mucus producing cells. In intestinal metaplasia (IM), a premaligant condition, the 40-50 kDa glycoprotein was expressed in the columnar cells. The 40-50 kDa glycoprotein was expressed in the intestinal, diffuse, mucinous, signet-ring cell and mucinous type gastric carcinomas. The intestinal type carcinoma was the predominant carcinoma in this study and it is considered to be an "epidemic type" of cancer in high-risk populations in the Western Cape region of South Africa. The pre-cancerous stage (IM) of the intestinal type carcinoma represents a prolonged and complex process, part of which results in the transformation of the normal mucosa into an intestinal type of mucosa. The diffuse type carcinoma, which is relatively more frequent in low-risk populations and is not preceded by well-defined precancerous lesions, was less observed in our study.

Using proteomics, we performed a more complete analysis of the 40-50 kDa glycoprotein. We performed two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) in combination with MALDI-TOF MS to define the protein components of the 40-50 kDa glycoprotein and to identify it. Purified mucin from gastric cancer specimens were separated by 2-D PAGE which allowed high resolution and reproducibility. Precision protein standards were used as molecular weight markers. Gels were stained with Coomassie Brilliant Blue and PAS. The 40-50 kDa glycoprotein separated as four spots (three on Coommassie Blue and one on PAS) on 2-D PAGE running side-by-side on the protein gel at M_r 40 to 50 kDa. The spots were excised from the gels and digested with trypsin. Fragments where measured by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-

TOF MS), to permit successful protein identification. The peptide mass fingerprint generated from the 4 spots was then used to search the NCBI database to identify the proteins. Three of the four proteins (from the Coommassie Blue stained gel) were identified as alpha-1-acid glycoprotein (AGP) also known as orosomucoid. We were unable to positively identify the fourth PAS positive spot on the PAS gel and this could possibly be due to interference in the MALDI analysis caused by the PAS stain or the extensive glycosylation of the protein. The identification of the 40-50 kDa glycoprotein as alpha-1-acid glycoprotein (AGP) confirmed previous findings based on an amino acid analysis of this glycoprotein being an N-linked one and not an O-linked glycoprotein, like mucin.

AGP or orosomucoid is an acute-phase protein with a high carbohydrate content (more than 40%) which could explain its fractionation with mucins in a CsCl density gradient. A number of activities of possible physiological significance have been ascribed to this protein, such as its immunomodulatory effects and activities related to the immune function of different blood cell types. AGP is produced mainly in the liver by hepatic cells, but extra-hepatic expression has been reported in several other tissues including human and bovine epithelial cells, stimulated alveolar macrophages, human endothelial cells and the prostate gland.

In cancer, AGP has been shown *(in vivo)* to have no effect on the basal activity of natural killer cells (NK), but inhibited the increase of NK cytotoxicicity induced by IFNy and IFNα. In colon cancer, evidence has been presented that AGP enhances the invasiveness and metastatic potential of malignant cells. It remains unclear what part AGP plays in the pathophysiology of stomach cancer. In light of what has been reported on AGP, the consistent and high expression of this glycoprotein (reported by us) at the tumour site then provides the rationale to exploit its presence in pre-malignant and malignant conditions to produce a clinically useful marker for gastric cancer, with potential use as a screening tool in areas such as the Western Cape region of South Africa where the risk of gastric malignancy is high.

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ABBREVIATIONS

A280	absorbance at 280nm
AMPS	ammonium persulphate
APES	aminopropyl-tri-ethoxysilane
AJCC	American Joint Committee on Cancer
BDH	British Drug Houses
BSA	bovine serum albumin
CHAPS	3-((3-cholamidopropyl)-dimethyl-ammonio))-1-propanesulfonate
CsCl	caesium chloride
DAB	3,3'-diaminobenzidine
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
ER	endoplasmic reticulum
GuHC	guanidinium chloride
H & E	haematoxylin and eosin
HID	high iron diamine
HRPO	horse radish peroxidise
HSA	human serum albumin
IAA	iodoacetamide
IEF	isoelectric focusing
IPG	immobiline pH gradient
kDa	kilo-Dalton
mA	miliamper
MALDI-TOF	matrix assisted laser desorption ionization time of flight
MUC	mucin
Mw	molecular weight marker
N/A	not available

N/D	not determined
NEM	N-ethylmaleimide
PAS	periodic acid schiff
PAS/AB	periodic acid schiff / alcian blue
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonylfluoride
RT	room temperature
S	resected stomach specimen
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide-gel electrophoresis
TAE	tris-acetate buffer
TBS	tris buffered saline
TBST	tris buffered saline-Tween
TEMED	N, N, N, N'-tetramethylethylenediamine
TNM	tumour lymph node metastasis
Tween 20	polyoxyethylene sorbitan monolaurate
VNTR	variable number of tandem repeats
V	voltage

CHAPTER 1

LITERATURE REVIEW

1.1 Background

Mammalian epithelial surfaces of the gastrointestinal, respiratory, and reproductive tracts are coated by a thin layer of mucus approximately 90 μm and 300 μm on the surface of gastric mucosa of the rat and humans respectively (Kerss *et al.,* 1982; Allen *et al.,* 1989). Mucus is a complex and viscous secretion synthesized by specialized mucous cells in the columnar epithelium that lines and adheres to all of the organs that are exposed to the external environment. The composition, properties, and functions of this dynamic secretion differ between epithelial tissues. Mucus serves many functions, among them lubrication for the passage of food and faecal material, maintenance of a hydrated layer over the epithelium, a barrier against pathogens, noxious substances and destructive hydrolases and as a permeable gel layer for the exchange of gases and nutrients with the underlying epithelium (Allen, 1981; Neutra, 1987; Forstner and Forstner, 1994).

Mucus is the first barrier with which nutrients and enteric drugs must interact and diffuse through, in order to be absorbed and gain access to the circulatory system and their target end-organs. Mucus itself can also act as a target with its binding sites for bacterial adhesions. Both non-specific and specific mucoadhesive formulations may be targeted to the mucus and this is likely to prevent targeting devices aimed at epithelial cellular ligands from reaching their goal. In addition to its protective functions, mucus is also involved in many disease processes. Regulation of mucus secretion has been coupled to a variety of factors such as pro-inflammatory cytokines (interleukin-4, interleukin-13, epidermal growth factor, and tumor necrosis factor-alpha), ATP, bacterial exoproducts, and host proteases (Forstner, 1995; Plaisancie *et al.,* 1998; Nadel and Burgel, 2001; Levine *et al.,* 1995).

In the cervical tract, the biological function of mucus varies with its physical properties during the ovulatory cycle. The thin mucus gel found at the periovulatory

period is penetrated much more easily by the spermatozoa than is the tenacious mucus plug of the luteal phase (Chantler, 1982).

Mucus in the tear film on the surface of the eye is secreted by conjunctival goblet cells and provides a protective covering that prevents pathogen penetration and desiccation of the underlying epithelium (Gipson, 2004).

The airway mucus gel entraps foreign particles and pathogens and also dissolves noxious gases, facilitating their clearance from the lung by means of ciliary transport or coughing (Knowles and Boucher, 2002). This highly hydrated gel, in conjunction with ciliated epithelial cells, is essential for the maintenance of sterile and unobstructed airways.

The high rate of mucus accumulation in the colon provides good lubrication, crucial to its function, and is also important in providing a suitable environment for the endogenous microflora (Allen and Pearson, 1993).

Mucus is composed primarily of water, but also contains salts, lipids such as fatty acids, glycoproteins, phospholipids and cholesterol (Allen, 1981) and proteins which have a defensive purpose such as lysozyme, immunoglobulins, defensins, growth factors and trefoil factors (Flemstrom *et al.,* 1999; Corfield *et al.,* 2000; Roberton *et al,*. 2005; Kouznetsova *et al.,* 2004).

Although most of mucus is water, high molecular weight glycoproteins known as mucins are the major component of mucus, giving it its protective, hydrophilic and rheological properties (Verdugo *et al.,* 1983). Mucins, characterized by their high density of O-linked oligosaccharide substitution, are responsible for the biological properties of a continuous, insoluble and adherent mucus gel that protects the gastric mucosa from the hostile environment in the lumen (Allen, 1981).

1.2 Gastrointestinal mucus

The stomach is normally protected by a layer of mucus, reportedly ranging in depth to as much as 500µm in the stomach (Bickel and Kauffman, 1981). This mucus layer

consists of two separable, physically distinct mucus gel layers termed shear resistant and shear-compliant (Figure 1.1). In the mammalian gastric mucosa, there are mainly two types of mucus-secreting cells, namely the surface mucous cells and the glandular mucous cells. The adherent gastric mucus gel (shear resistant), maintained by multiple interaction types (Taylor *et al.,* 2003), is distinctly a stronger secretion and provides a stable unstirred layer and maintains a pH gradient from acid in the lumen (approximately pH 2) to near neutral pH (approximately pH 7) at the mucosal surface (Schade *et al.,* 1994). This layer is considered to protect the mucosa from acid, pepsin and other damaging agents. The relative thickness of the two component layers of the mucus gel varies for different regions of the gut (Atuna *et al.,* 2001) (Figure 1.1).

The gastric mucosa produces two rheologically distinct gastric mucus secretions (Taylor *et al.,* 2004) composed of structurally distinct mucin populations with respect to the potential structure to function relationships (Fogg *et al.,* 1996) within the total mucus secretion. Atuna *et al.* (2001) observed the presence of two mucus layers *(in vivo)* in the rat gastrointestinal mucosa (Figure 1.1). The shear-resistant mucus gel has ideal rheological properties to form a firm protective barrier resistant to shear and maintaining the stable surface unstirred layer, essential for the mucus bicarbonate barrier. Pepsin, in *vivo* and *in vitro* has been shown to digest only the surface of the adherent mucus gel, being unable to penetrate the gel matrix (Allen *et al.,* 1993; Bell *et al.,* 1985). Thus the concentration of mucus glycoprotein (mucin) in the mucus may be the most important parameter which determines the special rheological features of mucus required in a special functional context.

Another clearly identifiable function of gastric mucus is that of a lubricant attenuating the shear stress on the mucosa that results from the mechanical forces associated with digestion (Allen, 1989). In stomachs full of food, the shear-compliant mucus is almost absent from the mucosal surface and is found forming strands of mucus visible on the food bolus (Taylor *et al.,* 2004). During digestion, the shear-compliant secretion, which changes into a viscous liquid when subjected to even low mechanical shear stress, acts as the ideal lubricant, facilitating the movement of solid matter through the gut during the digestive processes. Throughout the gut, the viscous mucus secretion acts as a lubricant facilitating the passage of digestive matter and protecting the underlying epithelium from excessive mechanical stress.

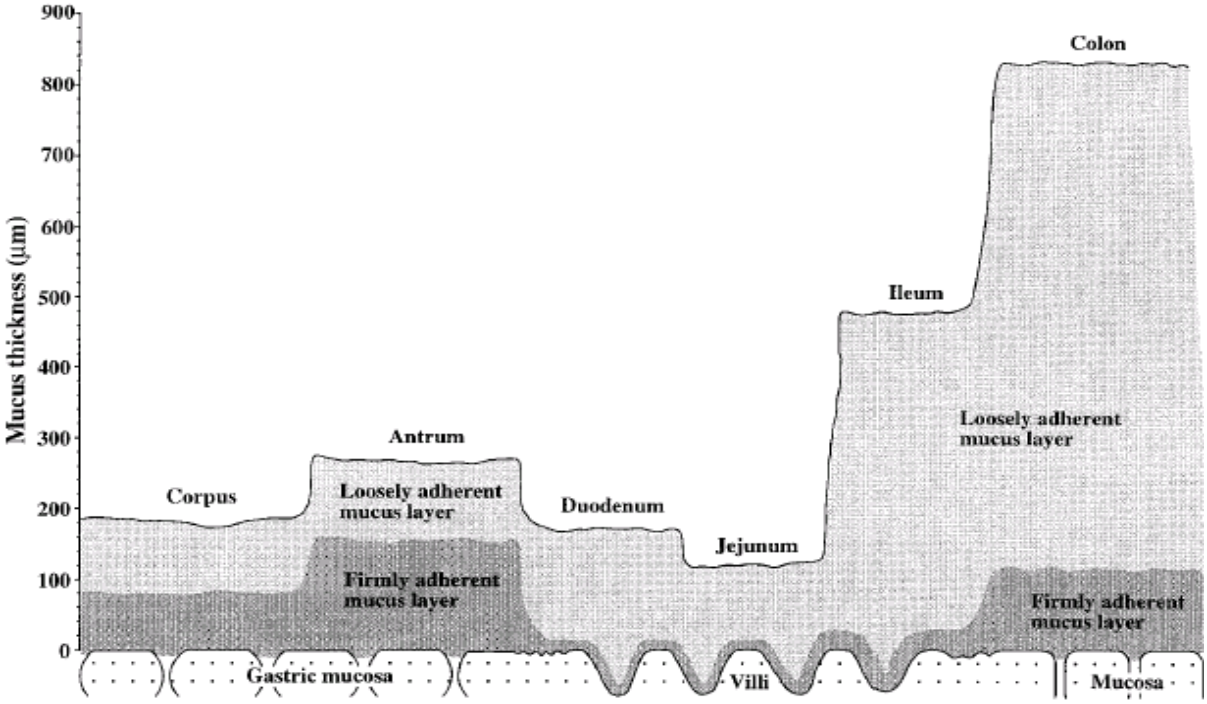


Figure 1.1 A schematic diagram showing the two mucus gel layers in the gastrointestinal tract in the corpus, antrum, duodenum, jejunum, ileum, and colon adherent to the surface of the epithelium providing an interface between the lumen and the mucosa. In the stomach a continuous layer of mucus forms a barrier between the erosive gastric contents and the underlyimg mucosa. The mucus layer in the antrum is thicker (due to the adherent mucus layer) compared with the corpus. The small intestine has a very thin discontinuous mucus gel layer. In the colon and ileum, the mucus layer is very thick (up to 4-fold thicker) compared with that in other regions of the gut. Most of the difference in mucus thickness is due to the loosely adherent mucus which is continuously renewed after removal while the firmly adherent mucus layer remains intact (reproduced from Atuna *et al.,* 2001).

1.3 Mucus renewal

Mucus is known to be continually produced from the epithelial surface in the gut, and lost to the lumen during renewal (Sababi *et al*., 1995; Plaisancie *et al.,* 1998). This turnover displaces microparticles specifically bound to the mucus surface. The loosely adherent mucus, which is rapidly replaced after its removal, has more lubricative properties *in vivo* while the firmly adherent gel, which forms a thick layer over the gastric and colonic mucosal surfaces, acts as a relatively stable protective barrier. In the colon the mucus layer provides an essential environment for the enteric microflora. The erosion of the mucus gel layer at its luminal surface by proteases and

mechanical sloughing (from a motile gut, passage of faeces, etc.) is normally balanced by secretion of new mucus to maintain the thickness of the adherent gel layer seen *in vivo* (Allen *et al*., 1998) (Figure 1.2), thus maintaining a dynamic balance between synthesis and degradation of the epithelial surface.

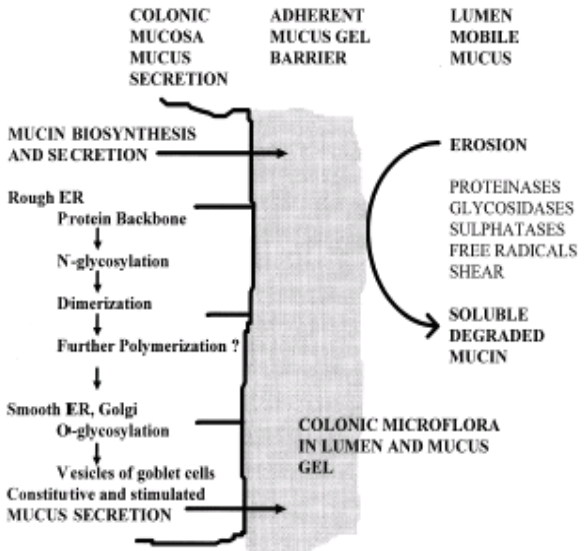


Figure 1.2 A diagrammatic representation of mucus renewal *in vivo*. The thickness of the adherent gastrointestinal mucus layer is a balance between its secretion rate and its erosion through enzymatic digestion by luminal proteases and mechanical shear. Normally, mucus secretion is sufficient to maintain a continuous barrier across the colonic epithelium. The removal of the loosely adherent mucus layer by mechanical shear forces and erosion by the mucolytic proteinases stimulates the accumulation of new mucus in the colon (reproduced from Allen *et al.,* 1998).

1.4 Definition of mucin

Mucins, the major and important components of all mucus secreted from epithelial cells covering the surface of epithelial tissues (Kim *et al.,* 1996), are complex O-linked glycoproteins characterized by the presence of at least one large region of the polypeptide rich in serine and threonine residues, termed the mucin domain. These hydroxylated amino acids are the sites of covalent attachment, via the linkage sugar N-acetylgalactosamine (GalNAc), of a diverse array of O-glycans, many of which are sialylated or sulfated. As a consequence mucins are polyanionic, and a high percentage (typically more than 70%) of their mass is carbohydrate arranged in a ‘‘bottle brush’’ configuration around the protein core (Gendler and Spicer, 1995).

1.5 Mucin biosynthesis

Making a mucin is a complex multistep process which starts after the MUC protein backbone (apomucin) is encoded by a MUC gene. In the endoplasmic reticulum the apomucin undergoes N-glycosylation and intramolecular disulfide bond formation (McCool *et al.,* 1994; Asker *et al.,* 1998; Dekker and Strous, 1990) (Figure 1.3). The apomucin is further maintained in an open conformation to expose the sugar acceptor sites to the Golgi enzymes (glycosyltransferases) which will then attach and elaborate the vast numbers of O-glycan chains (Hang and Bertozzi, 2005).

In the Golgi, GalNAc is the first glycan to be added to serine and threonine residues. This key step is catalyzed by polypeptide-GalNAc transferases (ppGal-NAcTs) and is important for the presentation of acceptor sites for other ppGalNAcTs and subsequent glycosyltransferases responsible for the elaboration of the O-glycan core. The addition of GalNAc to the apomucin is suggested to be completed prior to further elaboration of the glycan chains (Sheehan *et al*., 2004).

Glycosylation patterns are known to be tissue dependent and it is therefore likely that the structure of the carbohydrate chains will differ according to the tissue in which the mucin is expressed.

After glycosylation, the dimers are further polymerized to form mucins (Thornton *et al.,* 1990; Sheehan *et al.,* 1999, 2000) which then transit the trans- Golgi network (Perez-Vilar and Hill, 1999) where they may be packaged, in a highly condensed manner, into secretory granules (Perez-Vilar *et al.,* 2005) (Figure 1.3). The major steps in the synthesis of a polymeric mucin (steps 1–5) and the location of these events within the mucin-secreting cell are summarised (Thornton *et al.,* 2008) below Figure 1.3.

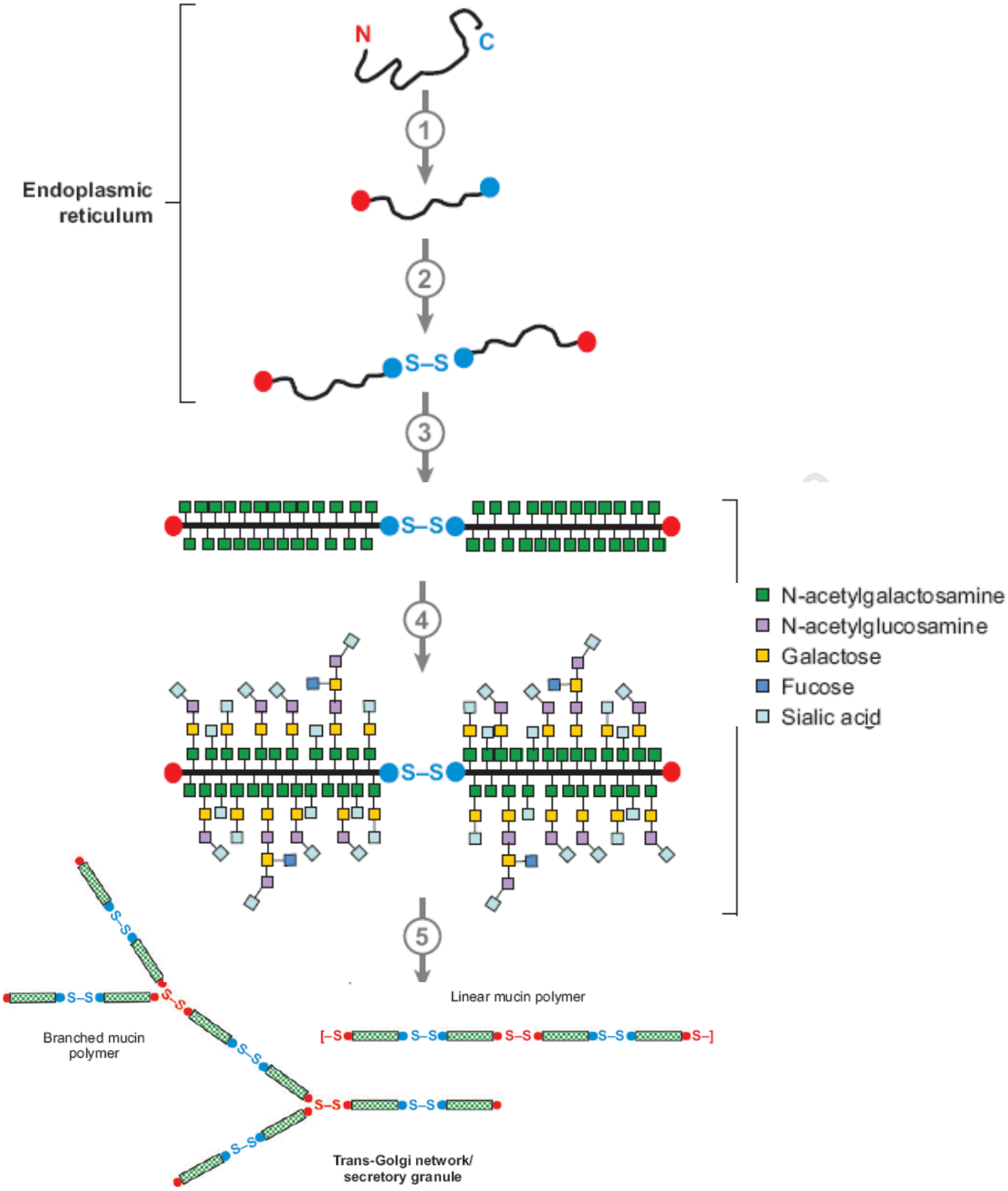


Figure 1.3 Diagrammatic representation of polymeric mucin biosynthesis. (1) In the ER, the translated mucin polypeptide undergoes N-glycosylation and (2) intramolecular disulfide bond formation. (3) In the cis Golgi, GalNAc is added to serine and threonine residues in the mucin domains. (4) These glycan chains are further elaborated as the mucin transits through the Golgi. (5) In the trans-Golgi network, or in the secretory granule, the mucin dimers polymerize and give rise to one linear polymer or branched structures (reproduced from Thornton *et al.*, 2008).

1.6 Mucin protein backbone (apomucin)

Mucins are classified by their MUC protein backbone, which is encoded by a MUC gene. The mucin protein backbone, making up 20% of the molecular mass, is made up of two regions. First, a central glycosylated region (mucin domain), which is comprised of a large number of tandem repeats (TRs) that are rich in serine, threonine and proline (STP repeats), which can make up greater than 60% of the amino acids. The number of TRs in a MUC protein determine mucin size as they elongate the protein backbone and thus increase mucin molecular mass. Serine (Ser) and threonine (Thr) are the sites for the attachment to the linkage sugar N-acetylgalactosamine (Verma and Davidson, 1994). The second region, located at the amino (N) and carboxy (C) terminals, and sometimes interspersed between the STP-repeats, are regions with relatively few O-glycosylation and N-glycosylation sites (Bell *et al.*, 1980), and with a high proportion of cysteine (>10%). Domains at the C- and or N-termini of mucin polypeptides have homology with domains within von Willebrand factor (Cao *et al.,* 1999), and are important for the disulfide-mediated polymerization of mucins (Sheehan., 2004; Voorberg *et al*., 1991).

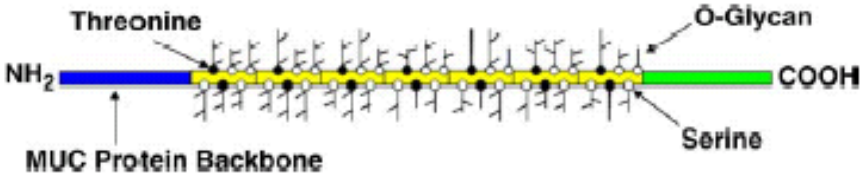


Figure 1.4 The structure of mucin. The protein backbone (apomucin) consists of an NH_2-terminal domain (blue), central domain with a high number of tandem repeat (TR) domains that contain proline and are high in serine and/or threonine residues (yellow), and a COOH-terminal domain (green). Numerous O-glycans are attached to threonine or serine residues in the TR domains (reproduced from Rose and Voynow, 2006).

1.7 Mucin O-glycans

More than 30 glycosyltransferases have now been identified, of which at least a dozen participate in the synthesis of mucin O-glycans (Toki *et al.*, 1997). In addition to N-acetylgalactosamine, mucin oligosaccharides also contain N-acetylglucosamine, galactose, fucose, sialic acids, and traces of mannose and sulphate. Terminal sugars, sialic acid and sulfates on Gal or GlcNAc, impart negative charges to mucins which account for their low pI values, whereas fucose imparts hydrophobicity (Allen, 1989; Forstner and Forstner, 1994; Corfield *et al.*, 1995). The glycan chains are clustered in specific, large, central domains and confer important structural and biological properties to the molecules, including protease resistance, pathogen sequestration, and ion and water binding. These properties are important for the formation, rheological properties, and function of the mucus gel.

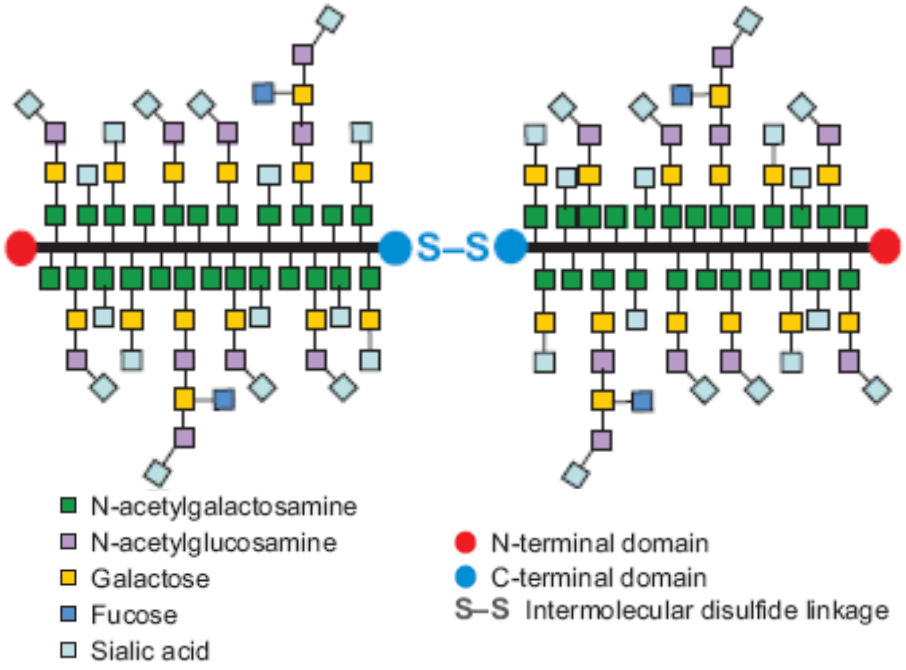


Figure 1.5. Diagrammatic representations of the five types of mucin O-glycans. O-glycan chains are synthesized by specific glycosyltransferases in a stepwise manner following transfer of GalNAc to a serine or threonine, elongated by addition of Gal or GlcNAc. The monosaccharide composition of mucins is comprised of five sugar residues namely: N-acetylglucosamine, N-acetylgalactosamine, galactose, fucose and sialic acid. Mucin can be terminally glycosylated by addition of fucose, sialic acid and sulfate (reproduced from Thornton *et al.*, 2008).

1.8 Proteolysis and reduction of mucin

Mucins are heavily glycosylated and play a major role in the protection of the epithelium from chemical and mechanical aggressions (Gum *et al*., 1992; Strous and Dekker, 1992; Lesuffleur *et al.,* 1994; Gendler and Spicer, 1995). Polymeric mucin from a variety of sources are made up of multiples of subunits (Figure 1.6) linked together into linear arrays (Harding *et al.,* 1983) to give the macroscopic mucins. Mucin subunits are linked together by disulfide bridges and these are susceptible to reductive disruption by thiols such as 2-mercaptoethanol or dithiothreitol (DTT) (Figure 1.6). The thiol reduction products are commonly referred to as 'subunits'. Regions of low or no glycosylation are prone to proteolysis by enzymes such as trypsin. The digestion products that result are commonly referred to as 'T-domains' (Figure 1.6). The glycoslyated mucin domain has limited sites of access for proteinases. Proteolytic enzymes and mucolytic drugs (e.g. N-acetylcysteine) dissolve the mucus gel (mucolysis), hydrolysing regions of accessible, non-glycosylated protein core and of inter-chain disulfide bridges, respectively, thereby fragmenting the polymeric mucins.

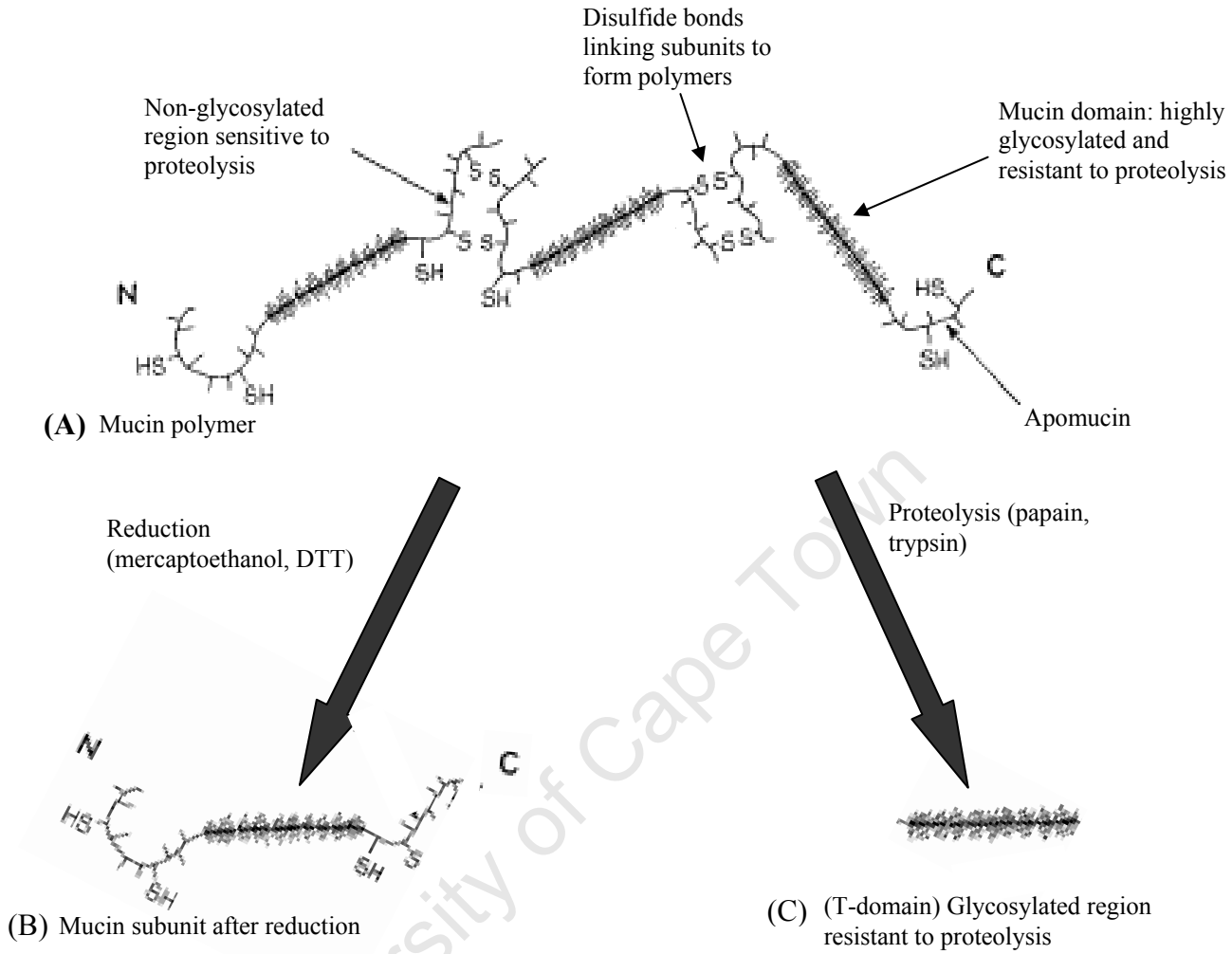


Figure 1.6. Diagrammatic representation of mucin proteolysis. (A) The whole mucin composed of three subunits linked together via disulfide bonds located on sparsely glycosylated N- and C-terminus. (B) Subunit obtained after reduction of disulfide bond and (C) carbohydrate side chains in the glycosylated region protect mucins from proteolysis (reproduced from Carlstedt *et al.,* 1983).

1.9 Mucin genes and classification

Mucin genes are independently regulated and their expression is organ-, tissue-, and cell-specific (Gambus *et al*., 1993; Ho *et al.,* 1993, 1995; Lesuffleur *et al.,* 1994). There are three classes of mucins which differ in biological structure and cellular location; those which are bound to the membrane (monomeric), those which are secreted and form extracellular gels (oligomeric) and those that are secreted and do

not form gels (soluble). Members from each family possess common characteristics (Figure 1.7).

MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC16, MUC17, and *MUC20* mucins have transmembrane domains in their carboxy terminus and are referred to as cell-tethered mucins (Dekker *et al*., 2002; Swallow *et al.,* 1987; Gendler *et al.,* 1990; Gum *et al*., 1990; Rose and Voynow, 2006) or trans-membrane mucins (Figure 1.7).

Secretory mucins are further subdivided into those that are cysteine rich and those that are cysteine-poor. *MUC2, MUC5AC, MUC5B, MUC6* and *MUC19* are large secretory mucins with cysteine-rich motifs while *MUC7, MUC8* and *MUC9* are non-polymeric mucins lacking the cysteine motifs. *MUC14, MUC15* and *MUC18* do not have tandem repeats and remain unclassified (Godl *et al.,* 2002; Lidell *et al.,* 2003; Moniaux *et al.,* 2001; Rose and Voynow, 2006).

1.9.1 Membrane-bound mucins

Membrane-associated mucins are bound by a transmembrane domain directly to the bilayer of a membrane (Figure 1.7). They serve as molecular sensors of the molecular environment at the cell surface, communicate information about the morphological condition or the differentiation status of the cell and signal the status of interaction between mucins and associated ligands and receptor (Hollingsworth and Swanson, 2004). They communicate information about the extracellular status via an intracellular signal transduction and associate with the secreted mucin layer by covalent and non-covalent bonds.

MUC1 is located on chromosome 1q21-q24; MUC3A, MUC3B, MUC11, MUC12 and MUC17 on chromosome 7q22; MUC4 and MUC20 on chromosome 3q29; MUC13 on chromosome 3q13.3 and MUC16 on chromosome 19q13.2 (Rose and Voynow, 2006). MUC1 is expressed in most epithelial tissues, including the breast and pancreas, as well as in the gastrointestinal, respiratory and urinary tracts (Zotter *et al.,* 1988). MUC3 is expressed predominantly in the colon (Ogata *et al.,* 1992).

1.9.2 Secreted and gel forming mucin (polymeric)

Genes for gel forming mucins MUC2, MUC5AC, MUC5B, and MUC6 are located in a gene complex on chromosome 11p15.5 (Gum *et al*., 1989; Winterford *et al*., 1999; Toribara *et al.,* 1993; Pigny *et al.,* 1996; Gendler and Spicer. 1995). The MUC19 gene is located on chromosome 12q12 (Rosseau *et al.,* 2008). There is very little data available on MUC19, which is expressed in the submucosal glands of the trachea (Rosseau *et al*., 2008). Only MUC2, MUC5AC, MUC5B, and MUC6 contain the cysteine-rich motifs in their C- and N-terminal domains necessary for disulfide-mediated polymer formation critical for the gel-forming properties of these macromolecules (Desseyn *et al.,* 1998; Toribara *et al.,* 1997). These domains are very similar to that of von Willebrand factor (Desseyn *et al.,* 1998). The covalent polymerisation (characteristic of secreted mucin) of the mucin molecules end to end through disulphide bridges is essential for mucins to form a gel (Allen and Pearson. 1993; Carlstedt *et al.,* 1983).

MUC2 is the first human secretory mucin gene to be cloned and completely sequenced (Gum *et al*., 1989, 1992). It is a major component of intestinal mucus gel secretions (Corfield and Gough, 1995) and is considered an insoluble mucin biochemically (Axelsson *et al.,* 1998). MUC5B has the largest central tandem repeat region (Desseyn *et al.,* 1997), among known mucins. It is the main gel forming mucin in saliva and one of the two major mucins of the respiratory mucus. MUC5AC and MUC6 are secreted into the stomach.

1.9.3 Secreted and non gel forming mucins (soluble)

Two genes fall into this class: MUC7 on chromosome 4q13.3 (Bobek *et al.,* 1993) and MUC8 on chromosome 12q24.3 (Shankar *et al.,* 1997). MUC7 is a small, secreted mucin that lacks cysteine-rich domains (does not form gels) while MUC8 is a large, secreted mucin that lacks the von Willebrand factor D4 or C1 and C2 domains typically present in the carboxyl end of large secretory mucins (Dekker *et al.,* 2002). There is little information on the MUC8 glycoprotein. However, MUC7 has been well characterized, particularly in saliva. The N terminus of MUC7 contains a histatin-like domain that has antifungal activity (Gururaja *et al.,* 1999; Situ and Bobek, 2000) and

interacts with bacteria (Reddy, 1998). MUC7 is clearly an important innate defense protein.

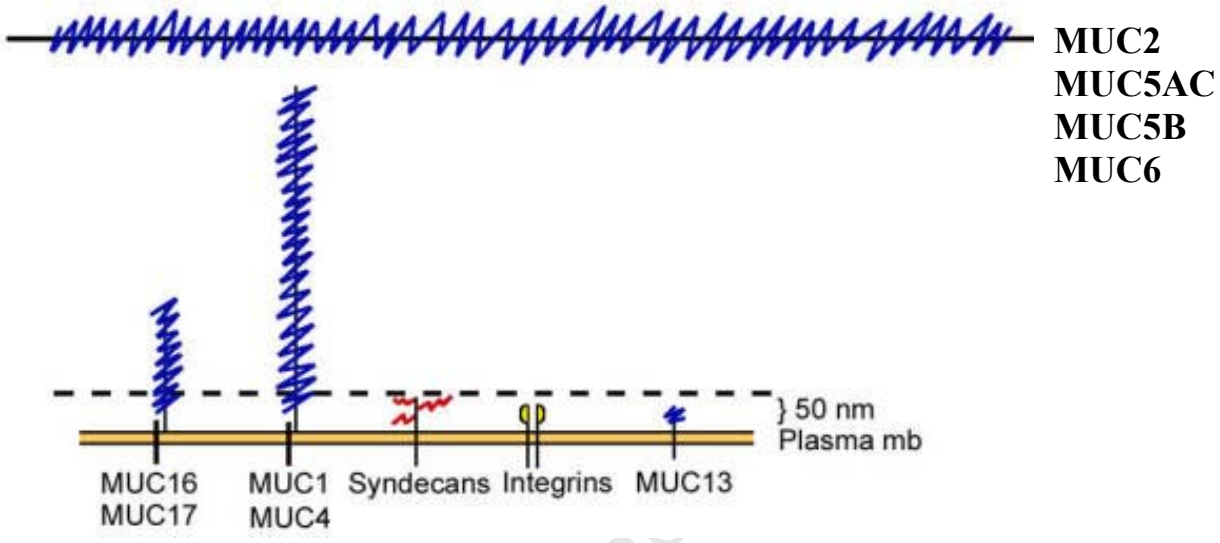


Figure1.7 Diagrammatic representation of three classes of mucins. Blue squiggles represent O-linked oligosaccharides. With the exception of MUC13, mucins extend much farther due to their extended 3D structures contributed by the proline-rich heavily O-glycosylated tandem repeat domains. MUC1 and MUC4 are the largest transmembrane mucins, extending >200 nm from the cell surface. The ectodomain structures of MUC16 and MUC17 are considerably shorter than those of MUC1 and MUC4, but still much larger than other surface glycoproteins. Secreted mucins, such as MUC2, MUC5AC, MUC5B and MUC19 are even larger, reaching 500–1000 nm in length. (Reproduced from Brayman *et al*., 2004)

1.10 Gastric mucin

Mucus of which mucin (mucous glycoprotein) is the gel forming component in the stomach protects the epithelium against acid and pepsin in the gastric juice, against exogenous damaging agents (pathogens, drugs), and mechanical damage (Bell *et al*., 1985; Allen *et al.,* 1986). Normal gastric mucosa expresses MUC1, MUC4, MUC5AC, and MUC6 (Audie *et al*., 1993; de Bolos *et al*., 1995; Ho *et al*., 1995; Byrd *et al*., 1997; Reis *et al.,* 1997), with MUC1, MUC5AC, and MUC6 being the most prominent (Ho *et al*., 1995). Other mucins are generally absent from the normal gastric mucosa (Carrato *et al.,* 1994; Ho *et al.,* 1993; Filipe *et al.,* 1996).

In the stomach, there is a separation of the regions of mucin production (Figure 1.8). Mucins present in the antrum are different from those in the body region (Ohara *et al.,* 1986). There is evidence that the two polymeric mucins, namely MUC5AC (surface) and MUC6 (glands) remain distinct within the mucus gel (Ho *et al*., 2004; Nordman *et al.,* 2002). MUC1 is widely expressed in mucous cells of the surface epithelium, and is focally expressed in oxyntic glands and parietal cells of the body and in the pyloric glands of the antrum (Burchell *et al.,* 1987; Girling *et al.,* 1989; Ho *et al*., 1993, 1995; Walker *et al*., 1995; Reis *et al*., 1998, 1999). MUC5AC is highly expressed in foveolar and neck cells of the body and the antrum (de Bolos *et al.,* 1995; Ho *et al*., 1995; Byrd *et al.,* 1997; Reis *et al*., 1997). MUC6 is expressed in mucopeptic cells of the neck zone of the body region of the stomach and in pyloric glands of the antrum (de Bolos *et al*., 1995; Tajima *et al.,* 2001; Ho *et al*., 1995, 2000; Byrd *et al*., 1997; Reis *et al*., 1999, 2000; Audie *et al.* 1993).

This characteristic cell- and tissue-specific distribution of mucin expression observed in the gastric mucosa may be related to the specific function of each mucin (de Bolos *et al.* 1995; Ho *et al*. 1995; Byrd *et al.* 1997). Glycosylation differences between gastric mucins isolated from the surface epithelium and the glands have also been shown (Nordman *et al.,* 2002). Therefore, in the stomach, it is possible that the two gel layers, the firmly adherent and that removed by shear, are two different mucin gene products (MUC5AC and MUC6) or different mixtures of two gene products, also perhaps with differing glycosylation patterns.

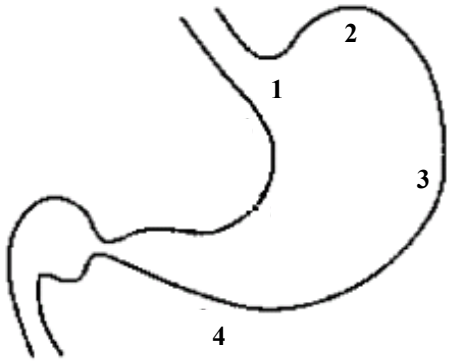


Figure 1.8. Diagrammatic representation of the stomach showing the different regions. (1) Cardia; (2) Fundus; (3) Body [MUC5AC, MUC1, MUC6] (4) Antrum and pylorus [MUC5AC, MUC1, MUC6] (reproduced from Marques *et al.,* 2005)

1.11 Gastric mucin: Protection against acid

The gelation of mucin is complex, involving the interplay of electrostatic and hydrophobic interactions. At neutral pH of 6–7 the non-glycosylated Cys-rich regions of mucins are in conformation with the hydrophobic domains. At acidic pH 2 the hydrophobic regions of protein are exposed (Figure 1.9). The hydrophobic domains on adjacent molecules are then able to associate and cross-link with mucin to form a stable adherent mucus gel. Bromberg and Barr. (2000) reported that the non-glycosylated regions of mucins were involved in the aggregation of human tracheobronchial mucin. The entanglement of the sugar side chains further contributes to the high viscosity of mucin solutions (Figure 1.9).

Only the firmly adherent layer is important in maintaining pH at a neutral level during luminal acid exposure (Phillipson *et al.,* 2002). Thus the combination of the firmly adherent mucus layer and HCO_3^- secreted from the surface epithelial cells maintain a neutral pH on the surface of the epithelial cells even though the luminal pH is acidic (Phillipson *et al.,* 2002, Synnerstad *et al*., 2001).

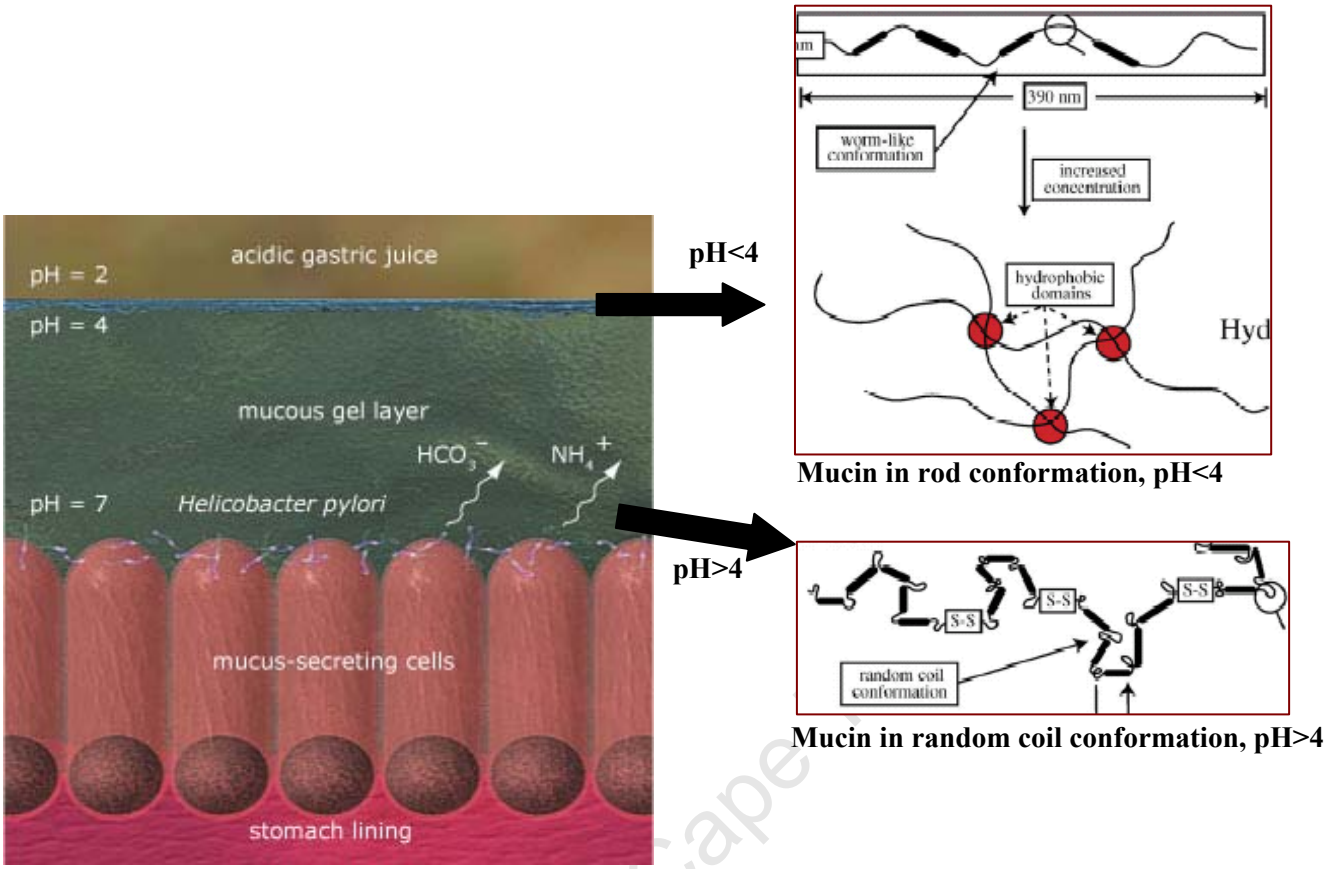


Figure 1.9 A diagrammatic representation of the protective mucus layer of the stomach and pH induced gelation. The breaking of electrostatic interactions around pH 4 and below produce a conformational change in pig gastric mucin from a random coil to a rod (or stiff worm-like chain) and lead to gelation at high concentrations and at low pH by exposing hydrophobic regions which are folded and thus sequestered in the interior at neutral pH (reproduced from Bhaskar *et al.*, 1992 and www.science.org.au/nobel/2005/05diagram3.htm).

1.12 Gastric mucin and trefoil peptides

Although mucins are the major molecular constituents of mucus, the secretion contains a host of other proteins as well as non-protein components and water as the main component (Creeth, 1978). One of the important roles for mucus is the retention of these non-mucin-secreted molecules in the immediate extracellular environment. There is evidence that some non-mucin proteins are either covalently or non-covalently associated with the extracellular mucin complex (Thornton *et al.*, 2001; Wickstrom *et al.*, 2000; Thim, 1997). These molecules may become associated with mucins during biosynthesis or packaging into secretory granules and remain integral

components of the secreted mucin complex, whereas other molecules may be co-secreted before becoming associated with the mucin complex.

Within the gastrointestinal tract, trefoil peptides and mucins are coexpressed in mucous cells in a closely related manner (Poulsom and Wright *et al.,* 1993; Thim *et al.,* 2002; Kjellev *et al.,* 2006; Wright *et al.,* 1997). Trefoil peptides are localised within mucin granules in mucous secreting epithelial cells (Ahnen *et al.,* 1994). Individual trefoil peptides are uniquely associated with specific mucin glycoproteins, possibly essential for mutual functional roles (Ahnen *et al.,* 1994).

TFF1 (pS2) is localised in the mucus producing superficial epithelial cells throughout the normal stomach (Rio *et al.,* 1988; Hanby *et al.,* 1993) and has been shown to be co-expressed with MUC5AC (Madsen *et al.,* 2007; Ruchaud-Sparagano *et al.,* 2004) Similarly, TFF2 or spasmolytic peptide (SP) is found in mucous neck cells and deep pyloric glands (Hanby *et al.,* 1993; Jeffrey *et al.,* 1994), co-expressed with MUC6. TFF3 or intestinal trefoil factor (ITF) is expressed predominately in the goblet cells of the small and large intestine (Suemori *et al.,* 1991; Podolsky *et al.,* 1993).

Non-mucin glycoproteins and proteins play key roles in mucosal protection and mucus organization. Tomasetto *et al.* (2000) showed that TFFs interact with the secreted gel forming mucins through binding with their cysteine-rich domains. Studies using TFF1 knockout mice revealed dysfunctional gastric mucus production; the mice developed gastric adenomas, and one third of them developed neoplasia (Lefebvre *et al.,* 1996). Thus trefoil peptides, together with their pattern of distribution and co-production with mucus in the gut, are involved in mucosal protection and repair (Kindon *et al.,* 1995; Playford *et al*., 1995; Babyatsky *et al.,* 1996).

1.13 Gastric mucin and *H. Pylori*

Helicobacter pylori, a gram-negative bacteria, is adapted to life in the mucus of the digestive tract of vertebrates. The acidic milieu in the stomach is lethal to most bacteria, but *H. pylori* not only survives but actually colonizes the stomach in 50% of the human population (Helicobacter and Cancer Collaborative Group, 2001). Due to

the polyvalent presentation of receptor analog structures, mucins are capable of binding bacteria through specific attachment sites (Schroten *et al*., 1992; Kubiet *et al.,* 2000; Reddy *et al.,* 1996). The expression patterns of gastric mucins MUC5AC and MUC6 are altered as a response to colonization (Byrd *et al.,* 1997, 2000).

The most comprehensive example of how mucin glycans protect the epithelium from pathogens is highlighted by the dual role of MUC5AC and MUC6 mucins in the defense of the gastric epithelium from *H. pylori.* The blood group Le^b oligosaccharides associated with MUC5AC are the primary receptor for *H. pylori* in the human (Van de Bovenkamp *et al.,* 2003; Linden, 2002) and compete with the bacterium for binding to similar ligands on the epithelial cell surface, whereas terminal α1–4-linked Nacetylglucosamine residues carried on MUC6 function as an antibiotic (Kawakubo *et al*., 2004). Sialylated Lewis antigens contribute to *H. pylori* binding in inflamed tissue (Mahdavi, *et al.,* 2002). *H. pylori* positive patients exhibit an increase in MUC6 in surface mucous cells with a reduction in MUC5AC (Byrd *et al.,* 1997). Reversal of the normal gastric pattern is corrected on elimination of infection (Byrd *et al.,* 1997). *H. pylori* has been shown to reduce the rate of renewal of the loosely adherent mucus layer (Atuma *et al.,* 1998).

H. pylori in humans are associated with gastritis, gastric ulceration, gastric adenocarcinoma and lymphoma of mucosal-associated lymphoid tissue (MALT lymphoma) (Morgner *et al.,* 2000). Its presence increases the risk of developing gastric cancer by 2–3-fold (Uemura *et al.,* 2001). Ilver *et al.* (1998) showed that there is a higher susceptibility to *H pylori* infection in individuals of O and Le^b blood groups, because they have a higher quantity of fucosylated antigens.

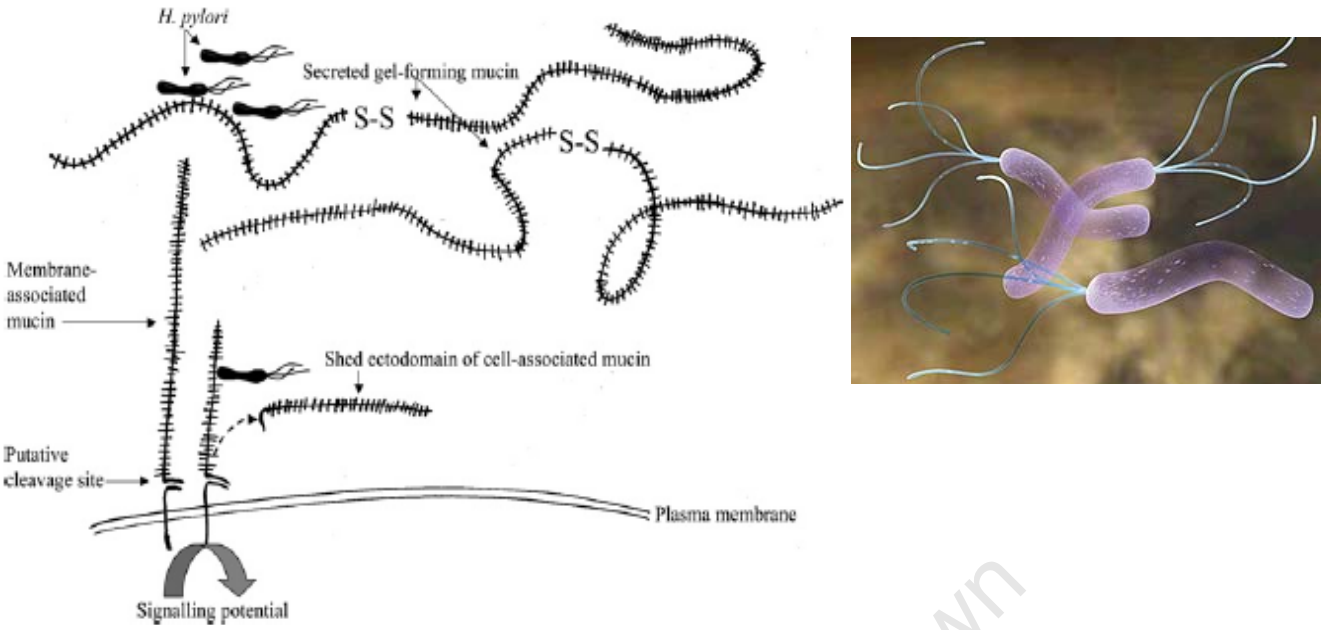


Figure 1.10. Diagrammatic representation of mucin- *H. pylori* interactions. *H. pylori* are Gram-negative, spiral-shaped microaerophilic bacteria that are involved in several gastric diseases. Binding occurs both to secreted and membrane-associated mucins. The mucin oligosaccharide diversity enhances the possibility that bacteria bind to mucus, thus facilitating their removal. By providing competing receptors for cell-surface glycoconjugates, mucins may trap bacteria and make them less successful in their attempts to colonize the epithelium. Thus, the array of oligosaccharides expressed on the mucins of an individual may play a key role in governing the susceptibility to infection. (reproduced from Linden *et al.*, 2004 and www.science.org.au/nobel/2005/interview.htm)

1.14 Mucin expression in intestinal metaplasia

Intestinal metaplasia (IM) of the stomach is the replacement of the gastric mucosa by an epithelium that histologically resembles the intestinal mucosa. The presence of goblet cells in the gastric mucosa is the most important single histologic parameter that characterizes intestinal transformation. Epidemiological and long-term prospective studies of patients with chronic gastritis and IM have provided strong evidence of a link between IM and intestinal-type gastric carcinoma (You *et al.*, 1999; Morson and Sobin, 1980; Correa, 1988; Chejfec *et al.*, 1992; Imai and Murayama, 1983; Boussioutas *et al.*, 2003).

Intestinal metaplasia is classified as complete (small intestinal or type I) and incomplete (colonic or types II and III). Specific subtypes of IM can be identified based on mucin secretion (Jass and Filipe, 1981; Jass, 1980).

The complete (type I) is characterized by the goblet cells secreting sialomucins and the incomplete type (encompassing types II and III) is characterized by the presence of columnar and goblet cells secreting sialo and/or sulfomucins. Mucins with O-acetylated sialic acids (O-acetylsialomucins) are not seen in type III IM but are observed in complete or type I IM (Filipe and Ramachandra, 1995; Jass and Roberton, 1994). Several studies have shown that type III intestinal metaplasia is associated with an increased risk of malignant transformation (Filipe *et al*., 1994).

The repertoire of mucins synthesized by gastric carcinoma cells is tightly associated with their differentiation program. Altered mucin expression patterns have been reported in intestinal metaplasia, including under-expression of MUC1, MUC5AC, and MUC6 and increased expression of MUC2 (Ho *et al.,* 1995; Filipe *et al.,* 1996; Reis *et al.* 1998). The complete form of IM (type I) contains the intestinal-type MUC2 in goblet cells. Incomplete variants (types II and III) exhibit MUC1 and MUC5AC in both goblet and absorptive cells and a mixture of MUC2 and MUC5AC and/or MUC6 (Reis *et al.,* 1999). Clinical and epidemiological observations, including follow-up studies indicate that the natural history of the process goes in the opposite direction: complete (type I) intestinal metaplasia may evolve to incomplete metaplasia (type III) (Silva *et al.,* 1990; Filipe and Ramachandra, 1995; Jass and Roberton, 1994).

Other markers for IM include sulphated carbohydrate antigen 91.9H (Ohe *et al.,* 1994), FU-MK-1 (Watanabe *et al.,* 1993), blood-group antigens (Sakamoto et al., 1989) and sialyl Tn and T (Carneiro and Santos *et al.,* 1994). Other important genes in the development of IM include TFF3, the goblet cell-associated trefoil factor that is induced in the stomach early in the development of IM in both humans and rats (Taupin *et al.,* 2001) that has recently been shown to be induced by Sonic hedgehog, a parietal cell morphogen, the expression of which is lost in IM in humans (van den Brink *et al.,* 2002). Indeed, atrophic gastritis, especially parietal cell loss is likely to be an important permissive event intestinal metaplastic development in the stomach.

1.15 Gastric cancer

Gastric cancer is the second most common cause of cancer-related death worldwide (Parkin *et al.,* 2001; 2005). Histologically, human gastric carcinomas have been classified into two major groups: the intestinal type/well differentiated and diffuse types/poorly differentiated (Jarvi and Lauren, 1951; Lauren, 1965).

Generally, the intestinal-type cancers, surrounded by IM, behave more passively than diffuse-type cancers (Davessar *et al.,* 1990). On the other hand, diffuse-type gastric carcinomas are composed mainly of tumor cells displaying gastric differentiation (Reis *et al.,* 2000; Lopez-Ferrer *et al.,* 2000) and show poorer survival compared to intestinal-type carcinomas. Mucinous carcinomas are marked by exuberant pools of mucin production and signet ring cell carcinoma, a rare subtype, is characterized by intracellular mucin accumulation.

1.16 Mucin alterations in gastric carcinoma

Alterations in mucin expression such as the pattern of loss of mucin expression, increased mucin heterogeneity, and changes in glycosylation including exposure of simple mucin-type carbohydrates have been reported in gastric carcinomas with suggestions that these mucin alterations can be regarded as "molecular" markers of malignant transformation of the gastric mucosa. (Bara *et al*., 1993; Gambús *et al*., 1993; Ho *et al.* 1993, 1995; Carrato *et al.,* 1994; Reis *et al.,* 1997, Sakamoto *et al.,* 1997; Baldus *et al*., 1998; Carneiro *et al*., 1994; David *et al.*, 1992; Mitsuuchi *et al.,* 1999). These changes may have an impact on cell growth regulation, immune recognition, and cellular adhesion, that in turn may influence the invasive and metastatic capabilities of a tumour (Bresalier, 1991; Mannori *et al*., 1995).

In normal gastric tissues, the type of mucin expressed is closely associated with the cell phenotype: MUC5AC is associated with foveolar cells and MUC6 with glandular cells. Gastric carcinomas have been found to contain a higher level of MUC1 expression than normal gastric mucosa and *de novo* expression of MUC2 (Ho *et al.,* 1995; Lee *et al.,* 2001; Pinto-de-Sousa *et al*., 2002; Baldus et al., 1998). In contrast to the higher expression of MUC1, MUC5AC and MUC6 expression is decreased during

gastric carcinogenesis (Ho *et al.,* 1995; Reis *et al.,* 2000; Pinto-de-Sousa *et al.,* 2002; Baldus *et al*., 1998).

MUC1 is the best known mucin antigen associated with malignant progression in gastric carcinomas (Yonezawa and Sato, 1997). MUC1 reactivity has been shown to be significantly enhanced in carcinomas exhibiting tubular/papillary or intestinal differentiation compared with signet-ring cell or diffuse types (Baldus *et al*., 1998). The co-expression of MUC1 and MUC5AC results in increased metastatic potential of gastric carcinoma cells (Ho *et al.,* 1995; Taylor, 1998). Populations at high risk of gastric cancer have been identified on the basis of the polymorphisms of the MUC1 and MUC6 genes. Shorter alleles of these two genes identify genotypes at increased risk of gastric neoplasia (Garcia *et al.,* 1997; Vinall *et al.,* 2002).

The expression of MUC5AC has significantly been shown to associate with carcinomas that usually display a "gastric" differentiation (Fiocca *et al.* 1987; Carneiro *et al.* 1992; Reis *et al*. 1997). MUC5AC was reported to correlate with infiltrative growth patterns (Pinto-de-Sousa *et al.,* 2002) associated with diffuse-type carcinoma (Pinto-de-Sousa *et al.,* 2002; Gurbuz *et al.,* 2002), which are characterized histologically by discohesive cells and the common presence of signet ring cells. Therefore, it would be expected that MUC5AC positive tumours have a worse prognosis than MUC5AC negative tumors. Kocer *et al.* (2004) reported that MUC5AC-positive tumours correlated with poor clinicopathological parameters and decreased overall survival. In contrast, Baldus *et al*. (2002) and Pinto de-Sousa *et al.* (2002) observed significantly better survival in patients who retained MUC5AC in their tumours.

MUC2 appears to be a hallmark of the mucinous type of gastric carcinoma (Reis *et al.,* 2000; Lee *et al.,* 2001; Pinto-de-Sousa *et al.,* 2002, Gurbuz *et al*., 2002; Kocer *et al*., 2004). Mucinous carcinoma is defined as an adenocarcinoma in which a substantial amount of mucin (more than 50% of the tumour) is retained within the tumour (Jass and Sobin, 1989). Detection of MUC2 in gastric carcinoma is an indicator of a suitable prognosis for the patient (Utsunomiya *et al.,* 1998).

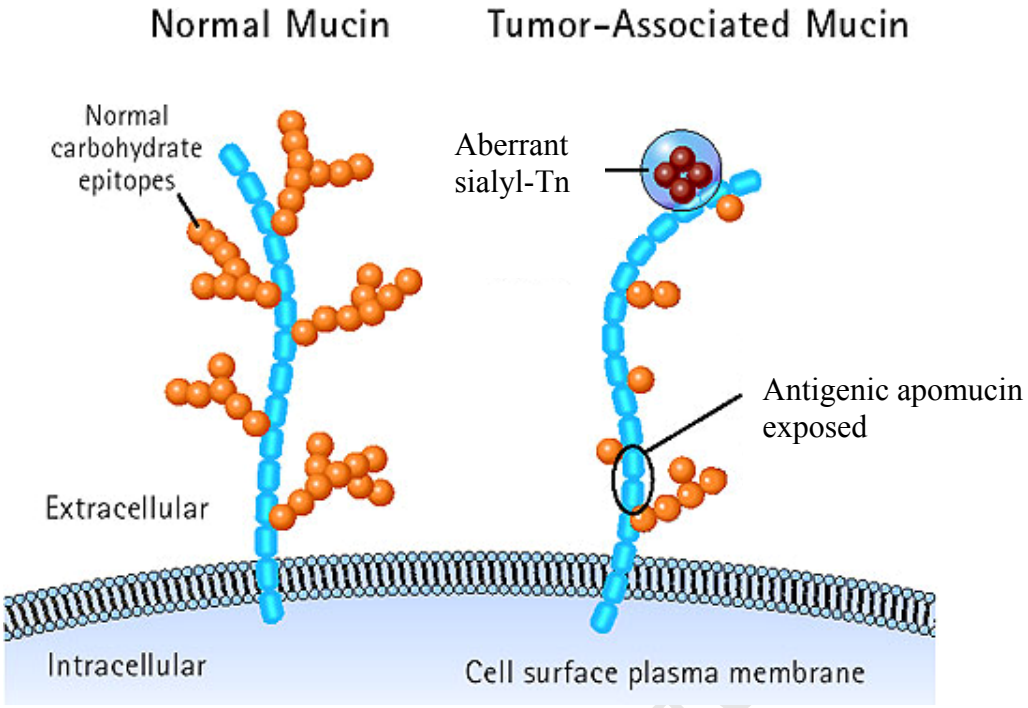


Figure 1.11. Mucin alteration in gastric carcinoma. An important feature of carcinoma is an altered biosynthesis of the carbohydrate structures on mucin. Cancer cells frequently exhibit incomplete glycosylation, which results in the synthesis of short oligosaccharides and hence the exposure of cryptic core region oligosaccharides and apomucin. (reproduced from www.merckoncologycol.com/medicos/brochure.htm)

1.17 Mucin and cell adhesion

Invasiveness and metastasis are the most important characteristics of malignant tumours and associated with mortality. Recently, many tumour makers have been identified which are used not only for diagnosis but also for the assessment of aggressiveness and the prognosis of tumours (Duraker and Celik, 2001). One such marker is MUC1, a membrane-bound mucin assumed to play various roles in tumour immunology, and involved in cell adhesion especially by interaction with β-catenin and the cell–cell contacts involving epithelial integrins (Wesseling *et al.,* 1995) (Figure 2).

The abnormal expression of E-cadherin and MUC1 has been shown in malignancy (Kimura *et al.,* 2000; Tanaka *et al.,* 2003, Zhang *et al.,* 2004). MUC1 contributes to tumour invasion via the impairment of E-cadherin (Kondo *et al.,* 1998; Ligtenberg *et al.,* 1992; Suwa *et al*., 1998). MUC1 expressed in tumours may function as an anti-

adhesion molecule, because of its large extended and rigid structure (Figure 1.12) that inhibits cell-cell adhesion, inducing the release of cells from the tumour (Ligtenberg *et al.,* 1992; Makiguchi *et al.,* 1996) thus enhancing metastasis. Therefore, the pattern of abnormal E-cadherin and MUC1-positive expression could be an unfavourable marker in a patient with advanced gastric cancer.

Mutations in the E-cadherin gene are associated with diffuse-type gastric cancer (Berx *et al.,* 1998). A single amino acid substitution in the extracellular domain of E-cadherin has been shown to result in functional consequences for cell adhesion (Handschuh *et al.,* 2001).

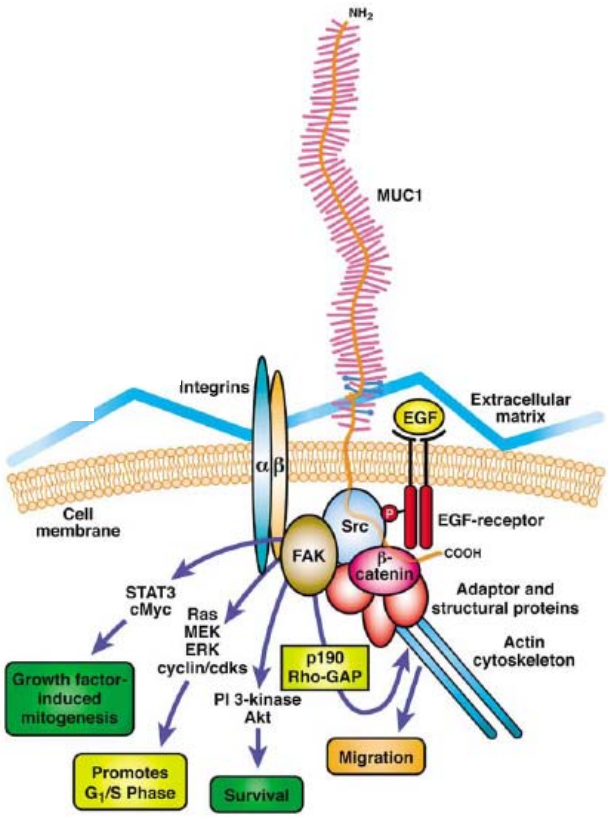


Figure 1.12. A diagrammatic representation of MUC1 interaction with structural proteins at cadherin-based adherens junction. MUC1 exerts anti-adhesive effects and is involved in signalling cascades. The cytoplasmic tail of MUC1 binds to actin filaments and in cancer cells interferes with cell-adhesion. Anti-adhesive effects can also be exerted by an inhibition of the integrin-mediated cell adhesion complex. The binding of MUC1 to β-catenin via a peptide motif in the cytoplasmic domain is regulated by various proteins including c-src tyrosine kinase and epidermal growth factor. The binding of MUC1 to β-catenin can represent an important downstream effect in tumour biology. (reproduced from Masri and Gendler, 2005)

1.18 Mucin and immunity

MUC1 is frequently over-expressed in carcinomas showing invasive growth and results in poor prognosis in patients with gastric cancer (Ho *et al*., 1993; Baldus *et al.,* 1998; Utsunomiya *et al*., 1998; Kocer *et al*., 2004).

It has been shown that an over expression of MUC1 on the membrane of cultured cells inhibit interaction between cytotoxic lymphocytes and tumor cells (van de Wiel-van Kemenade *et al.,* 1993). Agrawal *et al.* (1998) reported that higher levels of MUC1 expression are correlated with immunosupression in adenocarcinoma patients. Loss of polarity associated with the overexpression of MUC1 in cancer cells shields the tumour cell from immune recognition by the cellular arm of the immune system, thus favouring metastases. Tumour cells expressing sialomucin have been shown to be less sensitive to cytolysis by human lymphokine-activated killer lymphocytes (van Rinsum *et al.,* 1986; Moriarty *et al.,* 1990). Therefore, sialylation of the MUC1 mucin might be related to the invasiveness of gastric cancer cells and consequently, to the poor survival of the patients whose tumours express the sialylated MUC1 epitope. In addition to the impact of MUC1 expression on invasiveness and metastasis, this possible immunosupression in adenocarcinomas may affect poor outcome.

1.19 Mucin core glycans: Tn, sialyl-Tn and T epitopes

Mucins produced by diseased epithelia especially in cancer, are often modified in ways that are distinct from their normal counterparts, and these alterations are believed to contribute to the pathogenesis and progression of these diseases. Neoplastic tissues frequently show aberrant glycosylation of mucins leading to the expression of tumour-associated carbohydrate antigens which are often comprised of simple mucin-type carbohydrate antigens such as Tn, Sialyl-Tn and T (Thomsen-Friedenreich) (Hirohashi *et al.,* 1985; Kjeldsen *et al.,* 1988; Ho and Kim, 1991; Lesuffleur *et al.,* 1994; Kim *et al.,* 1996).

In normal mucin, this disaccharide core region coexists with many other long oligosaccharides on the mucin polypeptide backbone which act as a cover to prevent anti- Sialyl-Tn antibodies from binding to the “cryptic” Sialyl-Tn antigen of normal

mucin. However, in cancer the cryptic core region oligosaccharides are exposed (Figure 1.13) as a result of incomplete glycosylation synthesizing only short oligosaccharides (Hakomori, 1999) (Figure 1.13).

The linkage GalNAc-ά-O-ser/thr, is known as the Tn antigen (Figure 1.13) and was first described as a tumour-associated antigen by Springer *et al.* (1974). Tn is predominantly expressed in columnar cells (Carneiro *et al.,* 1994). Tn and T structures on mucin peptide cores expressed by adenocarcinomas contribute to adhesion, cell aggregation, invasion and metastasis (Gendler, 2001; Glinsky *et al.,* 2003). Thus, a prevalence of Tn antigens on a cancer cell usually denotes a highly aggressive and metastatic cancer irrespective of the type of organ affected.

Sialyl-Tn is a mucin-associated carbohydrate antigen which is not expressed by normal mucus-producing cells of the stomach but becomes expressed in metaplastic, pre-malignant and malignant gastric tissues. Sialyl-Tn is predominantly expressed in goblet cells (Carneiro *et al.,* 1994). It serves as an excellent marker of small intestinal mucins and is indicative of small intestinal-type differentiation in two thirds of gastric cancers. Addition of sialic acid forms the Sialyl-Tn antigen (Sia alpha2, 6GalNAcalpha-O-ser/thr), identified as a tumour-associated antigen by Kjelden *et al.* (1988). Sialyl-Tn on mucin plays a role in the adhesive interaction, which involves both the basement membrane and endothelial-associated ligands (Bresalier *et al.,* 1996).

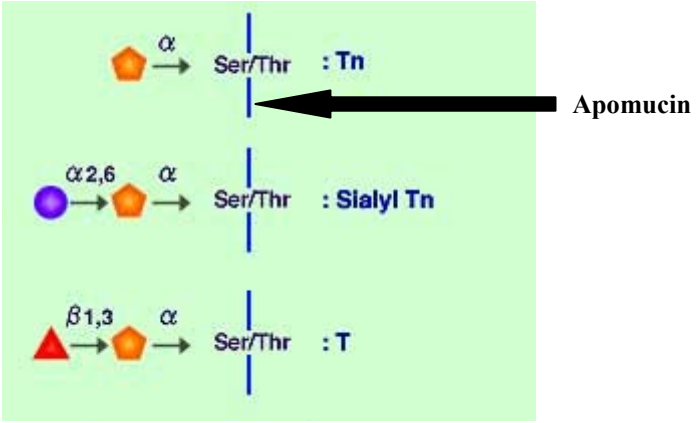


Figure 1.13 The initial step of mucin glycosylation showing Thomsen-Friedenreich epitopes. The first glycan GalNAc (orange) is added by GalNAc-transferases forming the Tn antigens. Using GalNAc as an acceptor, Gal (red) is added by β1,3Gal-transferase to form the T antigen. Alternatively, GalNAc can be sialylated (purple) by sialyl-transferase to form sialyl-Tn antigen. These antigens are masked in normal healthy cells, covered by polysaccharides, and only become unmasked and exposed as a cell moves towards malignancy where the ability to synthesize the polysaccharides which normally cover the T-Tn antigens is often lost (reproduced from www.dadamo.com/wiki/wiki.pl/Thomsen-Friedenreich (T-Tn) antigen).

1.20 Mucin terminal glycans: Lewis and sialyl Lewis antigens

Lewis antigens are carbohydrate structures (structurally related to determinants of the ABO blood group systems) expressed in many tissues throughout the body, especially in the epithelial cells of gastric mucosa. In the normal gastric mucosa, Lewis^{x} and Lewis^{y} antigens are co-localized with MUC6 in the deep glands and also expressed in the chief and parietal cells of the glands. Abnormal expression of MUC6 and Lewis^{x} in the superficial epithelium of the stomach from *H pylori* infected patients has been described (Boren *et al.,* 1993). Lewis^{x} has been related to an increased risk of metastasis and poor prognosis in gastric cancer patients. Lewis^{a}, Lewis^{b}, and sialyl-Lewis^{a} are co-localized in the superficial cells expressing MUC5AC (de Bolos *et al.,* 1995).

Lewis^{b}, expressed in the surface epithelial cells (Boren *et al.,* 1993) in a healthy stomach has been shown to mediate the attachment of *H. pylori* to the human gastric mucosa (Boren *et al.,* 1993) and to MUC5AC (Linden *et al.,* 2002), whereas sialylated Lewis antigens contribute to binding in inflamed tissue (Mahdavi *et al.,*

2002). More than 80% of *H. pylori* strains express Lewis blood group antigens, Lewis^x and/or Lewis^y, on their lipopolysaccharide (LPS) (Appelmelk *et al*., 2000; Simoons-Smit *et al*., 1996).

During the process of malignant transformation these antigens undergo important alterations (Coon and Weinstein *et al*., 1986; Sakamoto *et al*., 1989). Torrado *et al.* (1992) demonstrated that the simultaneous expression of Lewis^a antigen and sulfomucins indicate a greater risk of pre-neoplastic progression.

Sialyl-Lewis structures present on the surface of tumour cells are carried by the carbohydrate chains of N-glycans or O-glycans (Maemura and Fukuda 1992). High levels of sialylated antigens, such as sialyl-Lewis^x and Sialyl-Lewis^a antigens are carried by the abnormal mucins of cancer cells. Shimodaira *et al.* (1997) showed that sialyl-Lewis^a and sialyl-Lewis^x antigens on O-glycans are associated with the invasion of cancer cells. *Borsig et al.* (2001) showed that the removal of cancer mucin attenuated metastasis through the reduction of sialylated antigens. The expression of sialyl-Lewis^x and sialyl-Lewis^a in mucin-type O-glycans has been demonstrated to be highly correlated with venous invasion and lymph node metastasis (Shimodaira *et al.,* 1997) and is especially involved in the formation of liver and lung metastases by human gastric, colon, and pancreatic cancer cells (Nakagoe *et al.,* 2002; Opolski *et al*., 1998)

1.21 Objectives of this study

Our laboratory (Mall *et al.,* 1990, 1992, 1999, 2000) reported the presence of an albumin associated 55-65 kDa glycoprotein on 7.5%, 10% and 4-20% gradient polyacrylamide gel electrophoresis, extracted from crude mucus scrapings of gastric ulcer and cancer resection specimens and which was not present in normal gastric tissue. This fragment fractionated with purified gastric mucin at a density of approximately 1.39–1.42 g/ml in a CsCl density gradient after ultracentrifugation (Mall *et al.,* 1990, 1992, 1999, 2000). In the current study, using further extensive SDS-PAGE analysis (12% polyacrylamide) and a better quality molecular weight marker, we reproduced this finding, but the size of the fragment, previously reported to be M_r ~ 55-65 kDa, was now consistently 40-50 kDa on SDS-PAGE. There was

also a better separation of this fragment and albumin which appeared as a complex in previous studies (Mall *et al*., 1990, 1992, 1999, 2000). A similar finding was made by Hakkinen *et al.* (1991) who reported a structure associated with gastric mucins in carcinoma that could be useful as a clinical marker for gastric cancer.

The primary objective of our present study was to develop a rabbit polyclonal antibody against the 40-50 kDa glycoprotein found to co-fractionate with mucin purified from specimens resected for gastric cancer. The second objective was to compare, by immunohistochemistry, the tissue localization and expression of the 40-50 kDa glycoprotein with that of gastric mucins in the normal stomach, intestinal metaplasia and in cancer. Our final aim was to identify this glycoprotein by proteomic analysis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Ethics

The University of Cape Town Research Ethics Committee approved this study (ethics approval number REC REF: 084/2002).

2.2 Materials

This Chapter describes the general laboratory reagents and methods used during the course of this research project. Nitrocellulose membrane and dialysis tubing was from Kimix (Chemical and Laboratory Suppliers, SA). The ECLTM Western blotting detection kit was from Amersham Biosciences (Amersham UK). Monoclonal Armenian hamster anti-MUC1 raised against 17 amino acids (SSLSYTNPAVAATSANL) to the cytoplasmic tail of MUC1 was from Sandra Gendler (Mayo Clinic, Scottsdale, USA). Monoclonal mouse anti-MUC1 core raised against the tandem repeat epitope (TRPAG), anti-MUC2 raised against synthetic peptides NGLQPVRVEDPDGC, anti-MUC5AC raised against RNQDQGPKFMC and anti-MUC6 raised against synthetic peptide of the MUC6 tandem repeat sequence were from Novocastra (Newcastle, UK). Monoclonal mouse anti-MUC4 antibody raised against a synthetic peptide TSSASTGHATPLPVTD corresponding to the tandem repeat sequence of MUC4 and polyclonal goat anti-mouse horse radish peroxidise (HRPO) linked and biotinylated goat anti-Armenian hamster secondary antibodies were from Santa Cruz Biotechnology, Inc (Santa Cruz, California). Envision+® system labelled polymer-HRP anti-mouse, anti-rabbit and 3,3'-diaminobenzidine (DAB) substrate chromogen and polyclonal rabbit anti-Human Albumin were from Dakocytomation (USA).

Guanidinium chloride (GuHCl), caesium chloride (CsCl), pararosanaline, 3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propane-sulfonate (CHAPS), agarose, N-ethylmaleimide (NEM), N, N'-methylenebisacrylamide, 2-mercaptoethanol, iodoacetamide (IAA), poly-L-lysine, Ponceau S (C.I. 27195), ammonium persulphate

(APS), glycerol, N, N-dimethyl-meta-phenylenediamine-dihydrochloride (1,3), N, N-dimethyl-para-phenylenediamine dihydrochloride (1,4), glycine, thiourea, entellan, urea, aminopropyl-tri-ethoxysilane (A.P.E.S), acetone and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (UK).

Phenylmethylsulfonylfluoride (PMSF), N, N, N, N'-tetramethylethylenediamine (TEMED), Coomassie Brilliant Blue R-250, ethylenediaminetetra-acetic acid disodium salt (Na_2-EDTA), polyoxyethylene sorbitan monolaurate (Tween 20), acetone, acetonitrile, glacial acetic acid, hydrochloric acid, propan-2-ol (iso-propyl alcohol), magnesium sulphate, methanol, xylol, copper sulphate, citric acid, sodium iodate, aluminium ammonium sulphate, sodium di-hydrogen orthophosphate, formalin, hydrogen peroxide, folin reagent, thymol, glass slides and cover slips were purchased from Merck (UK).

Human serum albumin (HSA) was purchased from the Western Cape Blood Transfusion Service (Parow, South Africa). Ampholytes and Bradford assay solution were purchased from BIO-RAD. Mineral oil was purchased from GE Healthcare. Haematoxylin, eosin, hydrogen peroxide, hydrochloric acid (HCl), sodium metabisulphate, phloxine, periodic acid, acetic acid, sodium chloride, potassium chloride, alcian blue, potassium metabisulphite, charcoal hydrate and sodium bicarbonate were from British Drug Houses (BDH Chemicals Ltd). Alcohols were from Saarchem Chemicals (SA). Ammonium bicarbonate and trypsin were purchased from Promega (SA).

2.2.1 Standard equipment used throughout this project

A Hitachi U 2000 spectrophotometer, a Hitachi Himac high speed centrifuge, a Beckman L8-70m ultracentrifuge, a Christ ALPHA 1-5 freeze drier, a Hoefer SE600 slab gel system and a Janke and Kunkel Ultra-turrax were standard items of equipment used throughout the project. All equilibrium density gradients were performed in a Beckman 60 Ti titanium rotor using polyallomer tubes (capacity 40 ml). Pressure cooker for antigen retrieval, microtome for cutting sections, hot oven, waterbath set at 45ºC for floating tissue sections, Leica TP 1020 and EG 1140H/C

machines were used for processing and embedding tissue. Leica 2000R sliding microtome was used for cutting tissue sections from paraffin wax blocks.

2.3 Patients

Mucus scrapes were obtained from 27 patients who had undergone gastrectomy (16 partial and 11 total resections) between 2005 and 2008 from Groote Schuur Hospital (n=23) and the University of Cape Town Private Academic Hospital (n=4). Information collected from the patients' medical and pathological records about patient age, gender, specimen type, tumour type, stage and grade are summarised in Table 1.

Table 1. Clinical and pathological data of patients in this study

Lab number	Age	Gender	Hospital	Specimen	Type of cancer	TNM staging	AJCC histologic grade
S1	60	M	GSH	**Partial gastrectomy** Specimen size: lesser curvature 70mm, greater curvature 100mm	**Signet-ring cell** Tumour size: 20 x 20 x 15mm	T2N1M0	G3
S2	55	F	GSH	**Total gastrectomy** Specimen size: lesser curvature 100mm, greater curvature 220mm	**Signet-ring cell** Tumour size: 70 x 80mm	T4N2M0	G3
S3	59	M	GSH	**Total gastrectomy** Specimen size: lesser curvature 180mm, greater curvature 260mm	**Signet-ring cell** Tumour size: 70 x 50mm	T3N2M1	G3
S4	59	M	GSH	**Partial gastrectomy** Specimen size: lesser curvature 120mm, greater curvature 160m	**Signet-ring cell** Tumour size: **N/A**	T4N2M0	G3
S5	58	M	GSH	**Partial gastrectomy** Specimen size: lesser curvature 120mm, greater curvature 150mm	**Diffuse type** Tumour size: 30 x 150mm	T3N2M0	G2
S6	59	F	GSH	**Total gastrectomy** Specimen size: lesser curvature 160 mm, greater curvature 365mm	**Diffuse type** Tumour size: 45 x 120 x 12mm.	T3N2M0	G3
S7	43	M	UCT	**Partial gastrectomy** Specimen size: lesser curvature 55mm, greater curvature 90mm	**Diffuse type** Tumour size: 65 x 38 x 18mm.	T3N1M0	G4
S8	73	F	GSH	**Partial gastrectomy** Specimen size: lesser curvature 80mm, greater curvature 150mm	**Diffuse type** Tumour size: 150mm	T4N0M0	G3
S9	60	F	GSH	**Partial gastrectomy** Specimen size: lesser curvature 90mm, greater curvature 150mm	**Diffuse type** Tumour size 450mm	T3N0M0	G3
S10	74	F	GSH	**Partial gastrectomy** Specimen size: lesser curvature 100mm, greater curvature 270mm	**Diffuse type** Tumour size: **N/A**	T1N0M0	G3
S11	57	M	UCT	**Total gastrectomy** Specimen size: lesser curvature 40mm, greater curvature 150mm	**Diffuse type** Tumour size: **N/A**	T4N1M0	G3
S12	60	M	GSH	**Total gastrectomy** Specimen size: lesser curvature 150mm, greater curvature 200mm	**Mixed intestinal and diffuse** Tumour size: **N/A**	T3N2M1	G3
S13	55	M	GSH	**Total gastrectomy** Specimen size: lesser curvature 190mm, greater curvature 400mm	**Intestinal type** Tumour size: 40 x 30mm	T4N1M0	G3
S14	53	M	GSH	**Partial gastrectomy** Specimen size: lesser curvature 80mm, greater curvature 140mm	**Intestinal type** Tumour size: 25 x 20 x 7mm	T2N2M0	G3
S15	63	M	GSH	**Total gastrectomy** Specimen size: lesser curvature 160mm, greater curvature 300mm	**Intestinal type** Tumour size: 24 x 39 x 9mm	T3N1M0	G3
S16	63	M	GSH	**Total gastrectomy** Specimen size: lesser curvature 120mm, greater curvature 200mm	**Intestinal type** Tumour size: 90 x 90 x 30mm	T1N0M0	G2
S17	67	M	GSH	**Partial gastrectomy** Specimen size: lesser curvature 65mm, greater curvature 70mm	**Intestinal type** Tumour size: 45 x 30 x 12mm	T2N1M0	G3
S18	70	M	GSH	**Total gastrectomy** Specimen size: lesser curvature 45mm, greater curvature 85mm	**Intestinal type** Tumour size: 41 x 45 x 11mm	T2N1M0	G2
S19	63	M	GSH	**Total gastrectomy** Specimen size: **N/A**	**Intestinal type** Tumour size: **N/A**	T4N2M0	G2
S20	63	M	UCT	**Partial gastrectomy** Specimen size: lesser curvature 75mm, greater curvature 150mm	**Intestinal type** Tumour size: 80 x 45 x 45mm.	T4N1M1	G3
S21	61	M	GSH	**Partial gastrectomy** Specimen size: greater curvature 120mm	**Intestinal type** Tumour size: 40mm	T2N0M0	G1
S22	59	M	GSH	**Total gastrectomy** Specimen size: lesser curvature 120mm, greater curvature 150mm	**Intestinal type** Tumour size: **N/A**	T4N1M0	G2
S23	64	F	GSH	**Partial gastrectomy** Specimen size: lesser curvature 70mm, greater curvature 160mm	**Intestinal type** Tumour size: 65 x 72 x 6mm.	T2N1M0	G1
S24	67	M	GSH	**Partial gastrectomy** Specimen size: lesser curvature 105mm, greater curvature 130mm	**Intestinal type** Tumour size: 24 x 39 x 9mm	T3N0M0	G3
S25	51	F	GSH	**Partial gastrectomy** Specimen size: lesser curvature 70mm, greater curvature 140mm	**Intestinal type** Tumour size: 20 x 22 x 9mm	T2N0M0	G2
S26	60	M	UCT	**Partial gastrectomy** Specimen size: lesser curvature 90mm, greater curvature 130mm	**Mucinous carcinoma** Tumour size: 20 x 30 x 6mm	T1N1M0	G2
S27	64	F	GSH	**Partial gastrectomy** Specimen size: lesser curvature 115mm, greater curvature 175mm	**Mucinous carcinoma** Tumour size: 65 x 72 x 6mm	T2N2M0	G3

N/A: not available

AJCC: American Joint Committee on Cancer

2.4 Sample collection

2.4.1 Collection of gastric mucus

Crude mucus was obtained from resected specimens of patients with various cancers of the stomach. Total or partial gastrectomy specimens were provided by a surgeon within 20 min of a patient having undergone surgery for carcinoma of the stomach. The resected stomachs were opened along the greater curvature to expose the mucosal surface and the gastric mucus was collected by gently scraping the mucosa of these stomachs with two sterile glass slides. Mucus was collected into containers on ice containing 6 M guanidinium HCl containing a cocktail of protease inhibitors, 10 mM EDTA, 5 mM NEM and 1 mM PMSF pH 6.5. Specimens were then transported (on ice) from the operating theatre to the laboratory where further analysis was done. Some clinical and pathological features of the diseased stomachs are given in Table 1. Mucosal scrapes from human donor stomachs (used in previous gastric studies) were obtained from the -80°C freezer in our laboratory and used as controls.

2.4.2 Collection of human gastric tissue blocks

Formalin fixed paraffin wax embedded tissue blocks were obtained from the archives of the Division of Anatomical Pathology, National Health Laboratory Service (NHLS) - Groote Schuur Hospital. All resected tumours were histologically stained by haematoxylin and eosin and the diagnosis was confirmed by a pathologist (Professor D. Govender). These samples included 3 normal mucosa sampled away from tumour, 17 normal mucosa immediately adjacent to tumour, 13 intestinal type carcinomas, 7 diffuse carcinomas, 1 mixed intestinal type and diffuse carcinoma, 4 signet ring carcinomas and 2 mucinous carcinomas.

2.5 Sample preparation and solubilisation of mucus

Gastric mucus samples were subjected to homogenization with a Junkel and Kunkel Ultra-Turrax (1 min, 9500 rev/min at room temperature) to disaggregate mucus and increase its solubility. After homogenisation, gastric samples were solubilised by mixing overnight at 4^{o}C on a revolving rotor and mucins were extracted in the

following manner: samples were spun at high-speed centrifugation (Hitashi Himag Centrifuge) at 10 000 x g for 1 h at 4°C to remove the insoluble debris from the soluble mucus. After centrifugation, the soluble material was removed, and the pellet (if any) was re-extracted twice as described above. If an insoluble pellet still remained after the re-extractions, it was reduced with 10 mM DTT for 5 h in 6 M GuHCl, 10 mM EDTA and 5 mM NEM at 37ºC. Subsequent alkylation was with 25 mM iodoacetamide (IAA) for 18 h in the dark. Centrifugation (10 000 x g for 1 h at 4°C) was then performed to recover any soluble mucus from the reduced pellet and also to remove and discard the remaining insoluble material. Samples were analysed separately for each patient.

2.6 Gastric mucin purification

Gastric mucus samples obtained after extraction and/or reduction and alkylation of the insoluble pellet were prepared for purification by adding solid caesium chloride (CsCl) in 4 M GuHCl to adjust the density to 1.39-1.40 g/ml (Creeth and Denborough, 1970). Isopycnic density gradient ultracentrifugation was performed twice for 48 h at 105 000 x g (Beckman L45 centrifuge). After ultracentrifugation, tubes were demarcated into 9 equal fractions with the top fractions (1-3) containing proteins and lipids, the middle fractions (4-6) containing mucins and the lower fractions (6-9) containing nucleic acids. The density of each fraction was determined. The protein and glycoprotein content of each fraction was measured after dialysis (24 h) against distilled water to remove caesium and guanidinium chloride. The purified mucin fractions of each sample obtained after ultracentrifugation, were pooled, dialysed against distilled water and freeze dried.

2.7 Analytical procedures

2.7.1 Glycoprotein estimation

Mucins were estimated by the periodic acid Schiff (PAS) procedure of Mantle and Allen. (1978). Aliquots of each fraction after caesium chloride density gradient ultracentrifugation were dialysed against distilled water to remove excess caesium chloride. Samples were incubated with periodic acid for 60 min at 37°C. Decolourised

Schiff reagent was then added and after 30 min the sample absorbance was read at 555 nm.

2.7.2 Protein assay

After density gradient centrifugation and dialysis, the protein content of the fractions was determined by the Lowry assay (1951).

2.7.3 Bradford assay

Protein quantification was also performed using the modified Bradford assay method **(**Bradford, 1976), as modified by (Ndimba *et al.,* 2003), using BSA as a standard to generate the calibration curve. The absorbance was measured at a wavelength of 595 nm using the Genesis 5 Spectrophotometer (Milton Roy, Groton, CT, USA).

2.8 Dialysis of samples

Prior to any analysis or assay, the mucin rich fractions were pooled and dialysed overnight by constant stirring (using Spectrapore dialysis tubing prepared by boiling in distilled water containing 0.5% sodium bicarbonate/EDTA for 1 min) against four changes of distilled water at 4°C to remove GuHCl and caesium chloride salts which might interfere with biochemical procedures.

2.9 Freeze drying

Dialysed mucin samples were transferred into collecting jars which were immersed in liquid nitrogen and snap frozen. The jars were loaded into Virtis vacuum flasks which were connected to a Christ ALPHA 1-5 freeze drier and samples were freeze dried at -54^{o}C. Lyophilised mucins were stored at -20^{o}C until required.

2.10 One-dimensional Sodium dodecyl sulfate polyacrylamide-gel electrophoresis

Freeze dried, purified gastric mucin samples (300-500 µg each) were prepared in reducing gel loading buffer containing 2% sodium dodecyl sulfate (SDS), 10%

glycerol, 0.01% bromophenol blue and 5% 0.2 M 2-mercaptoethanol and boiled for 2 min prior to loading. One-dimensional (1-D) sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) 10% and 12% (w/v) was performed according to the method of Laemmli (1970) using the Hoeffer Mighty Small mini-electrophoresis system. Samples and molecular weight markers were electrophoresed simultaneously at a constant current of 20 mAmps per gel until the dye front ran out of the gel (2-3 h).

2.11 Staining gels

2.11.1 Coomassie Brilliant Blue R-250 gel staining

After electrophoresis, gels were immersed in Coomassie brilliant blue staining solutions 1, 2, and 3 prepared from stock solution 1.25% (w/v) Coomassie brilliant blue in distilled water and heated in a microwave for about 1 minute. Gels were stained for about 30 min in each solution with containers shaking on an orbital shaker. Staining solution 1 contained 10% (v/v) glacial acetic acid, 2% (v/v) Coomassie stock, 25% (v/v) propan-2-ol, staining solution 2 contained l0% (v/v) glacial acetic acid, 0.25% (v/v) Coomassie stock, 10% (v/v) propan-2-ol and staining solution 3 contained 10% (v/v) glacial acetic acid, 0.25% (v/v) Coomassie stock. Destaining solution contained 10% (v/v) glacial acetic acid, 1% (v/v) glycerol in water. Once the staining steps were completed, the gels were destained until the desired bands/spots intensity was achieved.

2.11.2 Periodic acid Schiff's gel staining

After electrophoresis, gels were fixed in 50% (v/v) ethanol and oxidized in 1% (v/v) periodic acid made up in 3% (v/v) acetic acid, then stained with PAS and destained in 0.1% sodium metabisulphite according to the method of Dubray and Bezard (1982).

2.11.3 Ponceau S gel staining

SDS-PAGE from which the 40-50 kDa glycoprotein was isolated for immunization was stained with Ponceau S (C.I. 27195) for 5 min. Gels were then immersed in distilled water until the high background was cleared and protein bands were visible.

2.12 Isolation of the 40-50 kDa glycoprotein

Gels were electrophoressed in duplicate. The first gel was stained with PAS to highlight the presence of the 40-50 kDa glycoprotein and the second with Ponceau S from which the glycoprotein was cut. The 40-50 kDa glycoprotein was cut as a band just below albumin and between M_r 40 and 50 kDa.

2.13 Antibody production

Immunization was carried out as described by Bellstedt *et al*. (1987). In brief, a three month old female rabbit was immunized intravenously with the 40-50 kDa glycoprotein complexed to acid-treated Salmonella minnesota R595 bacteria so that the ratio by dry mass of protein to bacteria was 1 to 5. The amount of 240 µg (40 µg of protein and 200 µg of naked bacteria) of the complex used at every immunization was suspended in 0.5 ml PBS and injected intravenously in the peripheral ear vein. The rabbit was immunized on days 1, 4, 7, 14, 17, 21, 28, 31 and 35 respectively. Boosts consisting of 3 immunizations within a week of the same amount of protein naked bacteria complex were administered intravenously in order to collect more serum. Blood (2.0 ml) was drawn from the central ear artery using a syringe prior to immunization as a negative control. Blood (2.0 ml) was drawn on day 28 to test whether the animal was responding. On day 42 when optimal antibody production was routinely achieved using this method, 24.0 ml of blood was drawn. A week after boosts, blood was drawn again (24.0 ml). The blood was allowed to clot, centrifuged and the antiserum separated from the blood. Antiserum was stored at -18ºC. Antibody activity was assessed by intensity of staining in western blot.

2.14 Dot blot

Mucin samples (crude and purified) and Bovine Serum Albumin in sample loading buffer were applied directly onto a nitrocellulose membrane. Samples were then vacuum blotted at pressure 40-50 bars for 1 h. Membranes were probed with anti-(40-50 kDa glycoprotein) antibody and the respective secondary antibody (goat anti-rabbit HRP). Detection was carried out using an ECL kit.

2.15 Western blotting

The reactivity of the polyclonal rabbit anti-(40-50 kDa glycoprotein) against the antigenic 40-50 kDa glycoprotein was determined by western blot. Gastric samples purified from cancer resected specimen were electrophoresed in both 10% and 12% (w/v) SDS-PAGE, and then transferred onto nitrocellulose membranes (Nitrocellulose, 0.22 Micron) using a transfer buffer containing 192 mM glycine, 25 mM Tris, 1.3 mM SDS and 20% (v/v) methanol and a semi-dry electroblotting unit at 0.8mA/cm^2 for 1h. After electro-blotting, non-specific binding was blocked by incubating the membranes in 5% (w/v) low fat milk powder in TBS, 0.05% (v/v) Tween-20 (TBST) at 4^{o}C for 1h. The membranes were then washed with TBST (3 × 5 min) and probed for 1 h with rabbit anti-(40-50 kDa glycoprotein) antibody.

The anti-(40-50 kDa glycoprotein) antibody was prepared in two ways, one with Human Serum Albumin (HSA, 20% (v/v) protein solution) and one without. The antibody was diluted in TBST and incubated with Human Serum Albumin in a 1 : 500 dilution at 37°C for 1 h. The cocktail was adjusted to a final dilution of 1 : 5000 with 5% (w/v) low fat milk powder made up in TBST. The membranes were washed (3 × 5 min) with TBST and incubated for 1 h with HRPO linked secondary antibody to goat anti-rabbit diluted in 5% (w/v) low fat milk powder in TBST at dilutions of 1 : 5000. After another TBST wash (3 × 5 min), bands were detected using an Enhanced Chemiluminescence (ECL; Amersham, Buckinghamshire, UK) kit.

2.16 Histology

2.16.1 Sample preparation

Formalin fixed paraffin wax embedded gastric tissue blocks were cut to 2 μm sections on a sliding microtome. These blocks were matched to the patients that had mucus scraped from their stomachs and used for the biochemical analysis.

2.16.2 Histochemistry

All the sections (2 μm) from paraffin wax embedded tissue blocks were stained with haematoxylin and eosin (H&E) as routine stain for morphology and diagnosis. Selected sections were stained with periodic acid-Schiff (pH 2.5)/Alcian blue (PAS/AB) to identify neutral mucins and sialomucins and with high iron diamine/alcian blue (HID/AB) to identify sialomucins and sulfomucins. Intestinal metaplasia was classified according to Filipe and Jass. (1986) as follows: complete (Type I) with mature absorptive cells (neutral mucins) and goblet cells (sialomucins); incomplete (Type II) with few or no absorptive cells (neutral and sialomucins) and goblet cells (sialomucins or little sulfomucins, or both); and incomplete (Type III) with columnar cells (predominantly sulfomucins) and goblet cells (sialomucins, sulfomucins, or both). All the slides were assessed with the assistance of a chief technologist and a pathologist (Professor D. Govender).

2.16.3 Immunohistochemistry

Tissue sections (2 μm) cut from paraffin wax embedded tissue blocks matched to the patients that had mucus scraped from their stomachs were fixed onto APES (amino-propyl-tri-ethoxy-silane) coated glass slides overnight in an incubator at 55ºC. Sections were dewaxed in xylol and rehydrated through descending graded alcohols to distilled water. Endogenous peroxidase was blocked by treating slides with 3% (w/v) hydrogen peroxide/methanol solution for 15 min. Heat-mediated antigen retrieval using citrate buffer (0.01 M, pH 6.0) was performed in a Presto pressure cooker (Amalgamated Appliances Holdings, Reuven, South Africa), for 2 min at full

pressure for sections probed with the respective mucin antibodies. No retrieval was applied for sections probed with anti-(40-50 kDa glycoprotein) antibody.

Non-specific binding for all antibodies was blocked with non-immune normal goat serum for 10 min (1 : 20) followed by optimally diluted primary antibody (Table 2) and the respective secondary antibody (Envision). Tissue sections fixed onto APES coated slides were incubated with primary rabbit anti-(40-50 kDa glycoprotein) polyclonal antibody, mouse anti-human monoclonal antibodies to (MUC1, MUC1core, MUC2, MUC4, MUC5AC and MUC6) and rabbit anti-human-albumin polyclonal antibody. The antibodies used in this study and their specificities are listed in Table 2. The anti-(40-50 kDa glycoprotein) antibody was diluted in PBS and HSA (1 : 500) and incubated at 37°C for 1 h. All mucin antibodies were diluted in PBS.

All batches included a negative reagent control and a negative as well as positive tissue control. For negative reagent control, the primary antibody was either replaced with immunoglobulins of the same class or with PBS buffer. For negative tissue control, we used tissue in which expression would be negative. Selection of a positive and negative control tissue for the anti-(40-50 kDa glycoprotein) antibody was based on reviewing different tissues (breast, lung, liver, small intestine, stomach, ovary, gall bladder) until the optimum staining tissue was obtained for the positive control and not obtained for the negative control. For positive controls, small intestine tissue was used for anti-(40-50 kDa glycoprotein) antibody, breast cancer tissue for MUC1 and MUC1 core, normal colon for MUC2, normal pancreas for MUC4, normal stomach for MUC5AC and MUC6 and normal liver for anti-human albumin. Sections were then incubated with the respective secondary antibody (Envision anti-mouse and anti-rabbit HRP) (see Table 2 for list of antibodies).

Finally, sections were developed using diaminobenzidine (DAB) as a chromogen (1 drop of DAB in 1 ml of cytomation substrate buffer) for 10 min. Sections were rinsed with PBS and water to remove chromogen (stop the reaction) prior to colour enhancement with 1% (v/v) copper sulphate for 10 min. Sections were counter stained with Mayer's haematoxylin and Scott's water for 1-2 min and dehydrated with 70%, 96% (v/v) and absolute alcohols for 10 sec each before being cleared in xylol. Finally slides were cover slipped using Entellan and viewed under the light microscope.

2.16.4 Scoring of immunostaining in gastric tissue

A semi-quantitative analysis was performed to evaluate the expression of the 40-50kDa glycoprotein and mucins. We examined 100 fields of each tissue (normal, intestinal metaplasia and cancer) at ×400 magnification and scored the proportion of staining as 0 (<5%) for non-stained cells, 1+ (5-25%) few positive cells; 2+ (26-50%) well defined areas with positive cells, 3+ (51-75%) extensive areas with positive cells and 4+ (>75%) most cells stained.

Table 2. Antibodies used in this study

Primary antibodies		
Antibody type	Antigen	Dilutions and incubation time
Polyclonal rabbit anti-(40-50kDa glycoprotein)	40-50kDa glycoprotein	1 : 500 (1 h)
Polyclonal rabbit anti-Human Albumin	Albumin isolated from human serum	1 : 500 (1 h)
Monoclonal armenian hamster anti-MUC1	17 amino acids to the cytoplasmic tail of MUC1 (SSLSYTNPAVAATSANL)	1 : 100 (2 h)
Monoclonal mouse anti-MUC1core	Synthetic peptide in tandem repeat region TRPAG of MUC1 core	1 : 100 (30 min)
Polyclonal rabbit anti-MUC2	Synthetic peptide NGLQPVRVEDPDG of MUC2	1 : 100 (1 h)
Monoclonal mouse anti-MUC4	Synthetic peptide in tandem repeat region TSSASTGHATPLPVTD of MUC4	1 : 100 (overnight, 4°C)
Monoclonal mouse anti-MUC5AC	Synthetic peptide RNQDQGPKFMC of MUC5AC	1 : 100 (30 min)
Monoclonal mouse anti-MUC6	Synthetic peptide in tandem repeat region of MUC6	1 : 100 (2 h)
Secondary antibodies		
Goat anti-armenian hamster (biotinylated) antibody		1 : 400 (30 min)
Goat anti-rabbit, HRPO linked antibody		Envision (30 min)
Goat anti-mouse, HRPO linked antibody		Envision (30 min)

2.17 Proteomic analysis

2.17.1 Reswelling of the IPG strips

Immobiline pH gradient (IPG) strips (7 cm; pH 3-10, BIO-RAD) were rehydrated overnight at room temperature in a total volume of 125 µl of rehydration buffer containing: 2 µl of 50% (w/v) DTT, 1.25 µl of ampholytes (pH 3-10), 100 - 200 µg of mucin sample solubilised in urea buffer (9 M urea, 2 M thiourea and 4% (w/v) CHAPS), and adjusted with urea buffer to constitute a total volume 125 µl in a rehydration/reswelling tray. The cover plastic was carefully removed from the IPG strips without damaging the gel and the IPG strip was gently laid on top of the sample in the tray channel with the gel side facing down and taking care to avoid trapping air bubbles under the strip. The IPG strips were overlaid with mineral oil in order to prevent drying out of the strips during the rehydration process. The strips were allowed to rehydrate overnight at room temperature on a perfectly horizontal surface. A standard temperature (20°C) was maintained as the rehydration buffer is mostly composed of urea. Urea at temperatures >37°C carbamolytes and at a temperatures <10°C crystallizes.

2.17.2 Isoelectric focusing (IEF) of the IPG strips

After overnight rehydration, the IPG strips were removed from the reswelling tray, rinsed with distilled and deionised water. By doing this, the excess rehydration buffer is removed preventing urea recrystallization, which could cause prolonged isoelectric focusing times and bad resolutions on the gels. The rinsed and blot-dried strips were then transferred to an IEF machine (Ettan^{TM}IPGphorII^{TM} IEF machine, GE Healthcare, Amersham, UK) with the gel side facing upward. The IPG strips were once again overlaid with mineral oil and the IEF machine was programmed in a step wise programme for a total of 12 000 Vhrs at 20°C for about 6 h.

2.17.3 Two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis

Following IEF, the IPG strips were removed, rinsed with distilled water and blotted on a wet filter paper. The IPG strips were then placed in a clean reswelling tray and equilibrated in an SDS containing buffer (6 M urea, 2% (w/v) SDS, 50 mM Tris/HCl, pH 8.8 and 20% (v/v) glycerol), containing 2% (w/v) DTT for 15 min. This was followed by a further 15 min equilibration in the same solution containing 2.5% (w/v) iodoacetamide instead of DTT. After the second equilibration step the IPG strips were removed and rinsed with 1 x SDS-PAGE running buffer. This step is essential for the transfer of proteins from the 1st dimension IPG strips to the 2nd dimension separation using the SDS polyacrylamide gel. Equilibration was followed by drying the strips and loading them on top of a 12% (w/v) SDS PAGE for separation of proteins according to their molecular mass. The IPG strips were finally overlaid with 0.5% (w/v) agarose sealing solution, which was allowed to solidify before the gels were mounted in the electrophoresis cells. Gels were run at 120 V for about 90 min (at a constant temperature of 25°C) until the bromophenol blue dye reached the bottom of the gel plates as described by Ndimba *et al.* (2005). Protein spots were visualized using Coomassie Brilliant Blue and PAS. Gels were scanned using a Canon scanner (CanoScan 4200F) and the BIO-RAD molecular multimager system**.**

2.17.4 In-gel digestion of protein spots from 2-D gels

Protein spots from Coomassie and PAS stained 2-D PAGE were selected and excised manually using a clean pipette tip or razor blade and put in 1.5 ml Eppendorf tubes. The gel spots were washed (2 x 5 min) in 50 mM ammonium bicarbonate. The solution was discarded at the end of every wash. The washing step was repeated using 50 mM ammonium bicarbonate but this time with vortexing for 30 min. The gel pieces were then washed (2 x 30 min) using 50% (v/v) acetonitrile and 50 mM ammonium bicarbonate with occasional vortexing. This step was repeated until the gel pieces were completely destained. Finally, 100 µl of 100% (v/v) acetonitrile was added to cover the gel pieces and left on the bench top for 5 min to allow the gel pieces to dry. The gel pieces were completely dried using a speed vacuum drier (Speed Vac SC100 (Savant), LASEC, Old Moll Road, Cape Town, RSA). About 10

µl of trypsin in 25 mM ammonium bicarbonate (10µg/ml) was added to the dried gel pieces and incubated at 37°C for 6 h for digestion. After the incubation zip tips were used to clean-up the sample and the quality of digestion was estimated by Matrix assisted laser desorption ionisation time of flight (MALDI-TOF) instrument (Voyager-DETM PRO Biospectrometry TM workstation, Applied Biosciences, Forster City, CA, USA). After the analysis, the digestion process was stopped by the addition of 50 µl of 1% (v/v) TFA. The samples were left for about 3 h at room temperature then stored at 4°C for further analysis.

2.17.5 Analysis using MALDI TOF mass spectrometry

Spots cut from the gel and digested with trypsin were analysed using the Matrix assisted laser desorption ionasation time of flight (MALDI-TOF) instrument (Voyager-DE^{TM} PRO Biospectrometry^{TM} workstation, Applied Biosciences, Forster City, CA, USA). Spectra were externally calibrated using a peptide calibration standard following manufactures instructions. The peptide mixture was analysed using a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) in 50% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid. Peak lists of the tryptic peptide masses were generated from spectrum and searched against the NCBInr databases using the Mascot software (Matrix Science, Ltd., London, United Kingdom; http://www.matrixscience.com). The MALDI-TOF MS identified polypeptide, its respective peptide coverage and a summary of other identified proteins was recorded. The whole protein identification process works in collaboration with database searching, utilizing databases such as SWISS-Prot, TrEMBL and the non-redundant collection of protein sequence at the US National Centre for Biotechnology Information (NCBI), SWISS-2D PAGE for protein identification, NCBI/BLAST and SWISS-PROT.

CHAPTER 3

THE PURIFICATION OF CANCER MUCINS AND THE ANALYSIS OF THE 40-50 kDa GLYCOPROTEIN FRACTIONATING WITH THESE MUCINS

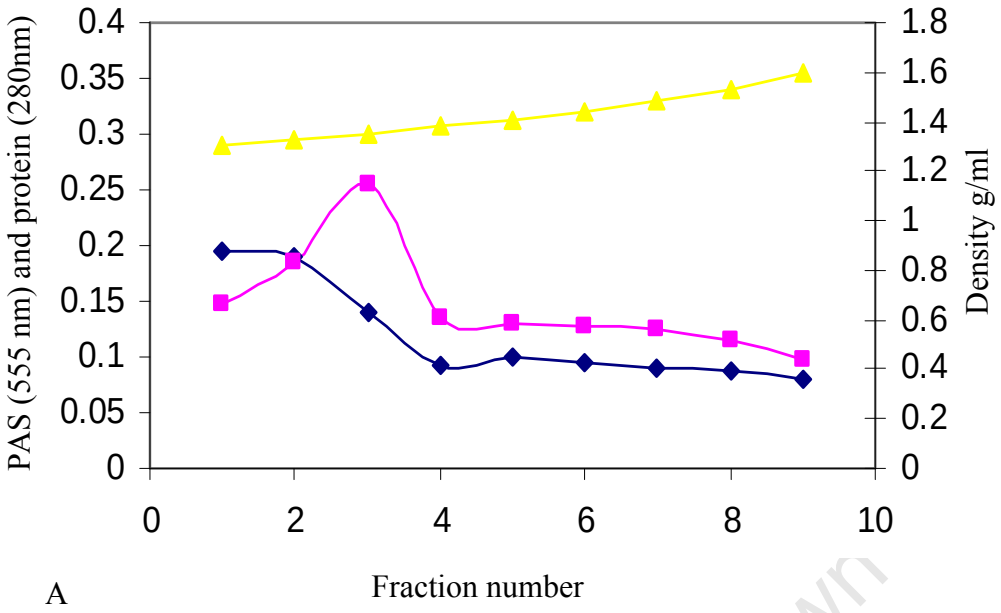
3.1 Introduction

The aim of the study reported in this chapter was to purify mucins from crude mucus scrapings of gastrectomy specimens from patients with carcinoma of the stomach, and then to separate the 40-50 kDa glycoprotein from the purified mucins by SDS-PAGE, and isolate it for the purpose of raising a polyclonal antibody to it. Mucins were purified by a two-step isopycnic density gradient centrifugation in caesium chloride (Creeth and Denborough, 1970). The gels were stained either with PAS to confirm the presence of the 40-50 kDa glycoprotein band or with Coomassie Brilliant Blue to assess the purity of these mucin samples, and with Ponceau S which would allow for the 40-50 kDa to be extracted and used for immunization of a rabbit. The antibody developed was then tested for homogeneity and cross reactivity by dot blot and western blotting methods.

3.2 Results

3.2.1 Purification of gastric cancer mucin

Mucins were purified by density gradient centrifugation, twice in CsCl / 4 M GuHCl at a density of 1.39 g/ml to remove contaminant proteins and nucleic acids (Creeth and Denborough, 1970; Carlstedt *et al.,* 1983). Mucins were estimated by the PAS method of Mantle and Allen, (1978) and protein by the Lowry method (Lowry *et al.,* 1951). The purification profile in Figure 3.1, was obtained after the second spin and demonstrates a clear separation and purification of mucin (PAS positive) from contaminating proteins (Lowry positive) which were successfully removed. The mucin positive fractions (3-6) were pooled and used for SDS-PAGE analysis.



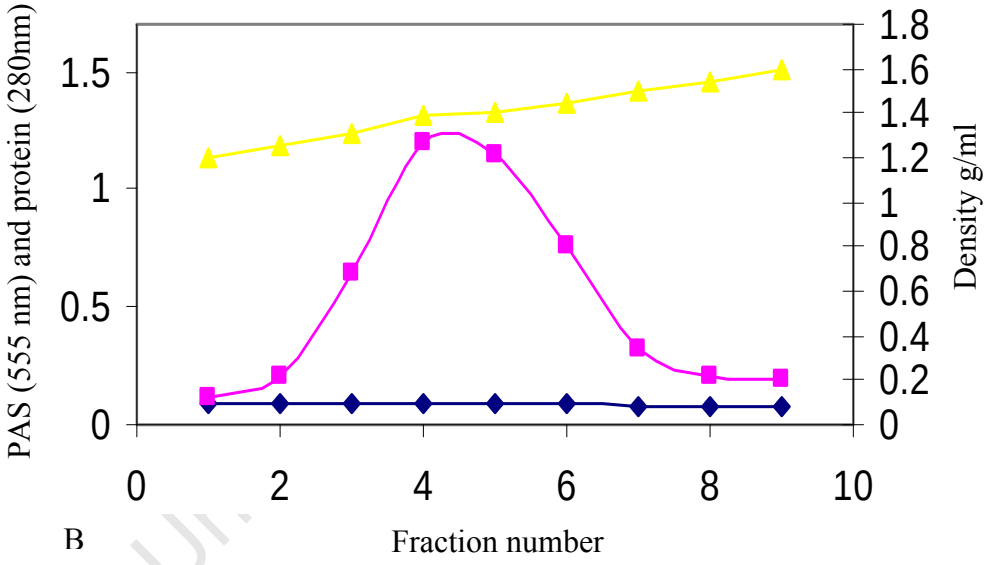


Figure 3.1 Caesium chloride isopycnic density gradient purification of gastric cancer mucin. Crude mucus scraped from stomachs resected for cancer was dissolved in 4 M GuHCl containing 10 mM EDTA, 5 mM NEM and 0.05% CHAPS pH 6.5 and adjusted to a density of 1.39 g/ml with caesium chloride. Density gradient centrifugation was for 48 h (Beckman L45 ultra-centrifuge) at 105 000 x g (4°C). Mucin positive fractions (■) at a density (▲) between 1.37-1.42 that still associated with some protein (♦) (A) were pooled and prepared for the second centrifugation step. Finally fractions (fraction number 3 to 6) clearly free of contaminating proteins (B) were pooled, dialysed against three changes of distilled water at 4°C overnight and freeze-dried.

3.2.2 Characterization of the 40-50 kDa glycoprotein by SDS-PAGE

Purified gastric cancer mucin samples were prepared in gel loading buffer containing 2% SDS, 10% glycerol and 0.01% bromophenol blue. Samples were electrophoresed after reduction with 2-mercaptoethanol. Following electrophoresis, gels were stained with PAS (Figure 3.2A) to confirm the presence of mucins (Figure 3.2A, lane 1-6, blue arrow) and the 40-50 kDa glycoprotein (Figure 3.2A, lane 1-6, black arrow) co-fractionating with these mucins, and with Coomassie Brilliant Blue R-250 (Figure 3.2B), to determine the presence of protein contaminants in the purified mucin preparation. The molecular weights of the components were evaluated by using pre-stained protein molecular-weight standards.

High molecular weight mucin bands with a size greater than 170 kDa were seen at the top of the stacking and running gel (Figure 3.2A, lane 1-6, blue arrow). A band of albumin staining with Coomassie Brilliant Blue R-250 was seen between 66k Da and 70 kDa (Figure 3.2B, lane 2 and 4, red arrow). Two smears were also seen below albumin in the region of 40-50 kDa (Figure 3.2B, lanes 2 and 4, green arrows). Low molecular weight protein bands below 40 kDa and 25 kDa were also seen (Figure 3.2B, lanes 2 and 4).

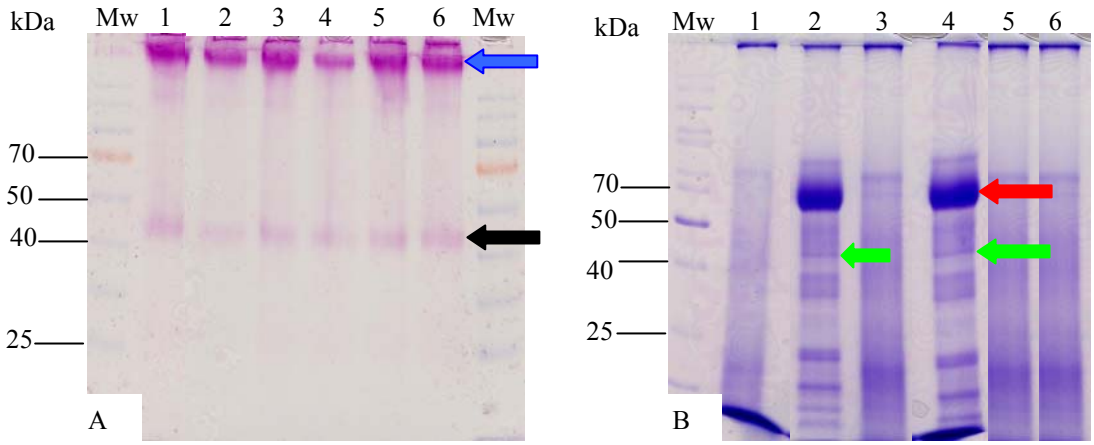


Figure 3.2. SDS-PAGE of mucin purified from stomachs resected for cancer. Freeze-dried samples (300 µg in A and 60 µg in B) of purified mucin were prepared in reducing gel loading buffer and separated on SDS-PAGE (12%). Gels loaded with the same samples were stained with PAS (A) and Coomassie Brilliant Blue R-250 (B). (A and B) in the 1-D gel stained with PAS, the black arrow indicates the 40-50 kDa glycoprotein and the blue arrow indicates mucin purified from gastric cancer specimens. In the 1-D gel stained with Coomassie Brilliant Blue, the green arrows indicate the faint 40-50 kDa protein band and the red arrow shows the band of albumin stained with Coomassie blue. Gels (A) and (B) contain samples in the following order: Lanes 1 (signet-ring cell carcinoma), lanes 2, 3, 5 and 6 (intestinal type carcinoma) whilst lanes 4 (diffuse carcinoma).

3.2.3 Isolation of the 40-50kDa glycoprotein by SDS-PAGE

Gels were electrophoresed in duplicate under the same conditions and stained with PAS (Figure 3.3A) and Ponceau S (Figure 3.3B). To isolate the 40-50 kDa glycoprotein (Figure 3.3A lane 6, circled), the Ponceau S stained gel was cut just below the region where albumin was visible (Figure 3.3B lane 1, red arrow). The illustration of the position of albumin and the 40-50 kDa glycoprotein can be seen in Figure 3.3C lane 11, blue arrow. The gel was loaded with mucin samples to which albumin was added. A clear separation by molecular weight between the 40-50 kDa glycoprotein and albumin was demonstrated by staining the gel with Ponceau S, for albumin (Figure 3.3C lanes 1-12, black arrow), and followed with PAS for the glycoprotein (Figure 3.3C lanes 1-12, blue arrow). From this we were able to confirm that the piece removed from the gel (Figure 3.3D, blue arrow) was indeed the PAS positive glycoprotein.

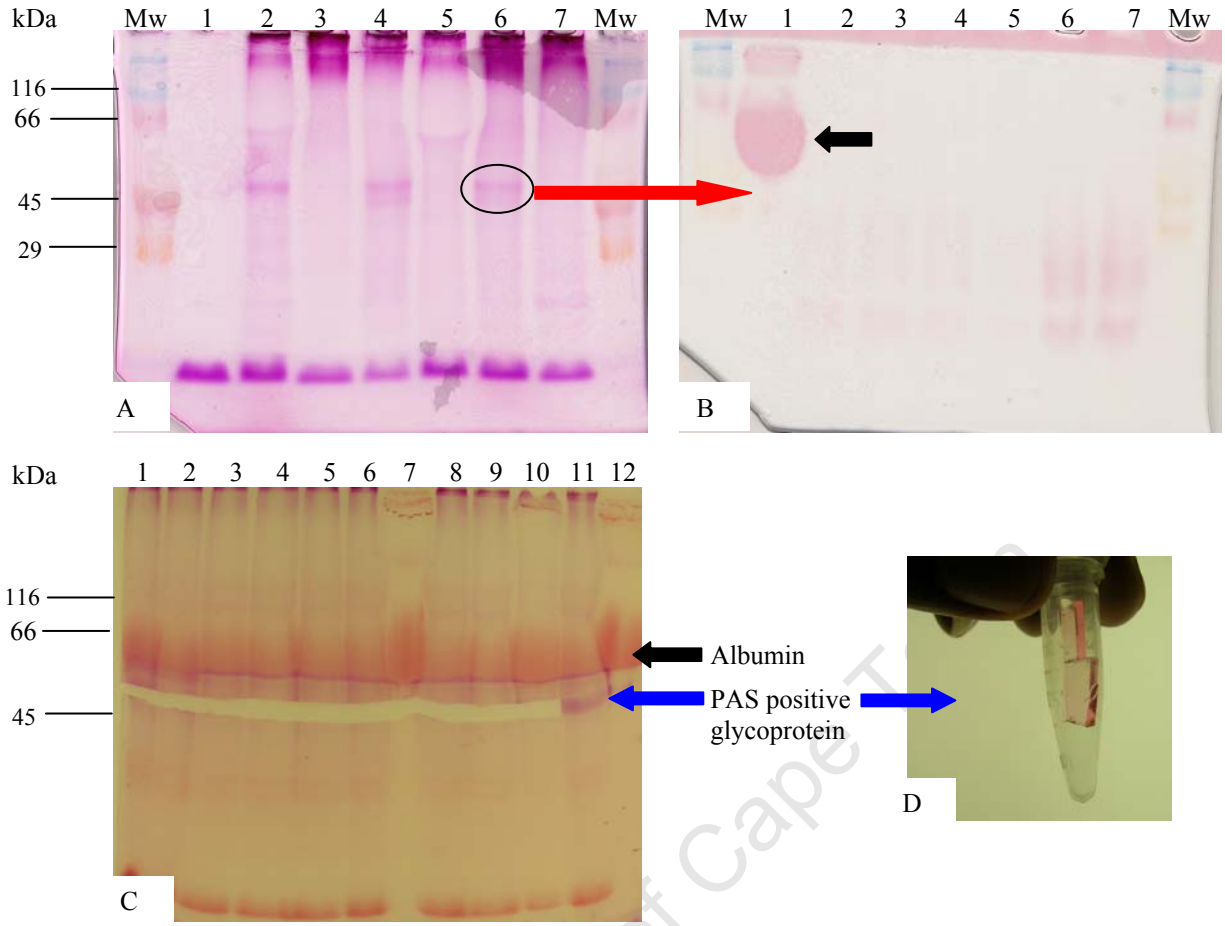


Figure 3.3 Analysis by SDS-PAGE of purified mucin obtained from specimens resected for gastric cancer. Freeze-dried mucin (400-500 µg) from patients with various gastric cancers was prepared in reducing sample application buffer and separated on 10% SDS-PAGE. (A) Gel stained with PAS showing the 40-50 kDa glycoprotein (circled). (B) Ponceau S with black arrow showing the "blob" of albumin. Lanes are numbered on the top of each gel. Gels (A) and (B) contain samples in the following order: Lane 1 (200 µg BSA), lane 2 (M, 55yr, partial gastrectomy, iac), lane 3 (M, 53yr, partial gastrectomy, iac), lane 4 (F, 74yr, partial gastrectomy, dac), lane 5 (M, 60yr, partial gastrectomy, srac), lane 6 (F, 57yr, total gastrectomy, dac), lane 7 (M, 60yr, partial gastrectomy, dac). (C) Gel double stained with Ponceau S and PAS. Lanes 1-6 (M, 55yr, partial gastrectomy, iac) and lanes 8-11 (F, 74yr, partial gastrectomy, dac) were spiked with BSA. Lanes 7 and 12 contained BSA only. (D) Eppendorf containing the 40-50 kDa glycoprotein cut from the gel (C) in the region indicated by the blue arrow, below albumin (black arrow) The cut gel was stored at -20°C until immunization.

(Abbreviations: M – male, F – female, iac – intestinal type carcinoma, dac – diffuse carcinoma, srac – signet-ring cell carcinoma)

3.2.4 Production of polyclonal anti-(40-50 kDa glycoprotein) antibody

Here we describe the production of a polyclonal antibody to the 40-50 kDa glycoprotein using an efficient immunization method described by Bellstedt *et al.* (1987). A three month old female rabbit was immunized intravenously with the 40-50 kDa glycoprotein (Figure 3.4), complexed to acid-treated *Salmonella minnesota* R595 bacteria. When the optimal antibody production was achieved using this method, 24 ml of blood was drawn from the central ear artery with a syringe and allowed to clot. The antiserum was separated from the blood clot by centrifugation and stored at -80ºC. Antibody activity was assessed by dot blot and western blot methods.

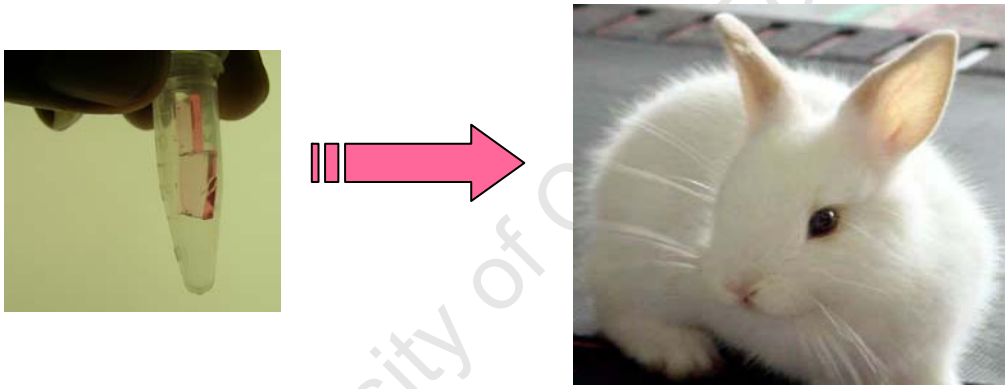


Figure 3.4. Immunisation with the 40-50 kDa glycoprotein. The 40-50 kDa glycoprotein excised from the gels (in sufficient quantities) was stored in Eppendorf tubes at -20°C until immunization. Upon immunization, the 40-50 kDa glycoprotein complexed to acid-treated *Salmonella minnesota* R595 bacteria (ratio by dry mass of protein to bacteria was 1 to 5) was intravenously introduced into a three month old female rabbit. Blood (2.0 ml) was drawn from the central ear artery using a syringe prior to immunization as a negative control. Boosts consisting of 3 immunizations within a week, of the same amount of protein- naked bacteria complex, were administered intravenously in order to collect more serum. When the optimal antibody production was achieved, 24.0 ml of blood was drawn and the immunoreactivity of the antiserum determined by dot blot and western blot methods.

3.2.5 Dot blot analysis

Samples where prepared in SDS sample application buffer and applied directly onto a nitrocellulose membrane. Membranes were then vacuum blotted at a pressure of 40-50 bars for 1 h and probed with the anti-(40-50 kDa glycoprotein) antibody followed by the secondary goat anti-rabbit HRP antibody. Detection was carried out using an ECL kit. Cross reactivity of the antibody with albumin (BSA) and mucin from various sources (PMP, PGC, FAP and CGC) was observed.

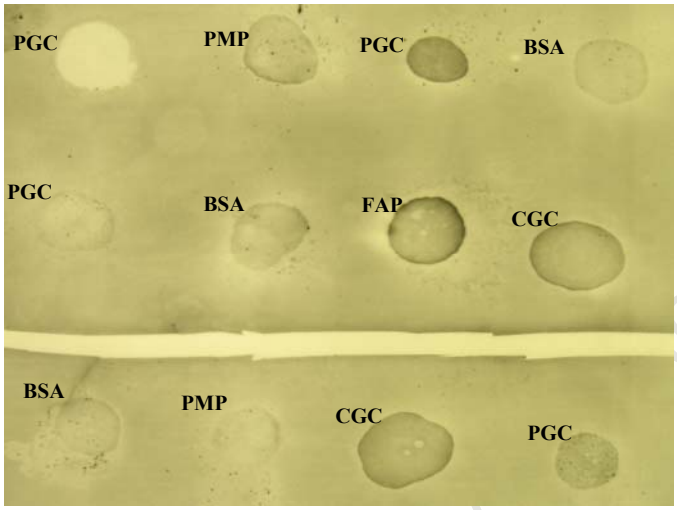


Figure 3.5. Dot blot showing antibody cross-reactivity. Samples purified by two spins of CsCl density ultracentrifugation were freeze-dried, dissolved in reducing SDS loading buffer and then boiled for 1 min. Crude samples suspended in 6M GuHCl containing protease inhibitors were centrifuged at 105 000 x g for 1 h at 4°C to remove insoluble debris. The supernatant containing mucin was mixed with SDS loading buffer at ratio 1 to 1 (v/v) and then boiled for 1 min. Samples were then vacuum blotted onto nitrocellulose membrane at a suction pressure of 40–50 bars for 1 h. The rabbit antiserum raised against the 40-50 kDa glycoprotein was used as the primary antibody without the addition of HSA followed by secondary goat anti-rabbit HRP antibody. ECL was carried out for the detection of antibody immunoreactivity.
(Abbreciations: PGC - purified gastric cancer, PMP - pseudomyxoma peritonei, BSA - bovine serum albumin , FAP - familial adenomatous polyposis coli and CGC - crude gastric cancer)

3.2.6 Western blot analysis

Western blotting is a method used to detect a specific protein in a given sample. Mucin samples were electrophoresed on a 1-D SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with the polyclonal antibody at 1 : 5000 dilution diluted only with TBS (without HSA) (Figure 3.6A) and with TBS (with HSA) (Figure 3.6B). After washing with TBST (3 x 5 min) membranes were incubated with goat anti-rabbit secondary antibody. Detection was by an ECL kit.

Some cross reactivity of the antibody with albumin was seen when the antibody to the 40-50kDa glycoprotein was prepared without HSA (added to the antibody dilution) (Figure 3.6A, lanes: 1-5 and 7-8). The strongest binding to our glycoprotein of interest can be seen in lane 6 (black arrow) which was a sample purified from a gastric cancer specimen. When the antibody to the 40-50 kDa glycoprotein was diluted in a buffer containing HSA, reactivity was observed with cancer mucin (Figure 3.6B, blue arrow) presenting as a smear at the top of the running gel and with specificity to the 40-50 kDa glycoprotein (Figure 3.6B, lanes 6 and 9, green arrow). In the lanes which were positive for the 40-50 kDa glycoprotein (Figure 3.6B, lanes 6 and 9), no reactivity was noted in the region of albumin at 66-70 kDa (red arrow). Very faint material was seen below the $M_r \sim$ 40-50 kDa band. The immunologically active fragments showing material above 67 kDa might suggest the existence of non-specific binding of this glycoprotein with mucins or the presence of familiar epitopes in both species (Figure 3.6B).

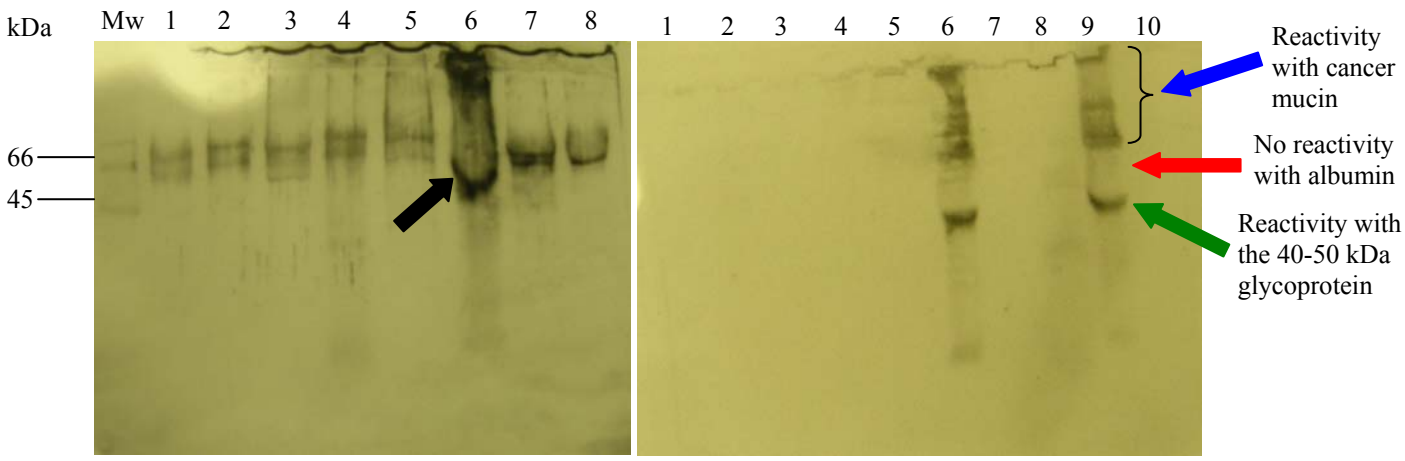


Figure 3.6. Western blot of the 40-50 kDa glycoprotein. Following two CsCl density gradient steps, purified mucins (300-500 ug) and BSA (190-200 ug) were electrophoresed on SDS-PAGE gel (3% stacking gel and 10% running gel). After electrophoresis, proteins were transferred onto a nitrocellulose membrane using a semi-dry electroblotting unit for 1h. Membranes were probed using the antibody to the 40-50 kDa glycoprotein. Immunoreactivity was detected using an ECL kit and films were processed using a standard X-ray developer. (A) Primary antibody prepared without human serum albumin (HSA) and showing high cross reactivity. Lane 1 (crude PMP), lane 2 (crude PCC), lane 3 (crude PMP), lane 4 (purified FAP), lane 5 (CGC), lane 6 (PGC), lanes 7 and 8 (BSA).
(B) Primary anti-(40-50 kDa glycoprotein) antibody incubated with HSA showing no cross-reactivity. Lane 1 (BSA), lane 2 (PCC), lane 3 (CGC), lane 4 (PGC), lane 5 (crude gastric cancer), lane 6 (PGC), lane 7 (purified PMP), lane 8 (purified FAP), lane 9 (PGC) and lane 10 (BSA).
(Abbreviations: PMP - pseudomyxoma peritonei, PCC - purified colon cancer, FAP - familial adenomatous polyposis coli, CGC - crude gastric cancer, PGC - purified gastric cancer and BSA - bovine serum albumin)

3.3 Discussion

Crude mucus, scraped from the gastrectomy specimen of patients with cancer, was diluted at a 1 : 5 ratio with ice cold buffer containing 6 M guanidinium chloride (GuHCl) and proteolytic inhibitors (PI) as a precaution against endogenous proteolysis during the preparation of crude mucus from all gastrectomy specimens (Mall, 1988). The use of denaturing solvents (GuHCl) and proteinase inhibitors to extract mucins has been shown to inhibit degradative enzymes during the extraction and purification of mucins (Carlstedt and Sheehan, 1982). The purification of gastric mucin by CsCl density gradient ultracentrifugation has been reported to be a method of choice and is widely used in mucin research to yield pure mucin (Pearson *et al.*,

1980; Creeth and Denborough, 1970). The samples had very little detectable levels of the protein after the second spin in CsCl.

Even in purified preparations, the 40-50 kDa PAS positive fragment was always associated with albumin on SDS-PAGE (Mall *et al.,* 1990, 1992, 1999). This glycoprotein reproducibly appeared as a smear of PAS positive material on gels, both in this study and that of Mall *et al.* (1999), in which mucins were purified from crude mucus scrapings. This is another indication of the extent of glycosylation of this fragment that would also have contributed to it fractionating with mucins in a CsCl gradient. A better separation of albumin and the 40-50 kDa glycoprotein than that reported by Mall *et al.* (1999) (who used the 10% acrylamide gels) was obtained on 12% SDS-PAGE in this study (Figure 3.2B). Mucins from the cancer patients were heterogeneous when analyzed on SDS-PAGE stained with PAS (Figure 3.2A), with some samples showing the PAS positive 40-50 kDa glycoprotein, and others not. Despite equivalent amounts of sample applied to the gels, the mucin prepared from the cancer specimens had varying amounts of polymer and subunit (Figure 3.2A) and the intensity of the 40-50 kDa glycoprotein varied from patient to patient. We cannot explain the absence of the glycoprotein in some samples. Mall *et al.* (1999) reported its presence in crude mucus in 8/9 gastric ulcers, 7/7 diffuse carcinomas, 6/7 intestinal type carcinoma, 3/3 poorly differentiated carcinoma and 1/1 mixed carcinoma (Mall *et al.,* 1999).

Purified mucin from post mortem specimens were taken from the previous study (Mall *et al.,* 1999), and used as controls. The 40-50 kDa glycoprotein did not appear in the control groups, a finding reported previously by Mall *et al.* (1999). This study has been constrained by a lack of suitable normal tissue due to the new forensic-legal regulations governing the use of tissue from cadavers and transplant donors. To confirm the presence of the 40-50 kDa glycoprotein in the samples prior to cutting it from the gel, gels were electrophoresed in duplicate and one gel stained with PAS (Figure 3.3A) to confirm the presence of the 40-50 kDa glycoprotein and the twin gel from which the 40-50 kDa glycoprotein was to be cut was stained with Ponceau S (Figure 3.3B), a reversible stain recommended for staining proteins required for immunization purposes. The 40-50 kDa glycoprotein was cut from the gel stained with Ponceau S and not from the PAS stained gel. This is because PAS is highly

antigenic and binds to the glycoprotein non-reversibly (D. U. Bellstedt, personal communication). In both gels, BSA was loaded in lane 1 and stained positively with Ponceau S (Figure 3.3B) and not with PAS (Figure 3.3A). This was another confirmation that this glycoprotein was not an artifact of albumin but a separate glycoprotein closely associated with albumin on SDS-PAGE, with albumin being associated with it even after purification (Mall *et al.,* 1999).

We could now safely assume that the 40-50 kDa glycoprotein was just below the albumin blob as an unstained protein on the Ponceau S stained gel (Figure 3.3B). We therefore cut the gel in this region and stored the pieces in Eppendorf tubes at -20ºC until further use. To confirm that the piece of removed gel (Figure 3.3B) was indeed the PAS positive glycoprotein, we prepared purified mucin samples and added albumin to them. After electrophoresis, the gel was stained firstly with Ponceau S followed by PAS (Figure 3.3C). This result showed the clear regional separation between the PAS positive 40-50 kDa glycoprotein and the Ponceau S positive albumin band. Although single staining was used to analyze the 40-50 kDa glycoprotein, double staining (Figure 3C) was used for documentation purposes only and the material on this gel was not used for immunological experiments. However, this result confirmed the close association pattern between the 40-50kDa glycoprotein and albumin, reported previously by Mall *et al*. (1999). Although stomachs of patients with gastric cancer are known to produce excessive amounts of albumin, these findings continued to intrigue us as they did previously (Mall *et al*., 1999) because of the failure to remove albumin from the sample preparations even after 2 and sometimes 3 CsCl/GuHCl spins (Mall – personal communication).

Prior to immunization, the 40-50 kDa glycoprotein stored in Eppendorf tubes was electro-eluted from the SDS-PAGE cut piece of gel and coupled to phenol treated bacteria in a ratio 1 : 5. The method, described by Bellstedt *et al.* (1987), was our preferred method as it allows for antibodies to be raised where the antigens retrieved from studies are only available in very small amounts. Indeed in our study, samples were obtained with difficulty with approximately 30 resections from 2006 to 2008. Our study was reliant on the scheduling of gastric cancer surgery for the collection of mucus specimens with less than a milligram of purified mucin obtained from each sample, not enough for our planned experiments and not even enough for raising

antibodies using conventional methods. Another important consideration is that we analysed each sample individually and did not pool the mucin samples. Dot blot assays where performed as a quick check for antibody cross reactivity against albumin (BSA). Faintly stained (BSA, PMP) as well as heavily stained (CGC, FAP, PGC) patches can be seen in Figure 3.5. This prompted us to do a more thorough investigation using Western blotting methods. The specificity of the antibody to the 40-50 kDa glycoprotein was determined by Western blotting. In the first experiment, the anti-(40-50 kDa glycoprotein) antibody was tested for reactivity against mucin from various sources which included crude PMP, PCC and purified FAP. It was also tested against BSA and against crude and purified gastric cancer mucin. It was seen that the anti-(40-50 kDa glycoprotein) antibody in Figure 3.6A also reacted with albumin, shown as bands around the region 66-70 kDa where albumin is usually found. This indicated that the 40-50 kDa glycoprotein was possibly contaminated with some albumin upon its retrieval from the gel.

In order to confirm whether the cross-reactivity presented by the 40-50 kDa antibody was associated with albumin contamination, we incubated the anti-(40-50 kDa glycoprotein) antibody with HSA for 1 h to block any antibody that might recognize albumin. The effect of treating the anti-(40-50 kDa glycoprotein) with HSA eliminated cross reactivity (Figure 3.6B). This result demonstrated that the cross reactivity observed in Figure 3.6A was indeed due to the presence of albumin as a contaminant or as a product complexed to the 40-50 kDa glycoprotein. In Figure 3.6B, the anti-(40-50 kDa glycoprotein) showed reactivity with cancer mucin. This could mean that some 40-50 kDa glycoprotein could be non-specifically bound with mucin. This explanation is likely considering this 40-50 kDa fragment actually fractionated with mucin in a CsCl gradient. In the study by Mall *et al.* (1999) this fragment was detectable on a Western blot using M1 monoclonal antibodies (1-13M1, 2-12M1, 9-13M1 and 58M1) at a similar molecular weight and range to the glycoprotein of interest. M1 antibodies are known to react with degraded fragments of MUC5AC (Bara *et al.,* 1998). However, bands were also positive for larger fragments suggesting non-specific binding with larger mucins (Mall *et al.,* 1999). However, we were successful in raising the polyclonal antibody to our 40-50 kDa glycoprotein, setting the stage for determining by immunohistochemistry, its localization and expression in human gastric tissue.

CHAPTER 4

IMMUNOHISTOCHEMICAL ANALYSIS OF THE 40-50 kDa GLYCOPROTEIN AND MUCIN IN HUMAN GASTRIC TISSUE

4.1 Introduction

Here we describe, using immunohistochemical techniques, the expression and location, in gastric tissue, of the 40-50 kDa glycoprotein using the polyclonal antibody raised against it as described in Chapter 3. Serial sections (2 μm) were cut from paraffin wax embedded tissue blocks and stained with haematoxylin and eosin (H&E) for diagnosis, periodic acid-Schiff (pH 2.5)/Alcian blue (PAS/AB) to identify neutral mucins and sialomucins and with high iron diamine/Alcian blue (HID/AB), to identify sulfomucins and sialomucins. The tissue distribution of the 40-50 kDa glycoprotein was investigated by immunohistochemistry using the anti-(40-50 kDa glycoprotein) antibody (we developed and described in Chapter 3). The expression of this glycoprotein was investigated in normal, intestinal metaplasia and gastric carcinoma tissue sections prepared from the same patients (Table 1) from whom the mucus gel was scraped for the isolation and purification of the 40-50 kDa glycoprotein. We also looked at the expression of mucin using anti-(MUC1, MUC1core, MUC2, MUC4, MUC5AC and MUC6) antibodies (Table 2) to be able to compare those with the expression of the 40-50 kDa glycoprotein. We evaluated the slides quantitatively by looking at 100 cells at ×400 magnification. The degree of positive staining for the 40-50 kDa glycoprotein and mucins was evaluated as follows: 1+ (5-25%) few positive cells; 2+ (26-50%) well defined areas with positive cells, 3+ (51-75%) extensive areas with positive cells and 4+ (>75%) most cells stained.

4.2 Results:

4.2.1 Normal stomach

The normal gastric mucosa expressed neutral mucins (PAS) in the surface epithelium and in the deep mucus glands with no HID/AB staining. The following results are summarised in Table 4.1 and Table 4.2, A to C. The 40-50 kDa glycoprotein was

consistently expressed in the cytoplasm of parietal cells (20/20) as follows: 2 cases 1+; 2 cases 3+ and 16 cases 4+ (Figure 4.1A). None of the mucin antibodies used in this study stained parietal cells. MUC1 was consistently expressed in the surface epithelium in the mucus neck cells and in the deep gastric mucus glands (26/26) (Figure 4.1B) with all cases showing 4+. There was inconsistent staining of MUC4 in the surface epithelium (10/26) in 16 cases <5%; 3 cases 1+; 5 cases 2+ and 2 cases 4+ and in the deep mucus glands (5/26) in 21 cases <5%; 3 cases 1+ and 2 cases 4+. MUC5AC consistently stained the surface epithelium (26/26) with 2 cases 3+ and 24 cases 4+ (Figure 4.1C). MUC6 was consistently expressed in the deep mucus glands (25/25) with 3 cases 2+; 3 cases 3+ and 19 cases 4+ (Figure 4.1D). MUC1 core and MUC2 were not expressed in the normal gastric mucosa.

The cytoplasmic expression of the 40-50 kDa glycoprotein in parietal cells of normal mucosa is shown at higher magnification in Figure 4.2. In comparison, human serum albumin (HSA) (Figure 4.2 B) MUC5AC (Figure 4.2C) and MUC6 (Figure 4.2D) were not expressed in parietal cells. The area of the stomach that did not contain parietal cells, such as the antrum, showed no staining with the 40-50 kDa glycoprotein. These cases (n=9) were excluded in tissue sections probed with the anti-(40-50 kDa glycoprotein) antibody. One slide with tissue sections probed for MUC6 repeatedly washed off the slide during antigen retrieval in the pressure cooker and therefore could not be evaluated. All mucin antibodies showed good staining in control tissues. A graphical comparison for the immunohistochemical results of the expression of the 40-50 kDa glycoprotein and mucins is shown in Figure 4.3.

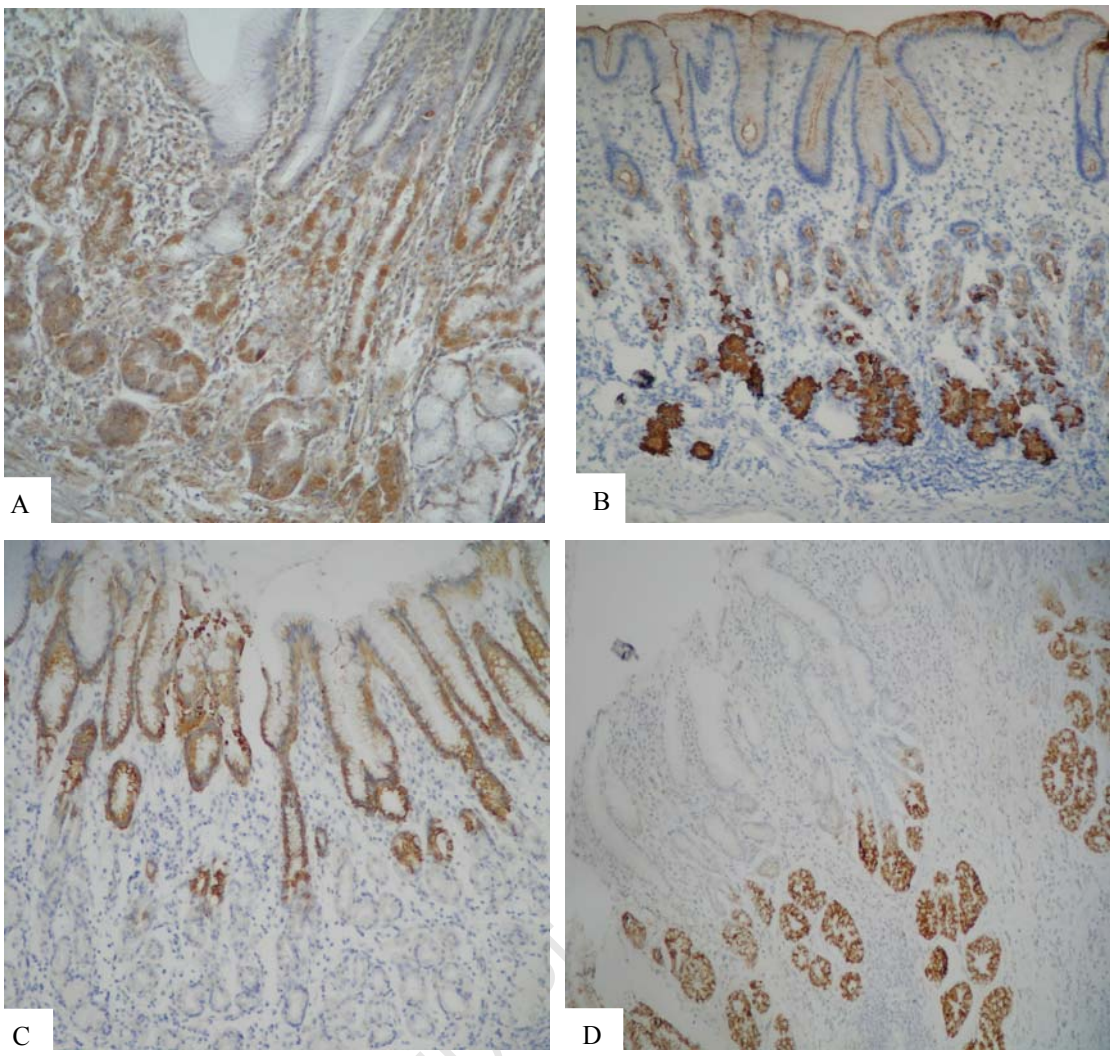


Figure 4.1 Expression pattern of the 40-50 kDa glycoprotein, MUC1, MUC5AC and MUC6 in the normal gastric mucosa. (A) The 40-50 kDa glycoprotein was highly expressed only in parietal cells. (B) MUC1 was expressed in both the surface epithelium and in the deep mucus glands. (C) MUC5AC was consistently expressed in the surface epithelium and mucus neck cells. (D) MUC6 was expressed in the deep mucus glands and in some cells of the neck zone.

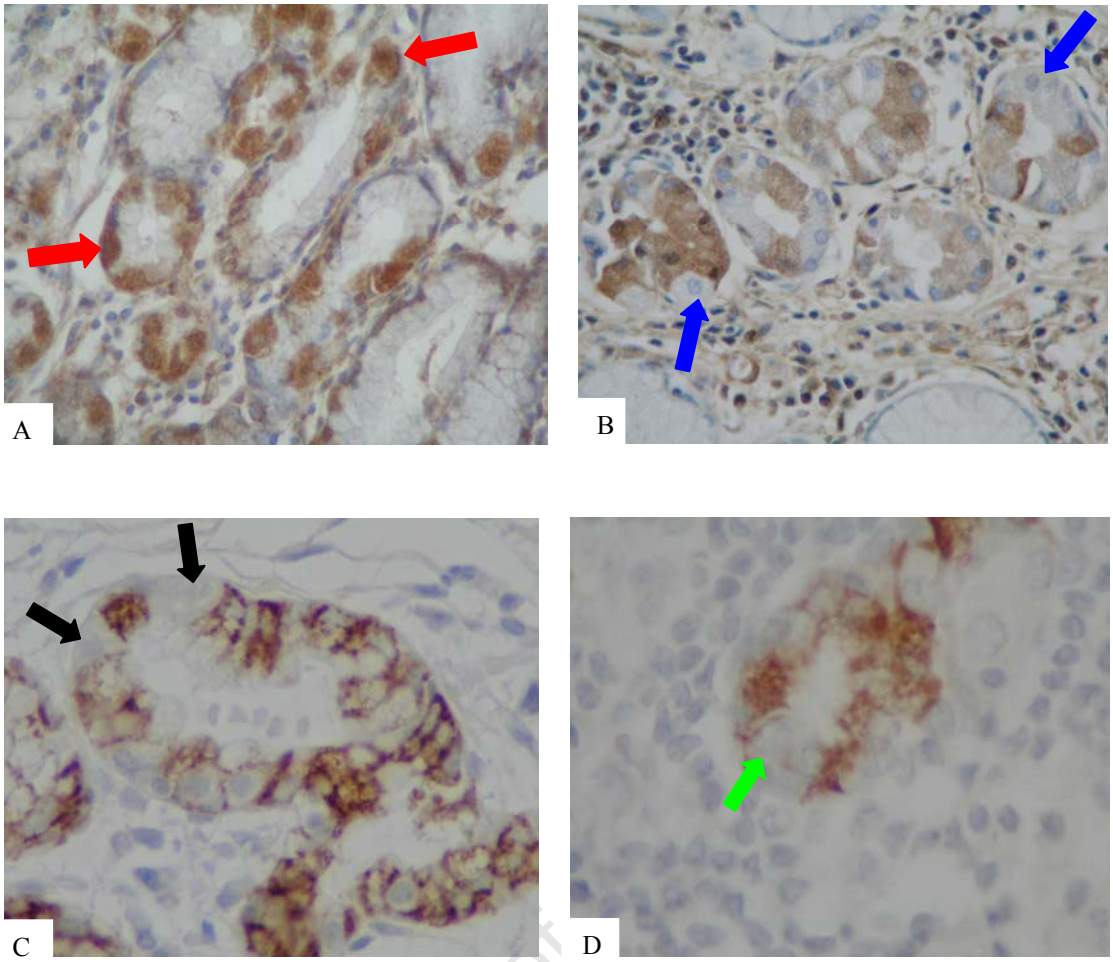


Figure 4.2 Immunodetection of the 40-50 kDa glycoprotein in the parietal cells of normal gastric mucosa. (A) Extensive staining of parietal cells shown by the red arrows, was consistently seen on tissue sections (normal adjacent to and away from tumour) stained with the anti-(40-50 kDa glycoprotein) antibody. This was also observed in the control tissues sampled away from cancer. (B) Albumin was expressed only in chief cells and not in parietal cells shown by the blue arrows. (C) MUC5AC was expressed in the mucus cells in the neck region and not in parietal cells shown by the black arrow. (D) MUC6 was expressed in the deep mucus glands and not in parietal cells shown by the green arrow.

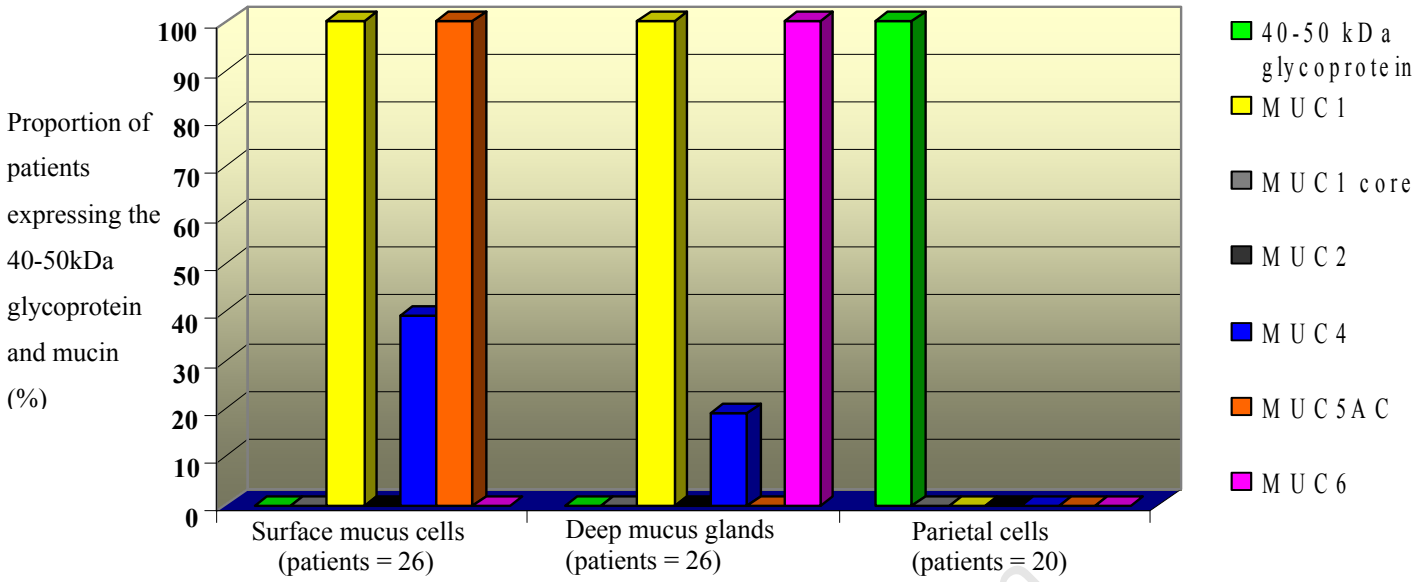


Figure 4.3 Expression of the 40-50 kDa glycoprotein and mucin in normal gastric mucosa. The 40-50 kDa glycoprotein was consistently expressed only in parietal cells, MUC1 consistently in both the surface mucus cells and the deep mucus glands, MUC4 in few surface mucus cells and the deep mucus glands, MUC5AC consistently in the surface mucus cells and MUC6 consistently in the deep mucus glands.

Table 4.1 Immunohistochemical expression of the 40-50 kDa glycoprotein and mucins in normal stomach tissue

Antigen	Superficial mucus cells (n=26)	Deep mucus glands (n=26)	Parietal cell (n=20)
40-50 kDa glycoprotein	0	0	20 (100%)
MUC1	26 (100%)	26 (100%)	0
MUC1 core	0	0	0
MUC2	0	0	0
MUC4	10 (39%)	5 (19%)	0
MUC5AC	26 (100%)	0	0
MUC6	0	25 (100%)*	0

***For reasons mentioned in 4.2.1, 25 cases were reviewed for MUC6**

Table 4.2 Degree of staining of the 40-50 kDa glycoprotein and mucin in normal gastric mucosa

Antigen	No. of cases	0(<5%)	1+ (5-25%)	2+ (26-50%)	3+ (51-75%)	4+ (>75%)
40-50 kDa glycoprotein	20	20	0	0	0	0
MUC1	26	0	0	0	0	26
MUC1core	26	26	0	0	0	0
MUC2	26	26	0	0	0	0
MUC4	26	16	3	5	0	2
MUC5AC	26	0	0	0	2	24
MUC6	26	26	0	0	0	0

A. Superficial epithelium

Antigen	No. of cases	0(<5%)	1+ (5-25%)	2+ (26-50%)	3+ (51-75%)	4+ (>75%)
40-50 kDa glycoprotein	20	20	0	0	0	0
MUC1	26	0	0	0	0	26
MUC1core	26	26	0	0	0	0
MUC2	26	26	0	0	0	0
MUC4	26	21	3	0	0	2
MUC5AC	26	26	0	0	0	0
MUC6	25*	0	0	2	4	19

B. Deep mucus glands

Antigen	No. of cases	0(<5%)	1+ (5-25%)	2+ (26-50%)	3+ (51-75%)	4+ (>75%)
40-50 kDa glycoprotein	20	0	2	0	2	16
MUC1	26	26	0	0	0	0
MUC1core	26	26	0	0	0	0
MUC2	26	26	0	0	0	0
MUC4	26	26	0	0	0	0
MUC5AC	26	26	0	0	0	0
MUC6	26	26	0	0	0	0

C. Parietal cells

4.2.2 Intestinal metaplasia

Intestinal metaplasia (IM) was classified according to Filipe. (1992). Mucin histochemistry was used to identify specific subtypes of IM based on the pattern of cell differentiation and mucin expression. Neutral mucins were decreased in intestinal metaplasia compared to normal gastric mucosa. An intense blue staining of sialomucins was observed in the complete IM (Figure 4.4A), whereas sialomucins and sulfomucins were expressed in the incomplete IM (Figure 4.4B).

The following results are summarised in Table 4.3 and Table 4.4. The 40-50 kDa glycoprotein was consistently expressed in the columnar cells in complete (type I) IM (12/12) (Figure 4.4C) in 6 cases 1+; 2 cases 2+ and 4 cases 3+, in the incomplete (type II) IM (5/5) (Figure 4.4D) in 3 cases 1+; 1 case 3+ and 1 case 4+ and in one case of the incomplete (type III) (1/1) as 4+.

The following immunohistochemical results for the expression of mucins are summarised in Table 4.3. Mucin expression in the complete (type I) IM was as follows: MUC1core (1/12), MUC2 (7/12) and MUC5AC (3/12). MUC1, MUC4 and MUC6 were not expressed in the complete (type I) IM. In the incomplete (type II) IM, mucins were expressed as follows: MUC1 core (1/5), MUC2 (2/5), MUC5AC (1/5) and MUC6 (2/5). MUC1 and MUC4 were not expressed in the incomplete IM. MUC2 was frequently expressed in the complete (type I) (Figure 4.4E) whereas in the incomplete (type II) IM, both MUC2 (Figure 4.4E) and MUC6 were expressed more frequently when compared to other mucins. MUC5AC (1/1) was the only mucin expressed in the incomplete (type III) IM.

In the normal small intestine (used as a positive control) the 40-50 kDa glycoprotein was consistently expressed in the cytoplasm of absorptive columnar cells (Figure 4.5). A graphical comparison of immunohistochemical results of the expression of the 40-50 kDa glycoprotein and mucins in intestinal metaplasia of the stomach is shown in Figure 4.6.

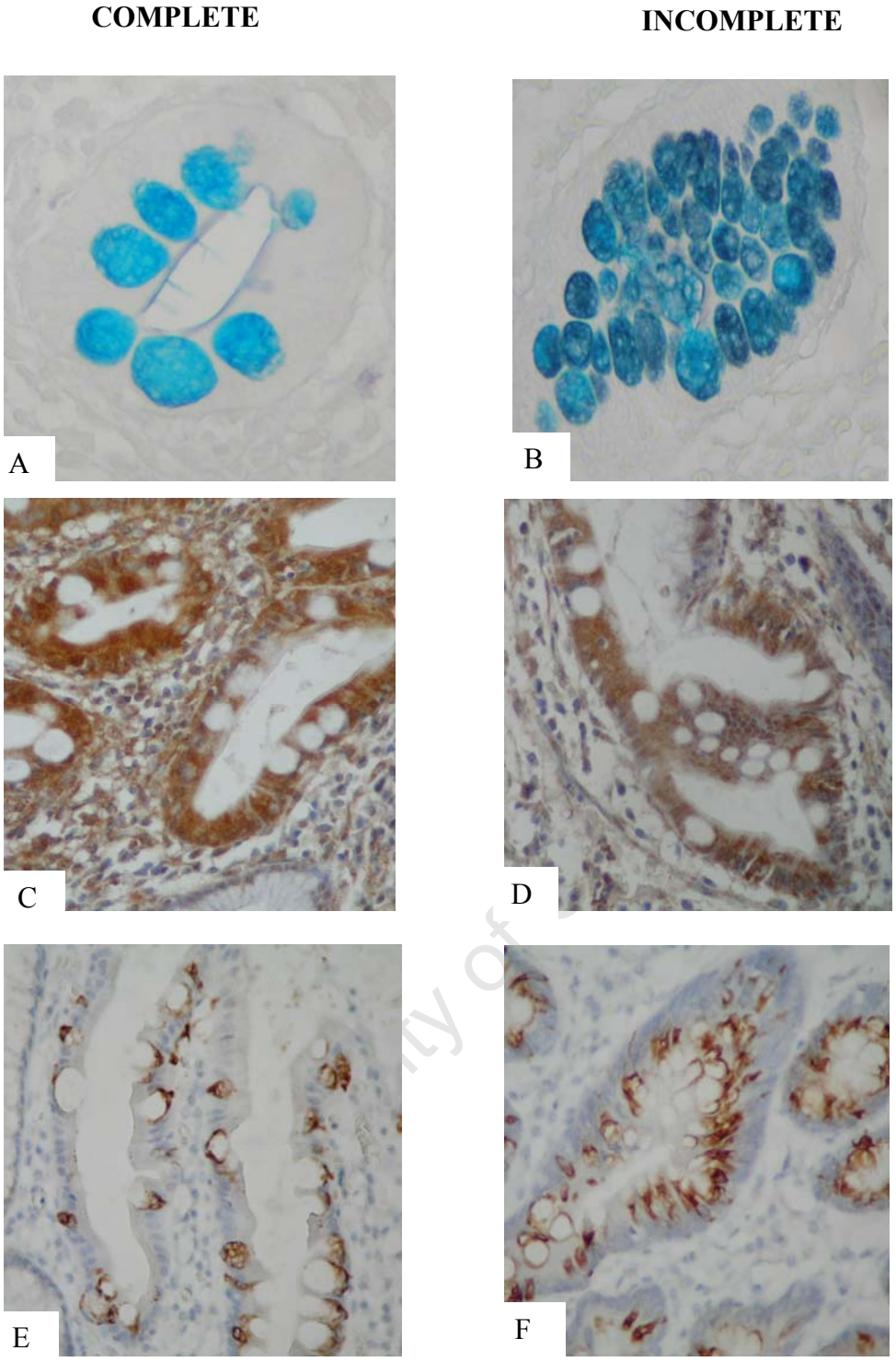


Figure 4.4. Histochemical (HID/AB) and immunohistochemical localization of the 40-50 kDa glycoprotein and MUC2 in intestinal metaplasia of the stomach. (A) HID/AB showing sialomucins in goblet cells of complete IM and (B) shows sialomucins and sulfomucins in goblet and columnar cells in incomplete IM (x40). The 40-50 kDa glycoprotein was expressed in the cytoplasm of columnar cells in complete (C) and incomplete (D) IM but not in the goblet cells (x40). MUC2, a typical intestinal mucin, was expressed in the supranuclear area of the goblet cells of both complete (E) and incomplete (F) IM.

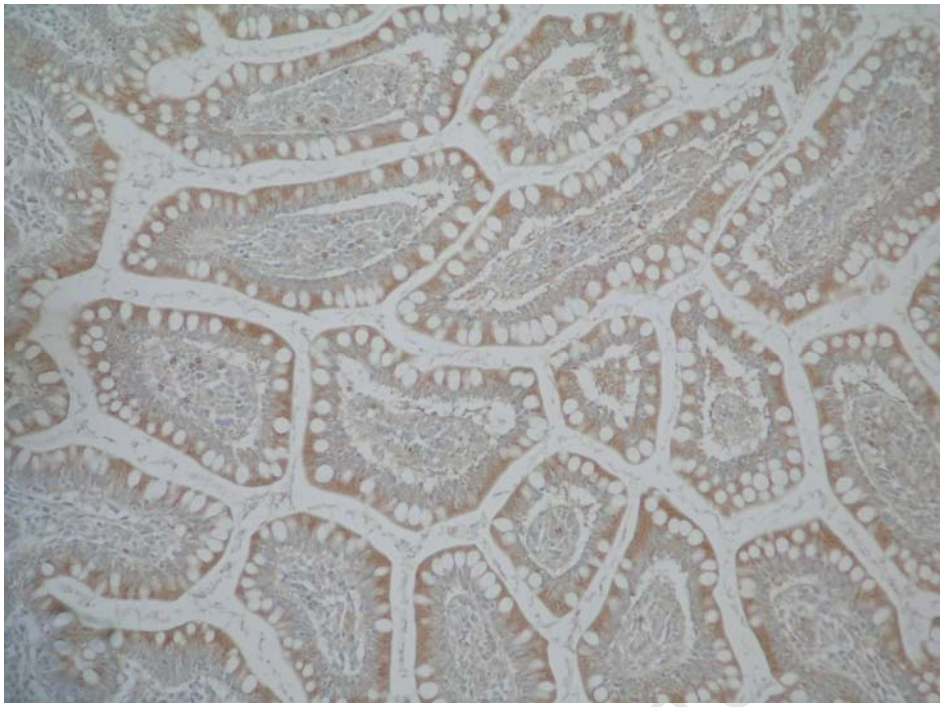


Figure 4.5. The 40-50 kDa glycoprotein expressed in columnar cells of the normal small intestine.
The 40-50 kDa glycoprotein was only expressed in the cytoplasm of columnar cells (x20).

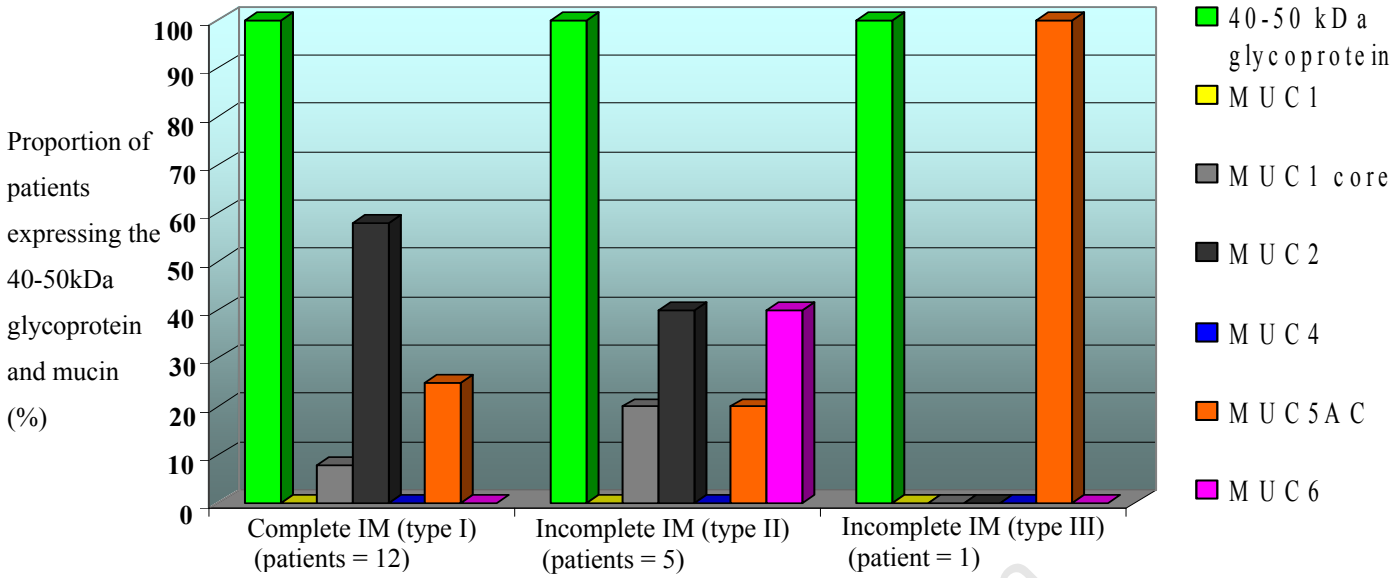


Figure 4.6 Expression of the 40-50 kDa glycoprotein and mucin in intestinal metaplasia of the stomach. The 40-50 kDa glycoprotein was consistently expressed in all types of IM, MUC1core in a few cases of complete (types I) IM and incomplete (type II) IM, MUC2 more in complete (type I) IM than incomplete (type II) IM, MUC5AC in a few cases of complete (types I) IM and incomplete (type II) IM and in 1 case of the incomplete (type III) IM, and MUC6 only in incomplete (type II) IM.

Table 4.3 Immunohistochemical expression of the 40-50 kDa glycoprotein and mucin in intestinal metaplasia of the stomach

Antigen	Complete (Type I) IM [n = 12]	Incomplete (Type II) IM [n = 5]	Incomplete (Type III) IM [n = 1]
40-50kDa glycoprotein	12 (100%)	5 (100%)	1 (100%)
MUC1	0	0	0
MUC1 core	1 (8%)	1 (20%)	0
MUC2	7 (58%)	2 (40%)	0
MUC4	0	0	0
MUC5AC	3 (25%)	1 (20%)	1 (100%)
MUC6	0	2 (40%)	0

Table 4.4 Degree of staining of the 40-50 kDa glycoprotein in intestinal metaplasia of the stomach

Metaplasia	No. of cases	0(<5%)	1+ (5-25%)	2+ (26-50%)	3+ (51-75%)	4+ (>75%)
Complete (type I)	12	0	6	2	4	0
Incomplete (type II)	5	0	3	0	1	1
Incomplete (type III)	1	0	0	0	0	1

4.2.3 Gastric cancer

We studied 13 cases of intestinal type, 7 diffuse, 1 mixed (intestinal and diffuse), 4 signet-ring cell and 2 mucinous type carcinomas. The well differentiated glands of the intestinal type carcinoma showed cytoplasmic staining for the 40-50 kDa glycoprotein (Figure 4.7A) and showed a similar expression in the diffuse type cancers (Figure 4.7B). The 40-50 kDa glycoprotein was also expressed in the cytoplasm of signet-ring cell (Figure 4.8A) and mucinous carcinomas (Figure 4.8B).

The following results are summarised in Table 4.5 and Table 4.6. The 40-50 kDa glycoprotein was consistently expressed in the intestinal type carcinoma (13/13) with the degree of staining as follows: 4 cases 1+; 2 cases 2+; 5 cases 3+ and 2 cases 4+. The 40-50 kDa glycoprotein was inconsistently expressed in the diffuse carcinoma (4/7) with 3 cases <5%; 3 cases 2+ and 1 case 3+. In the mixed (intestinal and diffuse) carcinoma (1/1), the 40-50 kDa glycoprotein was expressed in both components as 3+. In the signet-ring cell carcinomas, the 40-50 kDa glycoprotein showed inconsistency (2/4) with 2 cases <5%; 1 case 1+ and the other case 2+. The 40-50 kDa glycoprotein was expressed in both cases of mucinous carcinomas (2/2): 1 case 1+ and the second case 2+.

MUC1 was consistently expressed in the intestinal type carcinoma (12/13) with the degree of staining as follows: 1 case < 5%; 3 cases 1+; 1 case 2+; 5 cases 3+ and 3 cases 4+. In the diffuse type carcinoma, MUC1 was consistently expressed (7/7) with 2 cases 2+; 3 cases 3+ and 2 cases 4+. MUC1 was expressed in both components of

the mixed (1/1) (intestinal and diffuse) carcinoma as 2+. In the signet-ring cell carcinomas, MUC1 showed consistency (4/4) with 2 cases 1+; 1 case 2+ and 1 case 3+. MUC1 was expressed in the mucinous carcinomas with 1 case 2+ and the other case 3+.

In the intestinal type carcinomas (10/13), MUC1 core was expressed with the degree of staining as follows: 3 cases < 5%; 4 cases 1+; 1 case 2+; 4 cases 3+ and 1 case 4+. In the diffuse carcinomas (1/7) as follows: 6 cases <5% and 1 case 2+. In the mixed (intestinal and diffuse) carcinoma (1/1), MUC1 core was expressed in both components of carcinoma as 2+. In the signet-ring cell carcinomas, MUC1 core was expressed (2/4) in 2 cases <5%; 1 case 2+ and the other case 4+. MUC1 core was expressed in both cases of mucinous carcinomas (2/2): 1 case 2+ and the second case 3+.

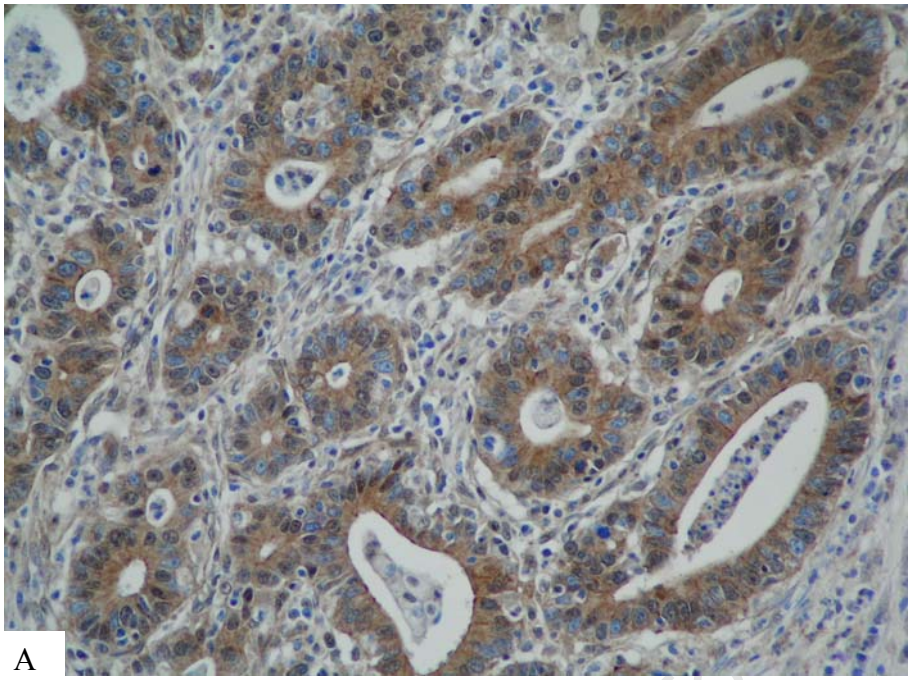
MUC2 was inconsistently expressed in the intestinal type carcinoma (2/13) with the degree of staining as follows: 11 cases < 5% and 2 cases 1+. In the diffuse carcinoma (2/7), MUC2 was expressed as follows: 5 cases <5%; 1 case 1+ and 1 case 2+. In the signet-ring cell carcinomas, MUC2 was expressed (3/4) in 1 case <5%; 1 cases 2+; 1 case 3+ and 1 case 4+. In mucinous carcinomas, MUC2 was expressed (1/2) as follows: 1 case <5% and 1 case 3+. MUC2 was not expressed in the mixed (intestinal and diffuse) carcinoma (0/1).

MUC4 was expressed in the intestinal type carcinoma (8/13) with the degree of staining as follows: 5 cases < 5%; 4 cases 1+; 3 cases 2+ and 1 case 3+. In the diffuse carcinoma (2/7), MUC4 was expressed as follows: 5 cases <5% and 2 cases 2+. MUC4 was expressed in both components of the mixed (1/1) intestinal and diffuse carcinoma as 2+. MUC4 was not expressed in the signet-ring cell carcinomas and mucinous carcinomas.

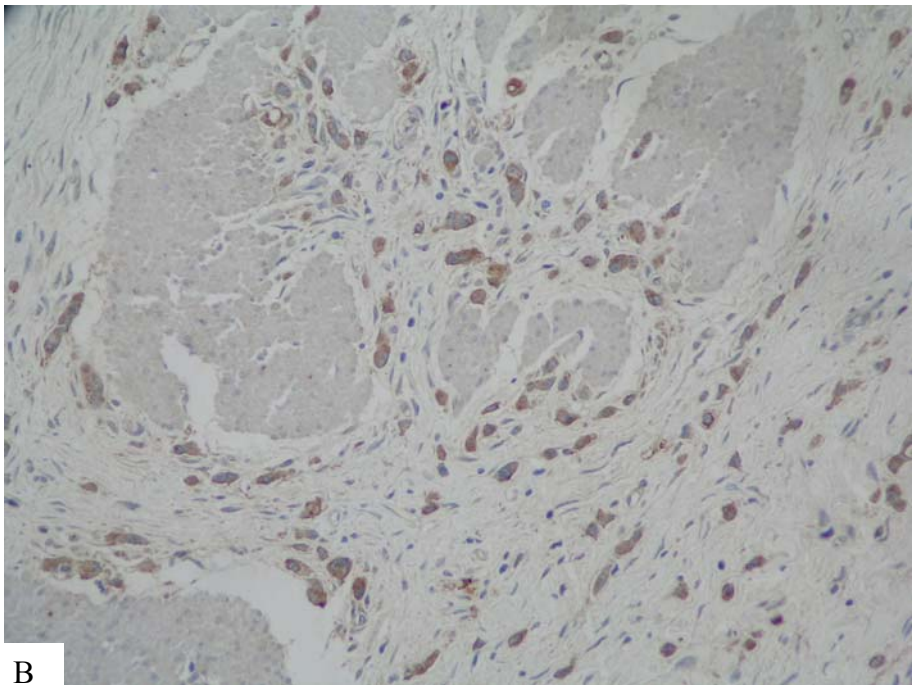
MUC5AC was expressed in the intestinal type carcinoma (6/13) with the degree of staining as follows: 7 cases <5%; 3 cases 1+; 2 cases 2+ and 1 case 3+. In the diffuse carcinoma (3/7), MUC5AC was expressed as follows: 4 cases <5%, 2 cases 2+ and 1 case 3+. In the signet-ring cell carcinomas, the MUC5AC was expressed (3/4) as

follows: 1 case <5%; 2 cases 3+ and 1 case 4+. MUC5AC was not expressed in the mixed (intestinal and diffuse) carcinoma and in the mucinous carcinomas.

MUC6 was expressed in the intestinal type carcinoma (4/13) with the degree of staining as follows: 9 cases <5%; 3 cases 1+ and 1 case 3+. In the diffuse carcinoma (1/7), MUC6 was expressed as follows: 6 cases <5% and 1 case 1+. In the signet-ring cell carcinomas, MUC6 was expressed (1/4) in: 3 cases <5% and 1 case 1+. MUC6 was not expressed in the mixed (intestinal and diffuse) carcinoma and in the mucinous carcinomas. A summary of the heterogeneous expression of the 40-50 kDa glycoprotein and mucins in gastric carcinomas is depicted in Figure 4.9.

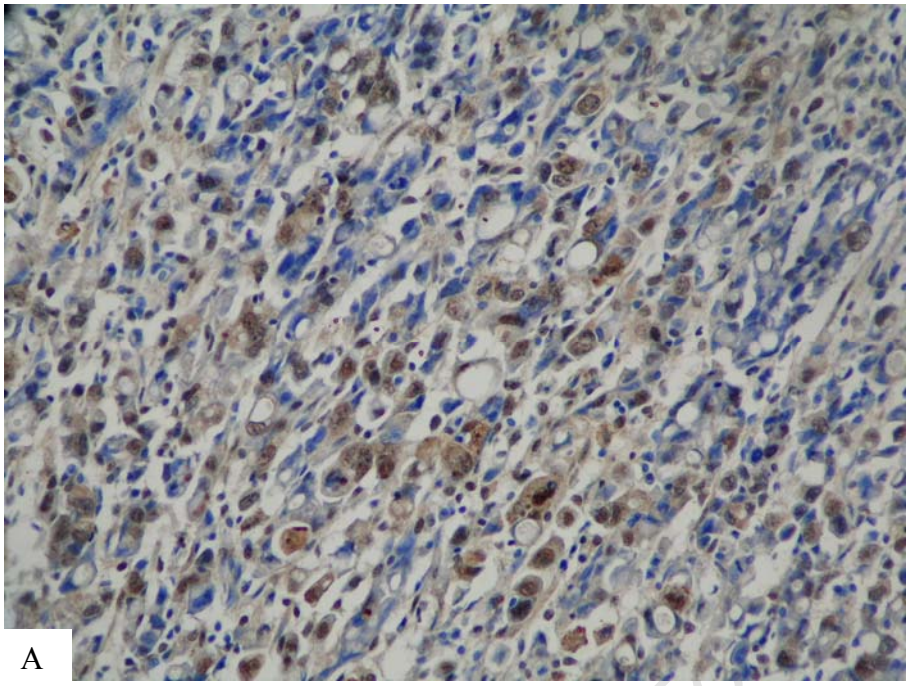


A

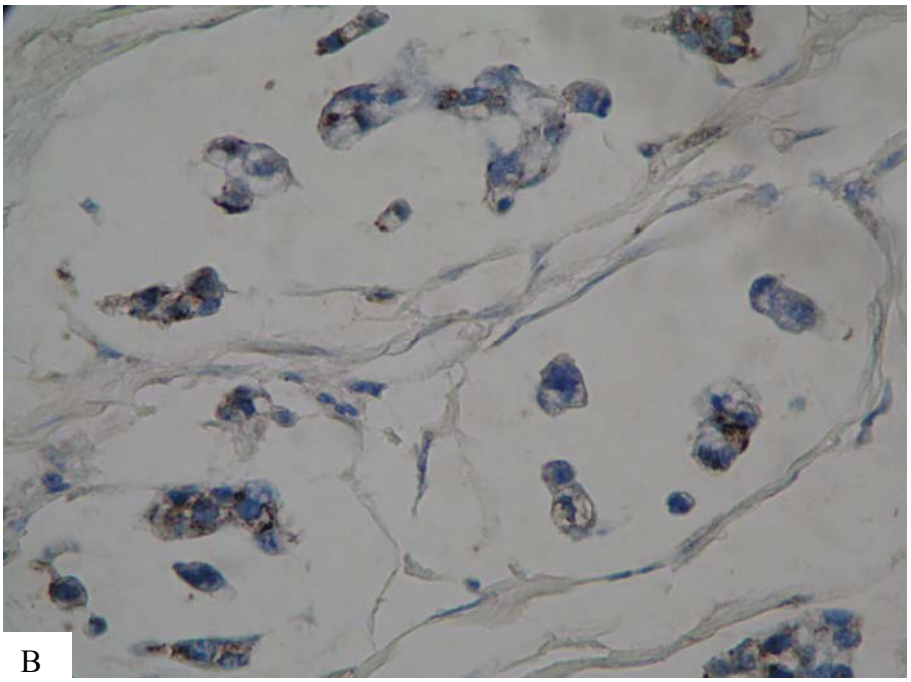


B

Figure 4.7 The 40-50 kDa glycoprotein expressed in the cytoplasm of intestinal and diffuse type gastric carcinomas. The 40-50 kDa glycoprotein was expressed in the cytoplasm of neoplastic glands in the intestinal type carcinoma (A) (x40) and by cancer cells of the diffuse type (B) shown invading the muscle wall of the stomach (x20).



A



B

Figure 4.8 The 40-50 kDa glycoprotein expressed in the cytoplasm of signet-ring cell and mucinous carcinomas. (A) The 40-50 kDa glycoprotein was expressed in the cytoplasm of infiltrative signet-ring cells (x20). (B) In mucinous carcinomas, the 40-50 kDa glycoprotein was expressed in cancer cells suspended in pools of the extracellular mucus (x40).

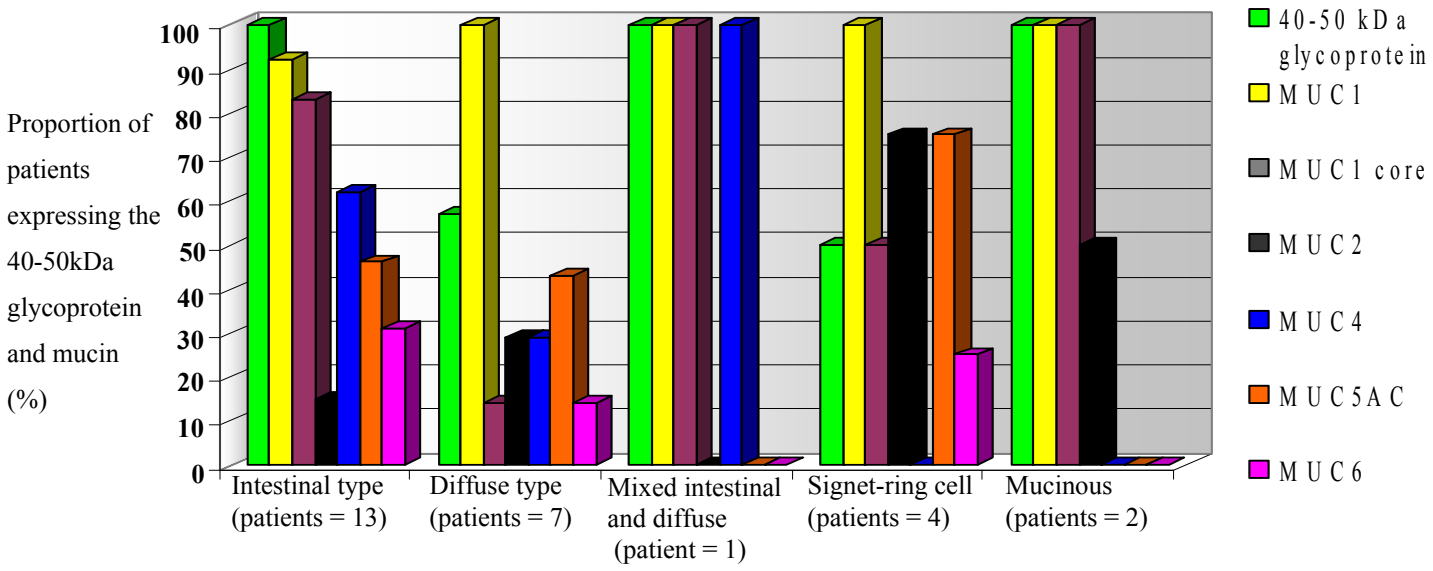


Figure 4.9 Summary of the immunohistochemical expression of the 40-50 kDa glycoprotein and mucin in human gastric carcinomas. The 40-50 kDa glycoprotein and MUC1 were consistently expressed in all carcinomas, more in the intestinal, mixed and mucinous type carcinomas. MUC1 was expressed more in the diffuse and signet-ring cell carcinomas than the 40-50 kDa glycoprotein.

Table 4.5 Immunohistochemical expression of the 40-50 kDa glycoprotein and mucins in gastric cancer

Antigen	Intestinal type (n=13)	Diffuse type (n=7)	Mixed Intestinal and Diffuse (n=1)	Signet ring (n=4)	Mucinous (n=2)
40-50 kDa glycoprotein	13 (100%)	4 (57%)	1 (100%)	2 (50%)	2 (100%)
MUC1	12 (92%)	7 (100%)	1 (100%)	4 (100%)	2 (100%)
MUC 1 core	10 (83%)	1 (14%)	1 (100%)	2 (50%)	2 (100%)
MUC1	12 (92%)	7 (100%)	1 (100%)	4 (100%)	2 (100%)
MUC 2	2 (15%)	2 (29%)	0	3 (75%)	1 (50%)
MUC 4	8 (62%)	2 (29%)	1 (100%)	0	0
MUC 5AC	6 (46%)	3 (43%)	0	3 (75%)	0
MUC 6	4 (31%)	1 (14%)	0	1 (25%)	0

Table 4.6 Degree of staining of the 40-50 kDa glycoprotein and mucins in gastric carcinoma

Antigen	No. of cases	0 (<5%)	1+ (5-25%)	2+ (26-50%)	3+ (51-75%)	4+ (>75%)
40-50 kDa glycoprotein	13	0	4	2	5	2
MUC1	13	1	3	1	5	3
MUC1core	13	3	4	1	4	1
MUC2	13	11	2	0	0	0
MUC4	13	5	4	3	1	0
MUC5AC	13	7	3	2	1	0
MUC6	13	9	3	0	1	0

A. Intestinal type carcinoma

Antigen	No. of cases	0 (<5%)	1+ (5-25%)	2+ (26-50%)	3+ (51-75%)	4+ (>75%)
40-50 kDa glycoprotein	7	3	0	3	1	0
MUC1	7	0	0	2	3	2
MUC1core	7	6	0	1	0	0
MUC2	7	5	1	1	0	0
MUC4	7	5	0	2	0	0
MUC5AC	7	4	0	2	1	0
MUC6	7	6	1	0	0	0

B. Diffuse type carcinoma

Antigen	No. of cases	0 (<5%)	1+ (5-25%)	2+ (26-50%)	3+ (51-75%)	4+ (>75%)
40-50 kDa glycoprotein	1	0	0	0	Intestinal = 1 Diffuse = 1	0
MUC1	1	0	0	Intestinal = 1 Diffuse = 1	0	0
MUC1core	1	0	0	Intestinal = 1 Diffuse = 1	0	0
MUC2	1	1	0	0	0	0
MUC4	1	0	0	Intestinal = 1 Diffuse = 1	0	0
MUC5AC	1	1	0	0	0	0
MUC6	1	1	0	0	0	0

C Mixed (intestinal type and diffuse) carcinoma

Antigen	No. of cases	0 (<5%)	1+ (5-25%)	2+ (26-50%)	3+ (51-75%)	4+ (>75%)
40-50 kDa glycoprotein	4	2	1	1	0	0
MUC1	4	0	2	1	1	0
MUC1core	4	2	1	0	0	1
MUC2	4	1	0	1	1	1
MUC4	4	4	0	0	0	0
MUC5AC	4	1	0	0	2	1
MUC6	4	3	1	0	0	0

D. Signet-ring cell carcinoma

Antigen	No. of cases	0 (<5%)	1+ (5-25%)	2+ (26-50%)	3+ (51-75%)	4+ (>75%)
40-50 kDa glycoprotein	2	0	1	1	0	0
MUC1	2	0	0	1	1	0
MUC1core	2	0	0	1	1	0
MUC2	2	1	0	0	1	0
MUC4	2	2	0	0	0	0
MUC5AC	2	2	0	0	0	0
MUC6	2	2	0	0	0	0

E. Mucinous carcinoma

4.3 Discussion

The expression of the 40-50 kDa glycoprotein was determined on 2 μm-thick histological sections of human gastric tissue using the anti-(40-50 kDa glycoprotein) antibody described in Chapter 3. We examined the expression of the 40-50 kDa glycoprotein in normal mucosa adjacent to the tumour site (n=17) and normal mucosa cut from uninvolved gastric mucosa (n=3). Immunostaining was regarded as positive, if greater than 5% of the cells expressed the antigen (Tables 4.2 and 4.5). The 40-50 kDa glycoprotein was expressed in the cytoplasm of all parietal cells (Figure 4.2A) but not in the surface epithelium and the deep mucus glands of the normal gastric mucosa.

When the anti-(40-50 kDa glycoprotein) antibody was prepared without HSA, a strong signal of background staining was observed, which we reported as cross reactivity in the western blot (see Chapter 3). The reactivity of background material due to non-specific binding was inhibited by diluting the anti-(40-50 kDa glycoprotein) antibody in HSA. We have used a similar approach here to that in western blotting whereby the antibody was incubated with HSA prior to staining and thus clear immunostaining was obtained. During optimization of the 40-50 kDa antibody, better results were obtained if sections were not subjected to antigen retrieval.

Mucin expression is regulated in a cell- and tissue-specific manner (Ho *et al.,* 1993, 1995). Our results confirmed the cellular and tissue distribution of MUC5AC in the surface epithelium as reported by de Bolos *et al.* (1995) (Figure 4.1B). In keeping with previous reports (Reis *et al.,* 1997, 2000; Bartman *et al.,* 1998; Byrd *et al.,* 1997), we observed MUC6 in the deep mucus glands and mucus cells of the neck (Figure 4.1C). One of the negative MUC6 tissue sections repeatedly washed off the slide during antigen retrieval in the pressure cooker. A new set of glass slides was coated with APES to ensure that the tissue section remained during antigen retrieval. However the tissue again washed off the slide after retrieval and therefore could not be evaluated. MUC1 was expressed in the mucus cells of the surface epithelium and in the glands of the body as reported by Reis *et al*. (1998) (Figure 4.1D). MUC4 was detected in normal gastric mucosa in agreement with Taylor *et al*. (1998) (Figure 4.3).

We found no expression of MUC1core and MUC2 in the normal gastric mucosa (Figure 4.3).

We could not use cadaver stomachs as normals because these were unfortunately unobtainable before 72 h post mortem due to forensic and legal implications. H&E staining of sections from these stomachs showed autolysis making them poor controls for this study. However, transplant donor stomachs obtained within 30 min of brain death were used by Mall *et al.* (1999) for mucosal scrapings and in which the 40-50 kDa glycoprotein was shown to be absent. Transplant donor stomachs are now also very difficult to obtain because of stringent new ethical regulations.

The surface epithelium of the gastric mucosa is normally lined by columnar cells and the presence of goblet cells in the gastric mucosa is an important histological parameter that characterizes intestinal metaplasia. In the healthy stomach, surface columnar cells produce neutral mucins (Jass *et al.*, 1984). In contrast to the neutral mucin produced by the normal columnar cells of the gastric mucosa, goblet cells in IM produce sialomucin that are normally secreted by the goblet cells of the small intestine, and/or sulphomucin, normally secreted by goblet cells in the large intestine (Filipe and Ramachandra, 1995). We found that goblet cells in the complete (type I) IM secreted sialomucin (AB) and the columnar cells secreted neutral mucin (PAS). In the incomplete (type II) IM, goblet cells secreted sialomucins and occasionally sulphomucins (HID/AB) with the columnar cells secreting both neutral and sialomucins. In the incomplete (type III) IM, goblet cells secreted sialomucins and/or sulphomucins with the columnar cells predominantly secreting sulphomucins. The tissue distribution of the 40-50 kDa glycoprotein in normal and intestinal metaplasia appears to be different to that of the mucins. In the normal tissue, the 40-50 kDa glycoprotein was consistently expressed in parietal cells. In IM, it was consistently expressed (18/18) in the columnar cells of all three types of metaplasia (type I, type II and type III) looked at in this study. We found varying degrees of staining of the 40-50 kDa glycoprotein within metaplastic tissue (Table 4.4). The 40-50 kDa glycoprotein was mostly present in the cytoplasm of columnar cells (Figure 4.2 C and D), additionally showing nuclear reactivity in some cases. The 40-50 kDa glycoprotein was weakly reactive (1+) in the majority of the cases, in type I (50%) and type II (60%) IM.

We found MUC2 expression in the goblet cells of complete type I (7/12) and incomplete type II IM (2/5). Ho *et al.* (1995) found MUC2 and MUC3 to be highly expressed in IM of the stomach in both goblet and columnar cells. Other studies have identified several antigens expressed both in gastric IM and in cancer, but not in normal gastric epithelium. These include large-intestine mucin antigen (LIMA) (Hertzog *et al*., 1991), FU-MK-1 (Watanabe *et al*., 1993) and 91.9H (Ohe *et al.,* 1994). Several studies raised the possibility that different types of IM may have different malignant potential. Jass (1980) and Filipe *et al.* (1994) associated the incomplete type II and III IM with intestinal-type gastric cancer.

We evaluated the expression of the 40-50 kDa glycoprotein and found it to be consistently expressed in the different types of gastric carcinomas (intestinal type, diffuse, mixed, signet-ring cell and mucinous) we looked at. The well differentiated glands of the intestinal carcinoma showed cytoplasmic staining for the 40-50 kDa glycoprotein, a similar expression seen in the diffuse type cancers. In the signet-ring cell and mucinous carcinomas, the 40-50 kDa glycoprotein was also expressed in the cytoplasm. We found that the 40-50 kDa glycoprotein was inconsistently expressed in the diffuse and signet-ring cell carcinoma and consistent in the intestinal type (13/13) carcinomas.

In the overall comparison, the 40-50 kDa glycoprotein and MUC1 were expressed in greater than 80% of the intestinal, mixed and signet-ring cell carcinomas (Table 4.4). The 40-50 kDa glycoprotein, MUC1 and MUC1 core were also expressed consistently in mucinous carcinomas. Other studies reported that the coexpression of MUC1 and MUC5AC increased the metastatic potential of gastric carcinoma cells (Ho *et al.,* 1995; Taylor *et al.,* 1998).

Overexpression of MUC1 in cancer cells has been reported to decrease cell-cell interaction by interfering with the integrin-mediated adhesion (Hilkens *et al.,* 1992; Wesseling *et al.,* 1995; Ligtenberg *et al*., 1992; Makiguchi *et al.,* 1996) thereby increasing the metastatic capacity of carcinoma cells and favouring invasion of tumour cells (Hiraga *et al.,* 1998; Utsunomoya *et al*., 1998). There is enough evidence to suggest that tumour cells expressing high levels of MUC1 may have increased invasive and metastatic potential. Agrawal *et al.* (1998) reported that

cancer-associated MUC1 mucin and synthetic tandem repeats of MUC1 mucin core peptide can suppress T cell proliferation and higher levels of MUC1 expression are correlated with immuno-suppression in adenocarcinoma patients. In our study, we found that gastric carcinomas expressed higher levels of MUC1 than normal gastric tissue as has been shown by Lee *et al.* (2001) (Figure 4.10). Baldus *et al.* (1998) reported that MUC1 was consistently expressed in intestinal type carcinomas than signet-ring cell or diffuse carcinomas. We found MUC1 to be consistently expressed in the diffuse and signet-ring cell and intestinal types. This combined expression of mucin and the 40-50 kDa glycoprotein may alter the biology of the cell, enabling it to develop greater potential for malignant transformation and metastases.

The *de novo* expression of MUC2 in gastric cancer reported by Utsunomiya *et al*. (1998) was confirmed in our study (Figure 4.10), with the exception of the mixed type carcinoma. In agreement with previous studies (Reis *et al.,* 2000; Gurbuz *et al.,* 2002), we observed consistent expression of MUC2 in mucinous carcinomas. MUC2 expression has been shown to be increased in mucinous carcinomas of the colon, breast and ovary (Hanski *et al.,* 1997). These researchers reported higher MUC2 expression in signet-ring cell carcinomas. Also, this study showed that MUC5AC and MUC6 expression decreased during gastric carcinogenesis (Figure 4.10), a feature reported by Pinto-de-Sousa *et al*. (2002). High levels of gastric mucins and relatively low levels of intestinal mucins have been reported in diffuse tumour cells (Lopez-Ferrer., 2000). MUC5AC has been shown to mostly associate with diffuse and signet-ring cell carcinomas (Reis *et al.,* 1997; Pinto-de-Sousa *et al.,* 2002). We found MUC5AC to be expressed more in the signet-ring cell carcinomas and less in the diffuse carcinomas. The absence MUC5AC in mucinous carcinomas of the colon has been suggested to be a prognostic factor for more aggressive colorectal carcinomas (Jass and Roberton, 1994). The absence of MUC5AC expression in mucinous carcinomas was confirmed in our study (Figure 4.10). However, Kocer *et al.* (2004) reported that in gastric cancer, MUC5AC positive tumours have worse prognoses than MUC5AC negative tumours.

In summary and based on the hypothesis that this glycoprotein might play a role in gastric carcinogenesis, we investigated by immunohistochemistry its expression in normal, intestinal metaplasia and carcinoma of the stomach. In the normal gastric

mucosa, the 40-50 kDa glycoprotein was expressed in parietal cells and not in the mucus producing cells. This pattern of expression was evident in both the uninvolved mucosa (n=3) and the mucosa next to the tumour site (n=17). The expression of the 40-50 kDa glycoprotein in parietal cells of normal gastric tissue adjacent to the tumour raises interesting questions. Akyurek *et al.* (2002) reported finding MUC1 in the canalicular system of parietal cells. We cannot say for sure that the observed extensive parietal cell staining with the anti-(40-50 kDa glycoprotein) is only in the transitional gastric mucosa as we were unable to obtain normal stomachs to include in this study. This 40-50 kDa glycoprotein may be an important factor in the pathogenesis of gastric cancer. Despite the assumed importance of the 40-50 kDa glycoprotein, there is still limited biochemical understanding of its association with gastric mucins, its identity and how this is altered during gastric disease.

We were unable to evaluate our findings statistically because of the small number of samples available to us for this study. Also, the location of the 40-50 kDa glycoprotein and its pattern of expression differed in the different groups, namely normals, intestinal metaplasia and cancer. In the next chapter (Chapter 5), we report how we used proteomics to perform a more complete analysis of the 40-50 kDa glycoprotein and by using two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) in combination with MALDI-TOF MS to define the protein components of the 40-50 kDa glycoprotein and to identify it.

CHAPTER 5

PROTEOMIC ANALYSIS AND IDENTIFICATION OF THE 40-50 kDa GLYCOPROTEIN IN GASTRIC MUCIN

5.1 Introduction

The purpose of the study reported in this chapter was to analyze the 40-50 kDa glycoprotein (resolved as a single band via SDS-PAGE in Chapter 3) using 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE), in order to produce a peptide sequence that would help identify it by using the Matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS). Two-dimensional gel electrophoresis (2-D PAGE), mass spectrometry (MS) and bioinformatics are the key components of current proteomics technology (Shevchenko *et al.,* 2002; Giorgianni *et al*., 2003). 2-D PAGE is a principal tool in proteomics that separates proteins according to their isoelectric point (pI) by isoelectric focusing (IEF) in the first dimension and according to their molecular weight (M_r) by SDS-PAGE in the second dimension. The principle behind MALDI-TOF MS is that the masses of the tryptic digested peptide fragments are measured and fragmentation of the peptide is generated by collisionally activated dissociation. Then the proteins can be identified because the sequence of the unknown peptide can be determined from the resulting fragmentation patterns using bioinformatic programs.

In the previous chapter (Chapter 4), we showed that the tissue localization of the 40-50 kDa glycoprotein was different from that of mucin, in that it was secreted by parietal cells in the normal gastric tissue. This raised the question of whether it was a mucin at all, and we hoped that the answer would be provided by the technique of proteomics. We isolated the glycoprotein from the crude mucus and separated it from purified mucin in patients with gastric carcinoma. We used 2-D PAGE for the analysis of the 40-50 kDa glycoprotein. We separated the 40-50 kDa glycoprotein using the first dimensional IEF technique, that separates proteins according to their pI, and then separated it in the second dimension according to its M_r on SDS PAGE.

We rehydrated strips (pH 3-10, BIO-RAD) with purified, freeze-dried gastric cancer mucin (100-200 µg) solubilized in urea buffer (9 M Urea, 2 M Thiourea and 4% (w/v) CHAPS). The IPG strips were overlaid with mineral oil and allowed to rehydrate overnight at room temperature. After IEF, proteins were electrophoresed on SDS-PAGE (12%, w/v) and stained with Coomassie Brilliant Blue or Periodic Acid Schiff (PAS). These separation parameters allowed for the resolution of proteins differing by a single charge, thereby allowing *in vivo* modifications such as post-translational modifications to be detected. Gels were scanned using a Canon scanner (CanoScan 4200F) and the BIO-RAD molecular multimager system.

5.2 Results

5.2.1 Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

The 40-50 kDa glycoprotein profile was better resolved on 7 cm strips (pH 3-10). Well resolved 2-D PAGE with clear separation of the 40-50 kDa spots was achieved by loading the optimized amount of mucin sample (100 µg mucin for gels stained with Coomassie Blue and 200 µg mucin for gels stained with PAS). Characterization of the 40-50 kDa glycoprotein in 2-D PAGE revealed three spots of varying abundance after Coomassie Brilliant Blue staining (Figure 5.1A, arrows 1-3) and one spot after staining with PAS (Figure 5.1B, arrow 4). Protein spots were observed to be migrating at M_r between 40 to 50 kDa with closely associated pI (estimated between pH 3 and pH 4). In chapter 3, we separated the 40-50 kDa glycoprotein on 1-D PAGE according to its molecular weight without the additional separation according to pI as compared to the 2-D PAGE discussed in this chapter.

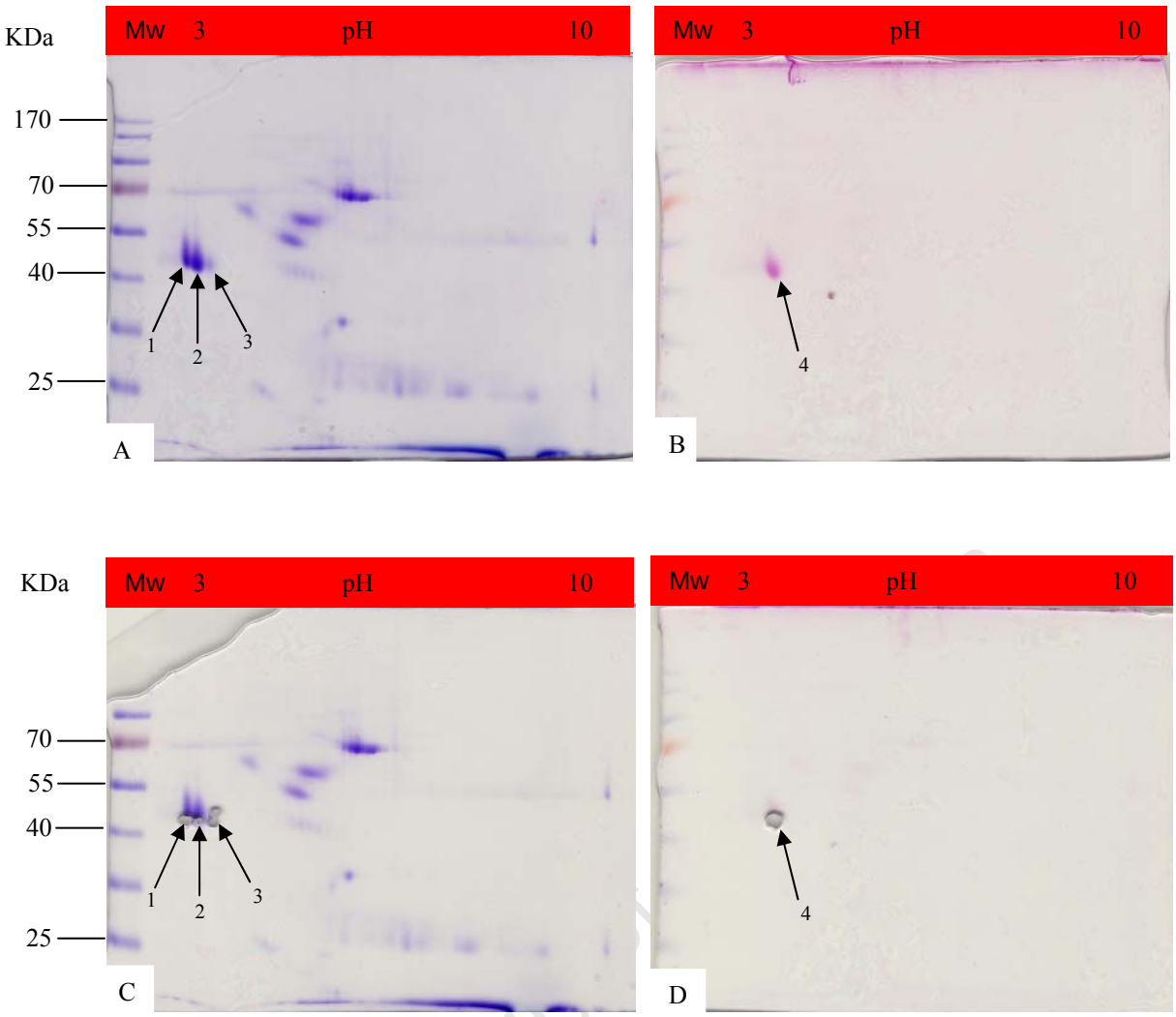
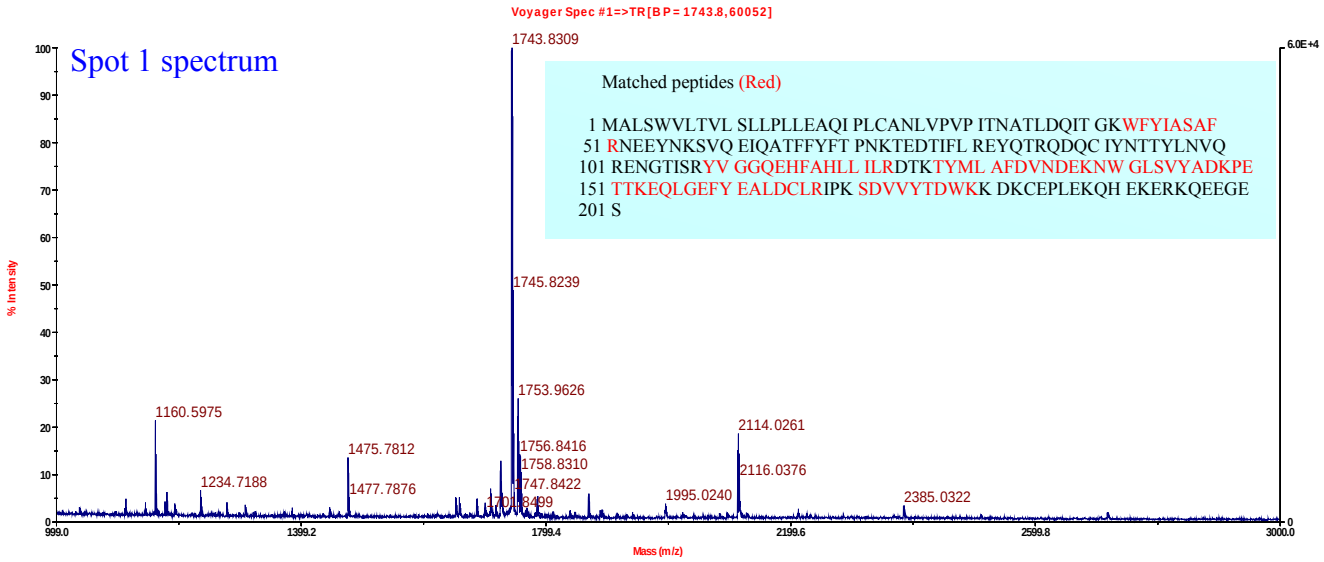
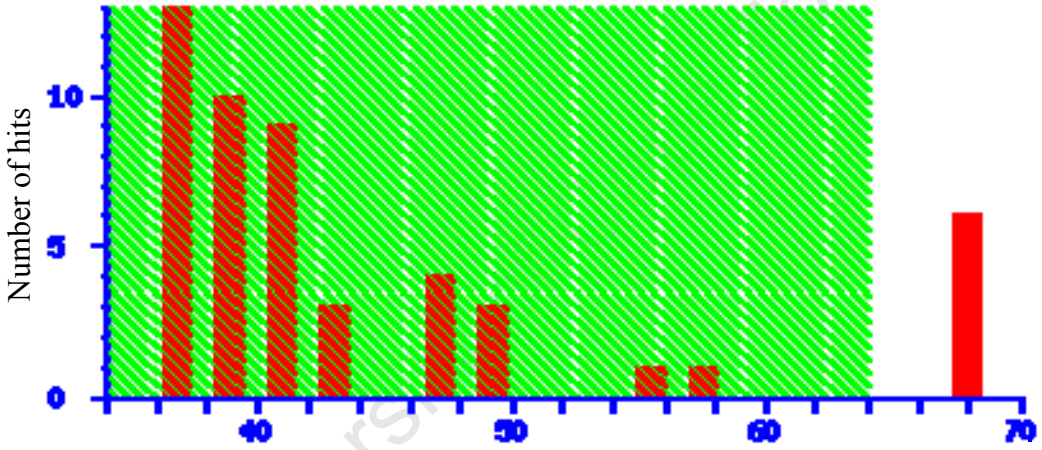


Figure 5.1 2-D PAGE expression profiles of the 40-50 kDa glycoprotein. Purified gastric mucin was used to rehydrate 7 cm, pH 3-10 IPG strips. Total volume of the rehydrate (sample with buffer) was 125 µl. IEF was applied to separate the proteins according to their isoelectric point (pI). The IEF gel was applied to an SDS PAGE (12%, w/v) which allowed the resolution of protein spots present at M_r 40 to 50 kDa. (A) Gel stained with Coomassie Brilliant Blue (100 µg mucin) showing three protein spots (arrows 1, 2 and 3). (B) Gels stained with PAS (200 µg mucin) showing only one spot (arrow 4). Gels C and D represent gels A and B after cutting. The protein spots were excised manually from the gels using a sterile pipette tip or razor blade and prepared individually for analysis by MALDI-TOF MS.

5.2.2 Identification of the 40-50 kDa glycoprotein by MALDI-TOF MS

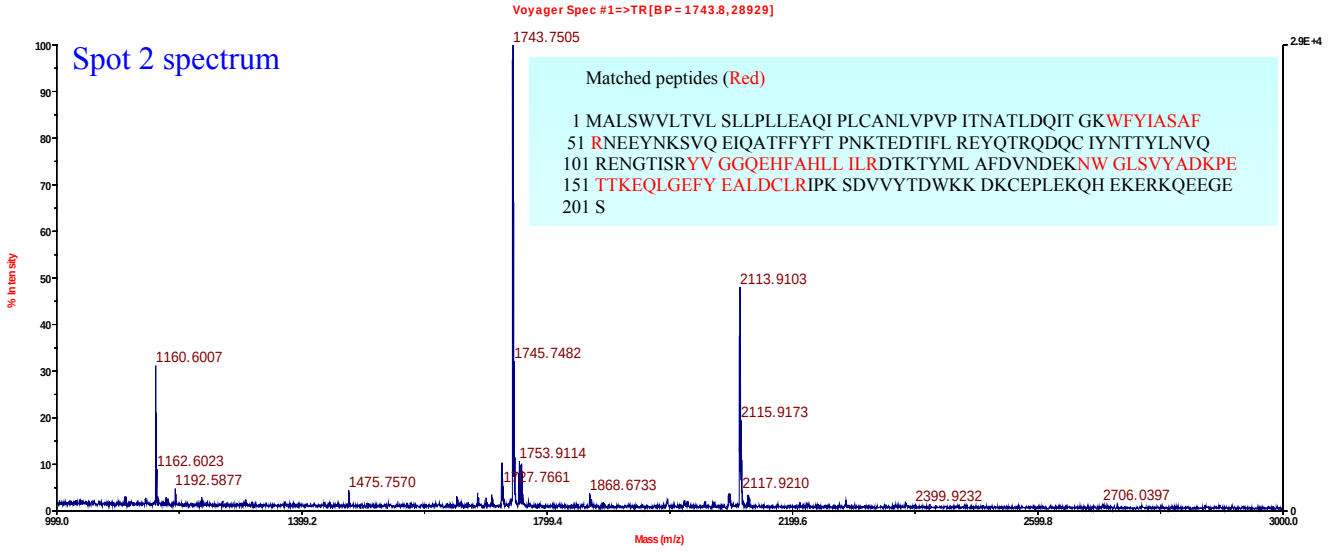
The gel spots excised from the 2-D PAGE (Figure 5.1, C and D) were prepared and digested (see Materials and Methods chapter 2) until a good spectrum defined by good peaks was detected. Spots corresponding to the 40-50 kDa glycoprotein were analyzed individually by MALDI-TOF MS and the resulting spectra and the corresponding histogram of the Mascot probability based score are shown in Figure 5.2 to 5.5. The protein sequence database (MASCOT) was searched for all proteins that matched the submitted masses of peptides generated from the trypsin digested 40-50 kDa glycoprotein. Peptides that matched the 40-50 kDa glycoprotein are inserted (red) in all spectra. The three Coomassie Brilliant Blue staining spots excised from the 2-D gel (Figure 5.1C) were identified as alpha-1-acid glycoprotein (orosomucoid) with a Mowse score greater than 64 (Figure 5.2 to 5.4) while the PAS staining spot (Figure 5.1D) could not be positively identified, confirmed by a low Mowse score of 40 (Figure 5.5). As can be seen in the spectra, there are similarities in the peak series for spots 1(Figure 5.2) spot 2 (Figure 5.3) and spot 3 (Figure 5.4), different from the peak series for spot 4 (Figure 5.5). The most abundant peptide at peak 1743.8309 (spot 1), 1743.7505 (spot 2) and 1742.8044 (spot 3) was common in all three Coomassie Brilliant Blue stained spots. The most abundant peptide for the PAS stained spot, at peak 1651.9033, differed from that shown by the Coomassie Brilliant Blue stained spots. A summary of the three Coomassie Brilliant Blue spots identified by MALDI-TOF MS is given in Table 5.1.





Probability Based Mowse Score for spot 1

Figure 5.2 MALDI-TOF mass spectrum and the histogram of the Mascot probability based score (the Mowse score) of spot 1. The Coomassie Brilliant Blue spot (spot 1) resolved on 2-D PAGE was excised from the gel, digested with trypsin and then analyzed by MALDI-TOF MS for the acquisition of the spectrum of the (40-50 kDa glycoprotein) peptides. The Mascot database was searched to detect all peptides that matched with the masses of the peptides from the digested spot (spot 1) (obtained during the spectrum acquisition). The spot was positively identified as alpha-1-acid glycoprotein with a high and significant ($p<0.05$) Mowse score of 68. Peptides matched from the database are inserted (in red) into the right hand corner of the spectrum.



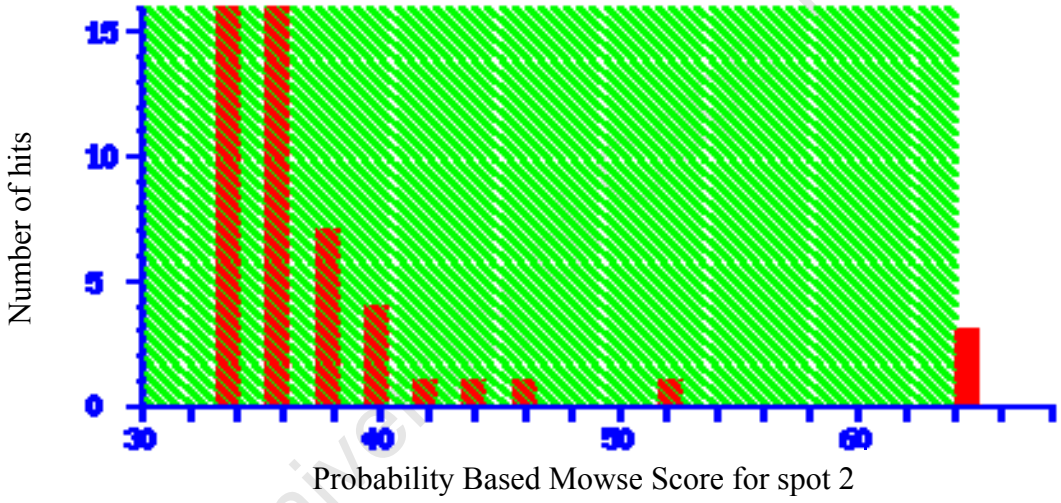
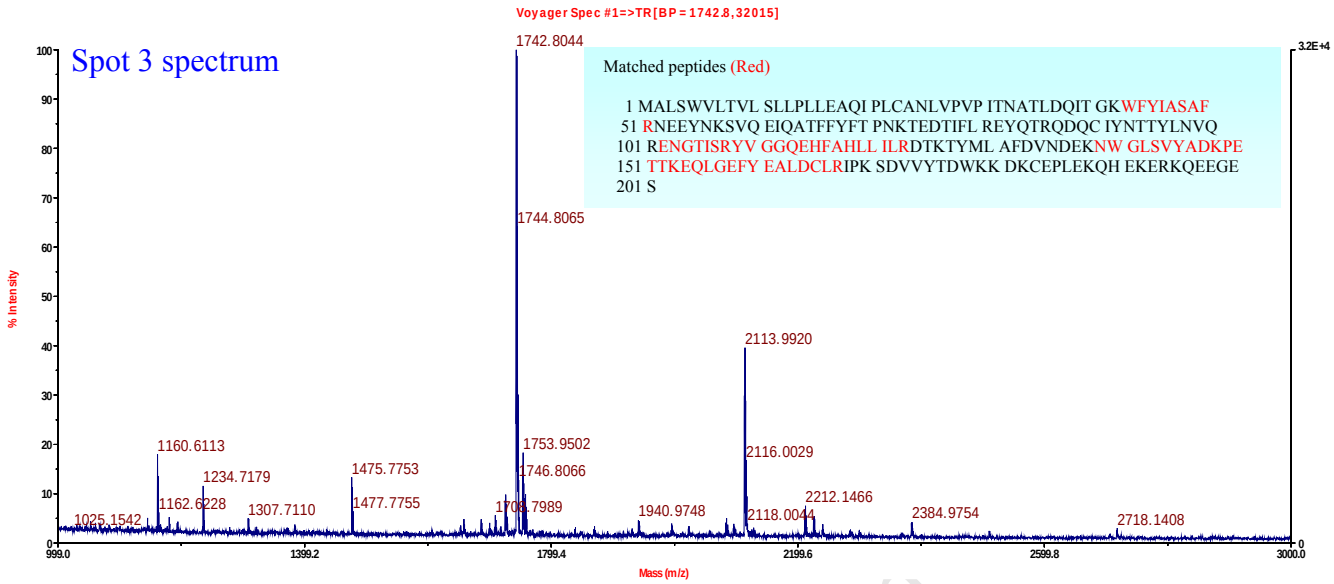


Figure 5.3 MALDI-TOF mass spectrum and the histogram of the Mascot probability based score (the Mowse score) of spot 2. The Coomassie Brilliant Blue spot (spot 2) resolved on 2-D PAGE was excised from the gel, digested with trypsin and then analyzed by MALDI-TOF MS for the acquisition of the spectrum of the (40-50 kDa glycoprotein) peptides. The Mascot database was searched to detect all peptides that matched with the masses of the peptides from the digested spot (spot 2) (obtained during the spectrum acquisition). The spot was positively identified as alpha-1-acid glycoprotein with a high and significant ($p<0.05$) Mowse score of 64. Peptides matched from the database are inserted (in red) into the right hand corner of the spectrum



C.

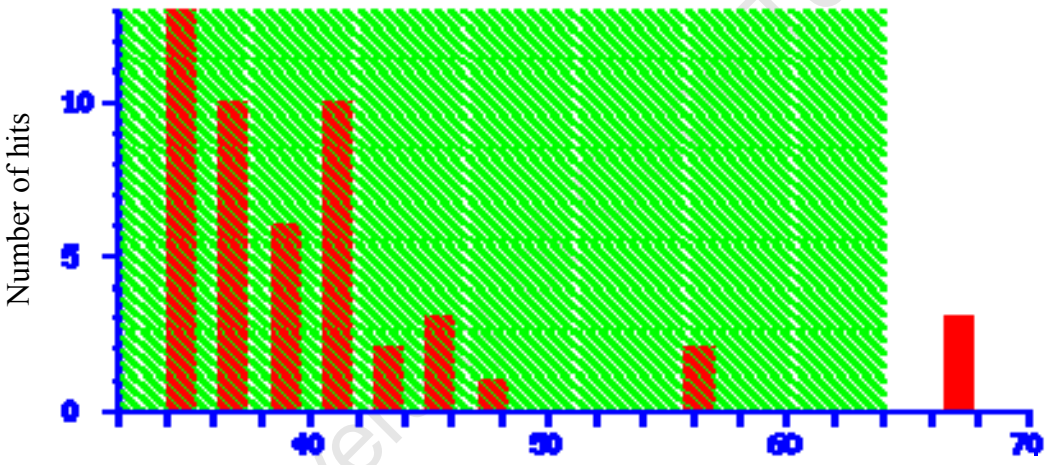
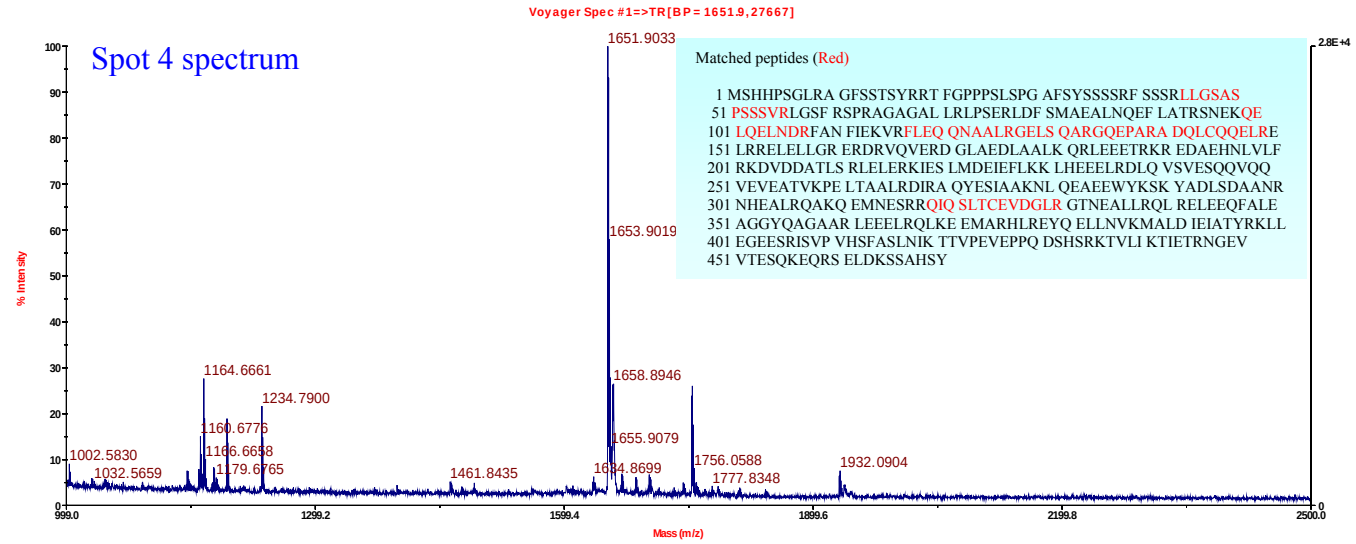


Figure 5.4 MALDI-TOF mass spectrum and the histogram of the Mascot probability based score (the Mowse score) of spot 3. The Coomassie Brilliant Blue spot (spot 3) resolved on 2-D PAGE was excised from the gel, digested with trypsin and then analyzed by MALDI-TOF MS for the acquisition of the spectrum of the (40-50 kDa glycoprotein) peptides. The Mascot database was searched to detect all peptides that matched with the masses of the peptides from the digested spot (spot 3) (obtained during the spectrum acquisition). The spot was positively identified as alpha-1-acid glycoprotein with a high and significant ($p<0.05$) Mowse score of 67. Peptides matched from the database are inserted (in red) into the right hand corner of the spectrum



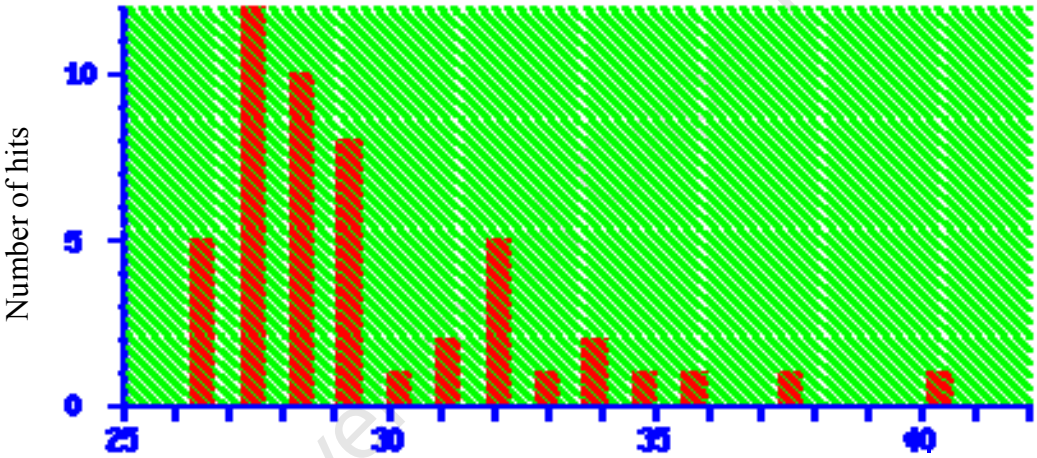


Figure 5.5 MALDI-TOF mass spectrum and the histogram of the Mascot probability based score (the Mowse score) of spot 4. The PAS spot (spot 4) resolved on 2-D PAGE was excised from the gel, digested with trypsin and then analyzed by MALDI-TOF MS for the acquisition of the spectrum of the (40-50 kDa glycoprotein) peptides. The Mascot database was searched to detect all peptides that matched with the masses of the peptides from the digested spot (spot 4) (obtained during the spectrum acquisition). The spot could not be positively identified as indicated by a low Mowse score of 40. Peptides matched from the database are inserted (in red) into the right hand corner of the spectrum

Table 5.1 The M_r 40 to 50 kDa Coomassie Blue spots identified by MALDI-TOF MS as alpha-1-acid-glycoprotein

Protein spot number	pI from database	pI from gel	Molecular weight from database (kDa)	Molecular weight from gel (kDa)	Mowse Score	Name of protein identified
1	4.93	3-4	46.994	40-50	68	alpha-1-acid glycoprotein 1 precursor [validated] - human
2	4.93	3-4	46.994	40-50	64	alpha-1-acid glycoprotein 1 precursor [validated] - human
3	4.93	3-4	46.994	40-50	67	alpha-1-acid glycoprotein 1 precursor [validated] - human

5.3 Discussion

This study was begun in the 90's based on a laboratory observation of the presence of a 40-50 kDa glycoprotein in mucins purified from patients with gastric cancer which was absent in the normal gastric mucosa. In Chapter 3 we described how the antibody was raised to this glycoprotein and in Chapter 4 we showed the location and expression of this glycoprotein in normal, intestinal metaplasia and carcinoma of the stomach. We then attempted to identify this 40-50 kDa glycoprotein using proteomics. A detailed analysis of the 40-50 kDa glycoprotein was carried out by MALDI-TOF MS. This technique is the most accurate method for the identification of an unknown protein and allowed for an in-depth analysis of the 40-50 kDa glycoprotein. The finding that the 40-50 kDa glycoprotein is AGP was not expected, although this discovery is supported by our previous findings in that the protein composition suggested that this was an N-linked glycoprotein (Mall *et al.,* 1999).

Some experimentation was carried out to determine the optimum conditions for 2-D PAGE to avoid horizontal and/or vertical streaking due to overloading and to improve focusing of spots into tight spots and not smears. We used 100 µg of mucin sample for 2-D PAGE stained with Coomassie Brilliant Blue and 200 µg for the PAS stained gels because Coomassie Brilliant Blue is a more sensitive stain than PAS. The optimum sample volume for loading was 125 µL (sample with buffer). The use of

high quality protein samples and loading the correct amount of protein where the key factors ensuring well resolved 2-D gels with a clear separation of the 40-50 kDa spots. The 40-50 kDa glycoprotein was well resolved (not overlapping) on the broad range IPG strips, (Figure 5.1 A and B, pH 3 to pH 10), thus the use of IPG strips with narower pH range (pH 3 to pH 4) was not necessary. IEF was performed on mucin purified from cancer and after equilibration with SDS buffer in the presence of DTT (used to reduce disulfide bonds for analyzing proteins as single subunits), the IEF gel was applied to an SDS gel (12%) which allowed the resolution of three protein spots between Mr 40 to 50 kDa.

The proteomics approach provided a more direct assessment of the nature of the 40-50 kDa glycoprotein. We confirmed the presence or absence of the 40-50 kDa glycoprotein in SDS-PAGE (Chapter 3) prior to analysis by 2-D PAGE. Mucin from post morten specimens and fresher tissues from transplant donor specimens did not contain the 40-50 kDa glycoprotein as confirmed by Mall *et al.* (1999). Only mucin samples containing the 40-50 kDa glycoprotein were analyzed by 2-D PAGE. In 1-D PAGE the 40-50 kDa reproducibly stained with PAS (Chapter 3, Figure 3.2A) and occasionally with Coomassie Brilliant Blue (Chapter 3, Figure 3.2B) as a single component "band" when separated by size. In 2-D PAGE, the 40-50 kDa glycoprotein was spread out across, effectively separating into three Coomassie spots (Figure 5.1A) and one PAS spot (Figure 5.1B). The four protein spots had a pI range between pH 3 and pH 4, with the molecular weight ranging between 40 kDa and 50 kDa.

Extensive care was taken when preparing and handling the protein samples to avoid contamination (keratin) prior to MALDI-TOF MS identification. Spots 1 to 4 excised from the gels were digested with trypsin and the resulting peptide fragments were extracted and identified by MALDI-TOF MS. Three protein spots (1 to 3) were successfully identified as alpha-1-acid glycoprotein and reconfirmed on numerous occasions (Table 5.1), presenting high confidence Mowse scores ($p<0.05$) (Figure 5.2, 68 for spot 1; Figure 5.3, 64 for spot 2 and Figure 5.4, 67 for spot 3) which indicate identity of extensive homology. Mowse scores >64 are highly significant, leaving little room for doubt. The Mowse score uses empirically determined factors to assign a statistical weight to each individual peptide match.

We were unable in the present study to positively identity the PAS positive spot. The MALDI-TOF MS spectra of the PAS spot showed a low Mowse score of 40 (Figure 5.5), not high enough to get a significant match. Given the M_r size and the pI of the PAS spot glycoprotein on 2-D PAGE (Figure 5.1B), it was possible to provide an objective measure of the significance of the result presented in Figure 5.5. The PAS spot matched Peripherin on the MS database which has a higher molecular weight (M_r 107.236 kDa) and a higher pI than that observed in the 2-D PAGE (Figure 5.1B) thus providing reason to conclude that this result is not significant.

We expected the fourth spot to be identified as the same protein as the other three spots stained with Coomassie Blue because of the similarity (in 2-D PAGE) in molecular weight and a pI within the range of pH 3 and pH 4. We therefore have reasons to believe that several drawbacks of using the PAS stain may have resulted in the possible impairment of the peptides thus interfering with the MALDI-TOF MS analysis. Countless staining procedures such as SYPRO Ruby and Deep Purple have been shown to combine high sensitivity and compatibility with mass spectrometry (Nishihara and Champion, 2002). Coomassie brilliant blue staining has been the dominating staining method in proteomics because it is highly compatible with mass spectrometry analysis and also non-expensive (Nishira and Champion, 2002). In contrast, the PAS stain as has not been tested for compatibility with MALDI-TOF and we suggest possible chemical modifications might have hindered our ability to positively identify the fourth spot. It has been reported that AGP has eight potential phosphorylation sites influenced by different physiological conditions (Founier *et al.,* 2000). Perhaps post-translational modification might be another possibility suggesting that the PAS spot is a glycoform of AGP thus different from the three Coomassie Blue spots.

A comparison of the MALDI spectra permits the immediate recognition of an overlap between several low intensity peaks, 1160.5975 for spot 1; 1160.6007 for spot 2; 1160.6113 for spot 3 and 1160. 6776 for spot 4, knowledge that leads us to think that the PAS positive spot (spot 4) could be related to the three Coomassie spots (spots 1, 2 and 3). However, we observed a discrepancy for the biggest signal peptides when comparing the Coomassie stained spots (1743.8309 for spot 1; 1743;7505 for spot 2; 1742.8044 for spot 3) and the PAS stained spot (1651.9033 for spot 4). The peak with

the highest intensity in spot 1, 2 and 3 (Figure 5.2 to 5.4) is missing in spot 4 (Figure 5.5) and this might suggest evidence of chemical modification by PAS, the limitation which was expected since certain stains are not compatible with MALDI-TOF MS.

However, a careful analysis of the PAS spot therefore appears to be an absolute requirement perhaps with the use of a glycoprotein stain that has been reported to be compatible with MALDI-TOF MS. The importance of comparing results obtained from the database and results from the gels has clearly shown predictions of MALDI-TOF to be accurate for the three Coomassie positive spots and to be false for the one PAS positive spot. Although 2D-PAGE and MALDI-TOF-MS based proteomics has proven powerful for the analysis of the 40-50 kDa glycoprotein, it still remains a costly and labor- and time-consuming process. The functional differences of the four spots identified by MALDI-TOF MS remains unclear as much as their functional importance with mucins in the stomach of patients with cancer.

CHAPTER 6

DISCUSSION, CONCLUSIONS AND THE FUTURE

Since 1975, the International Agency for Research on Cancer, in its estimate of the global burden of cancer (Parkin *et al.,* 1984) listed gastric cancer as a cancer of high fatality, with an estimated 934,000 new cases per year in 2002 (8.6% of the total of new cancer cases). Gastric cancer is ranked as the second most common cause of death from cancer, with 700,000 reported deaths annually (Parkin *et al.,* 2001, 2002, 2005) and once ranked as the fourth highest in the world at 69.9/100,000 (Bradshaw and Harrington, 1975).

Although it has been suggested that gastric cancer is rare in Africa (Holcombe, 1992), the incidence in Central Africa has been reported to be higher than previously suggested (Parkin *et al.,* 2003). In South Africa, gastric cancer is prevalent in the Western Cape region (Botha, 1972; Bradshaw and Harrington, 1975; Wyndham, 1985; Sitas and Isaacson, 1992) and is reported to be a fatal malignancy among so-called Cape Coloured (a hybrid race of Western European, Southern African and Asian origin) males (Botha, 1972).

Survival from stomach cancer is moderately good in Japan (52%), where mass screening by photofluoroscopy has been practiced since the 1960s. Survival is also relatively high in North America, possibly due to early diagnosis through rigorous endoscopic screening programmes for gastric disorders. Survival from gastric cancer is estimated to be 27% in Western Europe while it is as low as 6% in sub-Saharan Africa. There is a strong environmental component to the risk differences for gastric cancer. Factors such as *H. pylori* infection (Morgner *et al.,* 2000) and dietary factors, including high intakes of preserved salted foods and nitrates and deficiencies of vitamin C and E, certainly play an important role in increasing the risk of gastric cancer (Kelley and Duggan, 2003). There is a need for a biochemical marker for pre-malignant disease and early gastric cancer. The available markers such as carcino-embryonic antigen (CEA) increase in levels when the disease has spread considerably, and are therefore not useful for early detection (Fuchs and Mayer, 1995). Mucins have shown promise as diagnostic markers in disease, for example MUC4 in human

pancreatic cancer and MUC1 for breast cancer (Karanikas *et al.,* 1997). The inability to diagnose gastric cancer at an early, localized, and curable stage contributes to poor prognosis. Thus, gastric cancer remains a dismal disease and early diagnostic markers and therapeutic targets are urgently needed. Previous studies in this laboratory showed the 40-50 kDa glycoprotein to be present in crude mucus scrapings of both cancer patients and those with ulceration of the mucosa (Mall *et al.,* 1990, 1992, 1999). Thus the underlying aim of the isolation and identification of the 40-50 kDa glycoprotein reported in this study, has as a future goal, an investigation to determine its potential as a marker for pre-malignant and very early malignant disease (Mall *et al.,* 1992). Our plan is to assay blood levels of AGP in patients diagnosed with carcinoma of the stomach and compare these to blood levels in patients with gastric ulceration, the siblings of patients with gastric cancer and those who are normal, with no gastric disease whatsoever.

The 40-50 kDa glycoprotein was first described in 1990 by Mall *et al.* (1990), and was found to be associated with albumin, an intriguing find since the mucins were purified extensively prior to analysis by SDS-PAGE. It was also present in the gastric juice of a patient with gastric cancer (Hakkinen *et al.,* 1991; Mall *et al*., 2000). The unusual nature of the 40-50 kDa glycoprotein prompted us to identify it. We identified the 40-50 kDa glycoprotein as alpha-1-acid glycoprotein (AGP), also known as orosomucoid, a product of three adjacent genes (AGP-A, AGP-B and AGP-B′) (Dente *et al.,* 1987). AGP is predominantly synthesized by hepatic cells and was first described in 1950 (Fournier *et al*., 2000) as a 40-43 kDa glycoprotein (Fournier *et al.,* 2000) and a 50-60 kDa glycoprotein (Ceciliani and Pocacqua, 2007). Mall *et al.* (1990, 1992) reported the size of the 40-50 kDa glycoprotein to be 50-60 kDa on 4-20% gradient gels and later 55-65 kDa on 10% gels (Mall *et al.,* 1999), different from what was shown here on 12% SDS-PAGE gels (40-50 kDa). Thus, the identification (by proteomics) of the 40-50 kDa glycoprotein as AGP would now allow us to determine its levels in blood.

The identification of the 40-50 kDa glycoprotein as AGP confirms earlier suggestions by Mall *et al*. (1999) that the fragment was likely to be N-linked as opposed to an O-linked glycoprotein. The carbohydrate content of AGP represents >45% of the molecular weight (Schmid *et al.,* 1977) attached in the form of five to six highly

sialylated complex-type-N-linked glycans (Ceciliani and Pocacqua, 2007). The five N-glycosylation sites of human AGP are not conserved; each of the N-glycosylation sites can express any of the glycans. The common terminating carbohydrate is sialic acid, constituting 10-12% of the whole sugars. This unusual high content of sialic acid is responsible for the low pI (pH 2.8 to pH 3.8) of AGP. A clear decrease in size of the 40-50 kDa glycoprotein was seen after treatment with neuraminidase (Mall *et al.,* 1999), suggesting the presence of sialic acid on the carbohydrate composition of this glycoprotein. Using 2-D PAGE, we found the 40-50 kDa to have a low pI (pH 3 to pH 4), comparable to that reported for AGP.

Reduction of purified mucin obtained from gastric cancer samples in DTT and 2M 2-mercaptoethanol did not dissociate the 40-50 kDa glycoprotein suggesting it to be a single entity and not a polymeric structure of subunits joined by disulphide bridges (Mall *et al.,* 1990). This glycoprotein also failed to dissociate with boiling in 1% SDS, 4M guanidinium chloride and 3.5M CsCl (Mall *et al.,* 1990). Indeed, AGP is reported to be a single polypeptide chain of 183 amino acids in humans and 187 amino acids in rats (Nakano *et al.,* 2004).

AGP has been classified as a positive acute-phase protein (Hochepied *et al.,* 2003) as well as a member of the immunocalins (Ceciliani and Pocacqua, 2007; Fournier *et al.,* 2000). The biological function of AGP remains unkown; however, a number of activities of possible physiological significance, such as being a natural anti-inflammatory and immunomodulatory agent with respect to its anti-neutrophil, anti-complement activity (Williams *et al.,* 1997) and the ability to down regulate various phagocytic functions and T-cell-mediated activities (Hochepied *et al.,* 2003) have been described. The immunomodulatory as well as binding activities of AGP have been shown to be dependent mostly on its carbohydrate composition (Schmid, *et al.,* 1973).

The 40-50 kDa glycoprotein was not present in normal crude mucus scrapings (Mall *et al.,* 1999) whereas in this study we showed it to be expressed in parietal cells of normal human gastric tissue. Why then it is not secreted into the crude mucus of normal stomachs (transplant donors and cadavers) (Mall *et al.,* 1992, 1999) is not known. In addition to us showing the expression of the 40-50 kDa glycoprotein in

human gastric tissue, extra-hepatic expression of AGP has been reported in several other tissues (Sorensson *et al.,* 1999; Fournier *et al*., 2000). The presence of the 40-50 kDa glycoprotein in parietal cells reinforces the view that parietal cells produce a range of ligands that regulate gastric differentiation. Intrinsic factor is a glycoprotein secreted by parietal cells (humans) and it has an important role in the gastrointestinal absorption of vitamin B_{12} (Latner and Merrills, 1957). Failure to produce or utilize intrinsic factor results in pernicious anemia. High serum vitamin B_{12} concentration is seen in patients with chronic myelocytic leukemia (CML) (Pitney *et al.,* 1954; Beard *et al.,* 1954; Miller and Sullivan, 1958) and this is associated with plasma binding to AGP (Weinstein *et al.,* 1959). We are unable to explain the consistent expression of the 40-50 kDa glycoprotein in parietal cells of the normal gastric mucosa. It is possible that the intrinsic factor found in parietal cells might have binding sites to the 40-50 kDa glycoprotein. The fact that Weinstein *et al.* (1959) described the binding of vitamin B_{12} to AGP in sera (which is characteristically bound by the intrinsic factor in parietal cells) lends further support to our hypothesis.

We showed that the 40-50 kDa glycoprotein was consistently expressed in the columnar cells in both complete and incomplete metaplasia and not in the goblet cells. Molmenti *et al*. (1993) raised the possibility that columnar cells are involved in a local response to injury or inflammation by producing acute phase proteins under the control of cytokines (IL-1, IL-6, IFN and TNF). Geiger *et al*. (1988) reported that IL-6 *in vivo*, induced gene expression of AGP in rat intestinal epithelial cell lines while in this study we showed the expression of the 40-50 kDa glycoprotein in human small intestine.

Gendler *et al.* (1982) presented evidence for the active synthesis of AGP by human breast epithelial cells while Sorensson *et al.* (1999) reported expression of AGP by endothelial cells, required to maintain capillary permeability (Haraldsson *et al.,* 1987). Twining and Brecher. (1977) found AGP in malignant lung tissue, while Fournier e*t al.* (2000) and Crestani *et al.* (1998) reported AGP gene expression in human and rat lung tissue during inflammation. Several studies have reported an association of AGP with several neoplastic disorders, including breast and lung cancer, and immunologically mediated diseases, such as sarcoidosis (Duche *et al.,* 2000). We showed the consistent expression of the 40-50 kDa glycoprotein in the intestinal,

diffuse, mixed, signet-ring cell and mucinous gastric carcinomas. The stomach, like the liver, is involved in a local response to a local injury (intestinal metaplasia and gastric tumour) by producing AGP under the control of inflammatory mediators. Our results are in agreement with others who have demonstrated extra-hepatic expression of AGP. Indeed there is a growing body of evidence that the acute phase response may take place in extra-hepatic cells, notably the epithelial cells. To our knowledge, this is the first time AGP (40-50 kDa glycoprotein) has shown to be associated with mucus and mucins in gastric cancer. Ganz *et al.* (1984) reported AGP to be highly sensitive and specific in the detection of lung cancer.

Interestingly, both this study and the previous studies of Mall *et al.* (1999, 1992, 1990), showed the 40-50 kDa glycoprotein to be closely associated with albumin. This was shown even after extensive purification of the mucin in a CsCl/GuHCl density gradient. We are unable to explain this but are aware that albumin concentrations in the stomachs of patients with carcinoma are increased due to mucosal bleeding (Rossi *et al.,* 2003; Fletcher *et al.,* 2002). We suspect that the type of association between albumin and our glycoprotein of interest is non-covalent. We were successful once, in partially separating albumin from the 40-50 kDa glycoprotein in sodium dodecyl sulphate at 100°C for one to two minutes, followed by gel filtration (Mall *et al.,* 1992). However this was rather costly and laborious and was not pursued by us. It is possible that conformational changes either in the stomach of these patients or through the denaturing conditions in which the extraction of mucins occurs, results in this association between albumin and the 40-50 kDa glycoprotein, which is resistant to separation of mucin from the 40-50 kDa glycoprotein even in CsCl/4MGuHCl.

Our proteomic findings thus far have conclusively shown the 40-50 kDa glycoprotein to be alpha-1-acid glycoprotein which is distinguishable from both mucin and albumin. In our previous study, we used the anti-human albumin antibody to reveal that the 40-50 kDa glycoprotein was not albumin, but rather that it was associated with it. We went to great lengths to show that that this newly identified AGP was not an artefact of albumin. We used Coomassie Brilliant blue and Ponceau S stain to distinguish albumin from the 40-50 kDa glycoprotein. Mall *et al.* (1999) reported the 40-50 kDa glycoprotein to be found in gastric cancer mucus scrapings, fractionating

with mucin and associated with albumin, which persisted in being present even after 2 CsCl gradient purification steps (Mall *et al.,* 1992). AGP, together with albumin, are the most important binding proteins in plasma. Plasma AGP concentration is much lower than albumin, but during the acute phase the concentration of AGP is increased whereas the concentration of albumin, which is a negative acute phase protein, is decreased. Perhaps, this association between AGP and albumin, is similar to what would normally occur in the gastric mucosa (extra-hepatic expression) during an acute phase reaction. We showed albumin to be expressed in chief cells of the normal gastric mucosa while the 40-50 kDa glycoprotein now identified as AGP was expressed in parietal cells.

AGP found in the crude mucus fractionates with mucin in a caesium chloride density gradient due to its carbohydrate moiety that is greater than 40%. Cranmer *et al*. (1999) suggested in a von Willebrand factor molecule study that the tandem repeat units found in mucins originated to catch moving molecules or objects. Mucins have been shown to bind bacteria through specific attachment sites (Kubiet *et al.,* 2000). AGP has been shown to bind toxic molecules produced by bacteria, thus serving as a general protective agent (Moore *et al.,* 1997; Libert *et al.,* 1994). Perhaps AGP might be bound to material of similar density, such as mucin, via N-linked glycans. The large variations observed in the binding of AGP during physiological and pathological states are correlated to the variation in the carbohydrate moiety.

For years the presence of the 40-50 kDa glycoprotein in gastric mucus specimens has stimulated interest and the physiological importance of this glycoprotein is currently not understood. Since AGP was first identified, there has been a major effort to elucidate the occurrence and function of this molecule. Yet, we still have limited knowledge on the function of this glycoprotein *in vivo*. Based on our results, AGP is of great interest as a potential marker for the detection of gastric cancer.

Future investigations are recommended to compare the detectability and specificity of AGP in the sera of normal and cancer patients using the antibody we developed against it. Another project would be to address and circumvent the problems encountered with the identification of the PAS positive spot. The high variability of the glycan structure of AGP gives rise to several different glycoforms, and this could

prove to be very useful in investigating whether the PAS spot may show differences in the AGP glycoforms produced. The functional importance of AGP and mucin in the pathology of gastric cancer bears further investigation and therefore justifies the request for funding for future projects.

Finally, we need to expand this study with more samples that will enable us to verify statistically our findings and give us greater insight into the role of the 40-50 kDa glycoprotein in the pathogenesis of gastric disease. Unfortunately we were restricted by the scheduling of surgery in our attempts to secure samples for analysis during the three year period of this study. The other setback in the collection of samples was that a considerable number of patients were deemed by the surgeons to be inoperable because of the advanced nature of their disease. However, collection of samples is continuing and arrangements are being made for the preparation of samples for further analysis.

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