Expression of Secretory Mucins in the Human Upper Gastrointestinal Tract
The role of MUC5AC in the adhesion of *Helicobacter pylori*

The printing of this thesis was financially supported by:
Section Experimental Gastroenterology of the Netherlands Society of Gastroenterology (NVGE) and the Dutch Digestive Diseases Foundation (MLDS).

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"Expression of Secretory Mucins in the Upper Gastrointestinal Tract; The role of MUC5AC in the adhesion of *Helicobacter pylori*"

Thesis, Erasmus University Rotterdam – with references – with summary in Dutch
ISBN 90-6734-256-4

Cover: Adhesion of the bacterium *Helicobacter pylori* (red) to human antral gastric mucin MUC5AC (green).

Printed by Optima Grafische Communicatie, Rotterdam, The Netherlands (www.ogc.nl).

Expression of Secretory Mucins in the Human Upper Gastrointestinal Tract
The role of MUC5AC in the adhesion of *Helicobacter pylori*

Expressie van secretoire mucinen in het bovenste deel van het humane maagdarmkanaal
De rol van MUC5AC bij de binding van *Helicobacter pylori*

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof.dr.ir. J.H. van Bemmel
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 18 juni 2003 om 13.45 uur

door

Jeroen Henricus Bernardus van de Bovenkamp
geboren te Nijmegen

Promotiecommissie

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The study described in this thesis was performed at the Laboratory of Pediatric Gastroenterology & Nutrition of the Academic Medical Center, Amsterdam and at the Laboratory of Pediatrics, Pediatric Gastroenterology & Nutrition of the Erasmus MC / Sophia Children's Hospital, Rotterdam, The Netherlands.
Part of this study was financially supported by the Dutch Digestive Diseases Foundation (MLDS, Nieuwegein), Irene Children's Hospital Foundation (Arnhem), Jan Dekker/Ludgardine Bouman Foundation (Amsterdam) and Foundation "de Drie Lichten" (Leiden), all in The Netherlands.

Liens

Cordes faites de cris

Sons de cloches à travers l'Europe
Siècles pendus

Rails qui ligotez les nations
Nous ne sommes que deux ou trois hommes
Libres de tous liens
Donnons-nous la main

Violente pluie qui peigne les fumées
Cordes
Cordes tissées
Câbles sous-marins
Tours de Babel changées en ponts
Araignées-Pontifes
Tous les amoureux qu'un seul lien a liés

D'autres liens plus ténus
Blancs rayons de lumière
Cordes et Concorde

(…)

Guillaume Apollinaire,
in: *Calligrammes. Poèmes de la paix et de la guerre (1913-1916)*

Voor Arja

Bindingen

Linten van kreten

Geluid van klokken door heel Europa
Aaneengeregen eeuwen

Rails die volken aan elkaar vastketenen
We zijn maar met twee of drie mensen
Vrij van alle binding
Laten we elkaar de hand geven

Koorden van heftige regen die de rook kamt
Strengen
Touwen geweven
Kabels onder water
Torens van Babel veranderd in bruggen
Pauselijke spinnen
Alle geliefden verbonden door slechts één band

Andere vastere verbanden
Witte lichtbundels
Binding en Verbinding

(…)

Guillaume Apollinaire,
in: *Calligrammes. Poèmes de la paix et de la guerre (1913-1916)*
(vertaling van Arja Firet)

Contents

Chapter 1

General introduction

Upper gastrointestinal tract, *Helicobacter pylori*, and mucins

The upper gastrointestinal tract (GI) comprises the esophagus, stomach, and duodenum. These compartments of the GI tract function to transport and temporarily store ingested food and drink and initiate the digestive processes. Clinically, diseases of the upper GI tract form a major source of morbidity and mortality. Among these complaints are gastritis, gastric and duodenal ulcers, and gastroesophageal reflux disease (GERD), which each have a high incidence. Also cancer of the stomach and esophagus are among the most common in the Western world. It was not until 1983 that it became apparent that the presence of one particular bacterial pathogen might be linked to several of these diseases. This pathogen, the bacterium *Helicobacter pylori*, is now recognized as one of the most common human pathogens in the world, and is unique in that it can chronically infect the human stomach. The stomach is designed to kill bacteria that come down from the mouth. Yet *H. pylori* possess a number of intricate mechanisms to evade the killing fields of the stomach's digestive fluids and to even colonize the gastric mucus layer and epithelium. It is further able to largely evade the immune system, leaving only a very small chance for the infected individual for natural cure from the bacterium.

Infection with *H. pylori* inevitability leads to gastritis and chronic changes to the gastric mucosa. About 10-20% of the patients develop gastroduodenal ulcers, which are abnormalities closely correlated to chronic *H. pylori*-infection of the stomach. At the upper side of the stomach, GERD is one of the most common GI complaints. Here the acidic gastric fluid is forced back into the esophagus, giving symptoms often described as "heartburn". Twenty percent of the populations suffer from the symptoms of GERD at least once a week (Locke et al., 1997). The role of *H. pylori* in the pathology of GERD is uncertain. Other diseases of the upper GI tract with a high morbidity are esophageal and gastric cancer. Carcinoma of the stomach is the second most common cancer worldwide, and adenocarcinoma of the stomach accounts for nearly 95% of all gastric cancers (Parkin et al., 1999). The development of gastric adenocarcinoma has been correlated to *H. pylori*-infection of the stomach, leading to the classification of *H. pylori* as a class I carcinogen by the World Health Organization. Thus far it remains uncertain if *H. pylori* is involved in the occurrence of esophagus cancer.

Metaplastic tissue is generally considered to constitute a pre-cancerous condition, and is relatively common in the upper GI tract. In particular, the metaplasia of gastric epithelium, which is of the intestinal type, is closely correlated to gastric *H. pylori*-infection. Metaplasia of the esophagus, commonly referred to (as we will) as Barrett's esophagus (BE), is also a rather common condition. BE is considered to indicate an increased risk to progress into esophageal adenocarcinoma. It is still uncertain if *H. pylori*-infection of the stomach is related to the occurrence of BE or to its progression into adenocarcinoma. Although the prevalence of *H. pylori*-infection has been declining in developed countries (Parsonnet, 1995), it has been noted

that BE have been increasing along with cancer of the esophagus (Pera et al., 1993). We have closely recorded changes in the gastric epithelium and the metaplastic epithelia of the upper GI tract and studied their relation to *H. pylori*-infection. As the gastric as well as the metaplastic epithelial changes give rise to epithelia with many secretory characteristics, it is likely that secretory products of these cells form interesting markers to record the progress of the metaplastic progression. Moreover, these secretory products themselves may have impact on the disease processes, due to their diverse biological functions.

The "natural habitat" of *H. pylori* within the human body seems to be the gastric mucus and the mucus-producing epithelium (Shimizu et al., 1996a; Shimizu et al., 1996b). The question is; what is so special about the gastric mucus that *H. pylori* can establish itself there? This mucus-layer, like all the mucus-layers in the body, is composed of mucins; large glycoproteins that are able form watery gels to protect the underlying epithelium. Several questions immediately arise, each of which we will try to answer in this thesis: 1. What is the nature of the gastric mucin? 2. What is the nature of the interaction between the bacterium and the mucin? 3. Can the expression of this gastric mucin sufficiently explain the gastric tropism towards to stomach? 4. Which mucins, or other secretory proteins, are produced in the metaplastic epithelia within the upper GI tract, and can this knowledge help us understand the underlying pathology and the role of *H. pylori* herein?

This chapter will introduce some essential features of the three organs involved, esophagus, stomach and duodenum. It will describe the nature of *H. pylori* and its strategies to colonize the stomach. It will also describe two families of secretory proteins that are abundantly expressed in the Upper GI tract and its metaplastic epithelia, the mucins and the trefoil factor family peptides.

1.1. The upper gastrointestinal tract

1.1.1. Esophagus

Function and anatomy of the esophagus

The esophagus is a muscular tube that connects the oral cavity with the stomach. After food or drink is swallowed it passes into the esophagus, where it is rapidly propelled towards the stomach via the esophagus. Longitudinal and circular muscles move the swallowed bolus to the stomach by rapid peristaltic movements. The esophagus is lined by a stratified epithelium, not unlike the epithelium of the skin. The proliferation with this epithelium is solely situated at the most basal layer of the epithelium. Daughter cells migrate towards the lumen of the esophagus, forming multi-layered epithelium, until the cells are sloughed off at the surface, by erosion due to the passage of food. Besides this protection against mechanical erosion the epithelium does not seem to secrete proteins. Submucosal glands of the esophagus secrete

watery mucus, probably as a lubricant, although the esophagus epithelium does not seem to carry a mucus-layer like the stomach epithelium (see 1.1.2.).

Definition and diagnosis of Barrett's esophagus (BE)

The British surgeon Norman Barrett published in 1950 a report in which he defined the esophagus as "that part of the foregut, distal to the cricopharyngeal sphincter, which is lined by squamous epithelium"(Barrett, 1950). Today, BE is the condition in which an abnormal columnar epithelium replaces the stratified squamous epithelium that normally lines the distal esophagus (Spechler and Goyal, 1996). This condition is thought to develop when refluxed gastric juice damages the esophageal squamous epithelium, and the injury heals through a metaplastic process in which columnar cells replace squamous cells. Histologically, three different types of columnar epithelium are distinguished: (1) gastric-fundic type, (2) junctional type, and (3) specialized type characterized by intestinal metaplasia (Paull et al., 1976). Intestinal metaplasia, characterized by the presence of prominent goblet cells, is the epithelial type associated with cancer development (Reid et al., 1988).

The diagnosis of BE is established if the squamocolumnar junction (SCJ or Z-line) is displaced proximal to the gastroesophageal junction, and biopsy specimens of the columnar epithelium must reveal intestinal metaplasia, characterized histochemically by acid mucin-containing goblet cells. One often distinguishes short- from long-segment BE, depending on the extent of columnar lining (i.e., short segment <3 cm, and long-segment is >3 cm)(Sampliner, 1998). The junction between squamous and columnar epithelium (Z-line) is identified readily endoscopically. Columnar epithelium, with its reddish color and velvetlike texture, contrasts sharply with the pale, glossy squamous epithelium of the normal esophagus (Tytgat, 1997). Problems are recognized with the definition of the gastroesophageal junction. Anatomists, radiologists, physiologists, and endoscopists all have used different criteria to define this junction. The gastroesophageal junction is the imaginary line at which the esophagus ends and the stomach begins anatomically. Today, the most frequently used definition, given by endoscopists, takes the proximal margin of the gastric folds seen during endoscopy as landmark of the gastroesophageal junction (McClave et al., 1987). Still the traditional definition of BE (columnar epithelium with proven intestinal metaplasia) is subject to debate. Sharma and colleagues proposed the term short segment BE for the condition in which less than 3 cm of specialized intestinal metaplasia lines the distal esophagus (Sharma et al., 1998). Spechler and Goyal have suggested an alternative nomenclature for BE, the condition is simply designated as columnar-lined esophagus with or without specialized intestinal metaplasia (Spechler and Goyal, 1996). Important in the debate of defining BE, is that the replacement by columnar epithelium and the presence of goblet cells (i.e. intestinal metaplasia) is a pre-cancerous lesion, with a risk of development into adenocarcinoma of the esophagus, via the metaplasia-dysplasia-adenocarcinoma sequence (Jankowski et al., 1999).

Epidemiology of BE

BE is found in approximately 6-12% of patients undergoing endoscopy for symptoms of GERD, and in 1% or less in patients undergoing endoscopic examination for any clinical indication. BE is predominantly a disease of elderly (mean age 60-70 years) and Caucasian males (Cameron and Lomboy, 1992), and its incidence has increased markedly since the 1970s. However, this increase parallels the increased use of diagnostic upper endoscopy (Conio et al., 2001). It is generally accepted that patients with BE (with proven intestinal metaplasia) have a 30-fold increased risk of developing esophageal adenocarcinoma, and this risk is increased with the length of the metaplastic segment (Iftikhar et al., 1992). Recent data suggest that the risk to develop cancer in patients with BE is approximately 0.5% per year (Shaheen et al., 2000). Esophageal adenocarcinoma has a uniformly poor prognosis. Once diagnosed, patients have a median survival time of less than one year; fewer than 10% of patients survive for more than 5 years despite combined chemotherapy and surgery. The ideal requirement is to detect lesions at an early stage because surgical resection has proven survival benefits (Jankowski et al., 2000b).

Pathobiology of BE

BE is an acquired condition resulting from severe distal esophageal mucosal injury under the influence of gastric acid. From animal studies it was shown that excision of esophageal mucosa in conjunction with reflux of both acid and bile or acid alone resulted in re-epithelialization with primarily columnar epithelium. However, excision of the esophageal mucosa alone resulted in re-epithelialization primarily with squamous epithelium. Bile alone was not responsible for columnar re-epithelialization (Bremner et al., 1970; Gillen et al., 1988).

Three theories exist about the tissue of origin for BE metaplastic epithelium (reviewed by (Jankowski et al., 2000a). First, the novo metaplasia theory holds that the stem cells of inflamed squamous mucosa in the exposed papillae are damaged, and the resulting phenotypic or metaplastic change in these cells produces Barrett's stem cells. Second, the transitional zone metaplasia theory holds that cells at the gastroesophageal junction (transitional zone) colonize the distal esophagus in response to noxious luminal agents. Third, the duct-cell metaplasia theory holds that stem cells located in the glandular neck region of esophageal ducts might selectively colonize the esophagus when squamous mucosal damage occurs. The latter theory is supported by a recent histochemical study, which suggests that mucosal or submucosal gland epithelium may serve as a source of multipotential stem cells in BE (Glickman et al., 2001).

Intestinal metaplasia (IM) in BE can be diagnosed in biopsy specimens with conventional combined hematoxylin & eosin/alcian blue pH 2.5 (H&E/AB) staining. IM is recognized when mucin-positive goblet cells are present. Mucin histochemistry is usually used to determine the classification of IM. Specific dyes for mucin carbohydrates and their

modifications are used; these are alcian blue (AB), high-iron diamine (HID), and periodic acid-Schiff reagent (PAS). However, these dyes do not reveal the underlying mucin gene product. Only very recently, with the use of *in situ* hybridization and immunohistochemical techniques, different mucins in BE have been identified (Arul et al., 2000; Guillem et al., 2000). IM of the esophagus resembles IM of the stomach (Rothery et al., 1986). Depending on the cell types that are present and the types of mucins being secreted, IM can take several forms: (a) complete, or small intestinal type, also designated type I; which consists of mature absorptive cells and goblet cells, the latter secreting sialomucins; (b) incomplete, or colonic type, also designated types II and III. Type II consists of columnar "intermediate" cells secreting neutral and acid sialomucins, and goblet cells secreting sialomucins or, occasionally, sulfomucins, or both. Type III contains columnar or-"intermediate" cells secreting predominantly sulfomucins and goblet cells secreting sialomucins or sulfomucins, or both (Filipe and Jass, 1986). Complete IM has the lowest risk of developing an esophageal adenocarcinoma, whereas incomplete type III has the highest (Jass, 1981).

1.1.2. Stomach

Functional anatomy of the stomach mucosa

The human stomach consists of four anatomically separated zones, fundus, cardia, corpus (or body), and antrum. The gastric mucosa basically consists of two types. One type is present in the fundus and corpus, designated as fundus-type or oxyntic mucosa, and is characterized by rather straight, composite glands lined by mucous neck cells secreting mucus glycoprotein, chief (or zymogenic) cells secreting pepsinogen, parietal cells secreting hydrochloric acid (HCl), and neuroendocrine cells like histamine releasing enterochromaffin-like (ECL) cells and D-cells which release somatostatin. The second type of mucosa is found in the antrum. This antrum-type mucosa is characterized by more branched, predominantly mucus glycoprotein secreting glands with neuroendocrine G-cells secreting gastrin and D-cells secreting somatostatin. Besides different glands, fundus-type and antrum-type mucosa differ also with respect to the luminal openings of their glands, the foveolae or pits. In the antrum, pits comprise about one-half of the mucosal thickness, whereas in the fundus and corpus, the pits length occupies only about one-quarter of mucosal thickness. The gastric pits throughout the stomach are lined by cylindric mucus-producing epithelium (Owen, 1997).

Secretory proteins of the stomach epithelium

The normal antral stomach expresses different groups of secretory proteins. The first group are the secretory mucins. Secretory, gel-forming mucin is the most important structural component of the mucus-gel layer and plays an important role in the protection of the underlying epithelium (Van Klinken et al., 1995). The second group of secretory proteins consists of TFF peptides (formerly P-domain peptides or trefoil factor (Wright et al., 1997)), which are often expressed in close association with secretory mucins (Wong et al., 1999), and very likely play a role in gastrointestinal defense and repair (Hoffmann et al., 2001). The third

group of secretory proteins comprises endocrine cell markers, gastrin and chromogranin A (CGA). Gastrin is produced as a prohormone by G-cells located within the gastric antrum. This prohormone is processed into shorter COOH-terminally amidated peptides, gastrin-34 (G34) and gastrin-17 (G17), which consist of 34 and 17 amino acid residues, respectively (Dockray, 1999). Under fasting conditions, G34 is most abundant. However, post-prandially, the mainly released gastrin is G17 (Mulholland et al., 1993). The most important role of gastrin is the regulation of gastric acid secretion. In addition, in the fundus and corpus gastrin stimulates epithelial proliferation. CGA is a 48-kilodalton acidic protein specifically expressed in neuroendocrine and neural cells of the gastrointestinal tract (Facer et al., 1985). In the human stomach, enterochromaffin-like (ECL) cells of the corpus have been identified as the main source for CGA expression (Dockray et al., 1996). Besides ECL cells, CGA expression has been demonstrated in antral G-cells (Van den Brink et al., 2000a; Wiedenmann and Huttner, 1989). CGA is thought to influence granule stability, prohormone processing and peptide sorting into the regulated secretory pathway (Hocker et al., 2001).

Tissue dynamics of the stomach epithelium

Gastric mucosa differs from other mucosea in the digestive tract with respect to its proliferative zone. In any location of the stomach, the proliferating stem cells are localized in the neck of gastric glands, and epithelial migration occurs bidirectionally. One part of the daughter cells migrate within few days upward to the luminal surface, and cells differentiate into columnar mucus-secreting epithelial cells that line the pits and surface. Other daughter cells migrate downwards into the gastric glands. In the antrum, they differentiate within a few days to mucus-secreting cells, whereas in the fundus and corpus they differentiate within a few weeks to chief cells, parietal cells, and neuroendocrine cells (Owen, 1997). In the gastric fundus and corpus, gastrin effectively stimulates the proliferation of epithelial cells. However, in the antrum, gastrin hardly influences epithelial proliferation (von Herbay and Rudi, 2000).

Cell proliferation is balanced with a process named programmed cell death, or apoptosis (Kerr et al., 1994), to maintain gastric epithelial homeostasis. Apoptosis comprises different stages, initiation by a stimulus (intrinsic or extrinsic), detection and signal transduction, effector (caspase) activation, and a post-mortems phase (Staunton and Gaffney, 1998). Extracellular signals can either inhibit or activate apoptosis. For example, Fas and tumor necrosis factor-α (TNF-α) stimulate apoptosis via targets on the cell membrane, whereas growth factors can stimulate or suppress apoptosis. Endogenous apoptosis may be activated by the detection of DNA damage by wild type p53. Other major intracellular regulators are members of the Bcl-2 protein family. Bcl-2, Bcl-X_L, Mcl-1 and Bfl-1 suppress apoptosis, whereas Bax, Bak, Bad, and Bcl-X_S promote apoptosis (Adams and Cory, 1998). Disturbance of the balance between apoptosis and proliferation could interfere with the normal mucosal homeostasis (Thompson, 1995) and could lead to gastric pre-neoplastic process, including atrophic gastritis, intestinal metaplasia, gastric dysplasia and gastric carcinoma (Cahill et al., 1996).

Pathology of the stomach, *H. pylori* and metaplasia

The sequence of events leading to gastric carcinoma, via chronic gastritis, atrophic gastritis, intestinal metaplasia and dysplasia were placed by Correa *et al.* in a hypothetical temporal sequence known as Correa's cascade (Correa, 1984). This model was updated by the same group (Correa, 1992) and is now a well-accepted model for human gastric carcinogenesis. In the same year as Correa's cascade was published, 1984, a revolutionary discovery was reported by Marshall and Warren (Marshall and Warren, 1984), with comprehensive corollaries. They isolated a bacterium that was responsible for the occurrence of gastritis and peptic ulceration. This bacterium, *Helicobacter pylori*, is nowadays identified as the major etiologic factor of gastritis, gastric ulcer, gastric atrophy, and gastric carcinoma (Parsonnet et al., 1991).

A few years after the discovery of *H. pylori*, gastrointestinal pathologists searched for universal diagnostic criteria to describe the different stages, which lead to gastric carcinoma. The most controversy among pathologists was found in classification for the onset of disease. Therefore, a meeting between gastrointestinal pathologists was planned. This resulted in the classification of gastritis, that combine topological, morphological, and etiological information into a scheme, that was designated as "The Sydney System" (Price, 1991), and four years later updated (Dixon et al., 1996). The Sydney System is a histological division of gastritis. The five recommended biopsy sites are the antrum (one biopsy from the lesser and one from the greater curvature), corpus (one biopsy from the lessen and one from the greater curvature) and the incisura anglularis. The System recognizes three morphological patterns: acute gastritis, chronic gastritis and special forms, and has a standard grading scale applicable to a selected number of morphological variables. This grading scale include mild, moderate and marked. The graded variables are: inflammation, activity, metaplasia and density of *H. pylori*.

1.1.3. Duodenum

Functional anatomy of the duodenum

After the lower sphincter of the stomach, the pylorus, the stomach empties into the duodenum, the first part of the small intestine. The duodenum has basically the same anatomy as the other consecutive parts of the small intestine; the jejunum and the ileum. The epithelium of the duodenum is intricately folded to increase its surface, as degradation and absorption of nutrients is its main purpose. The small intestinal epithelium is a fast proliferating epithelium. The stem cells are located into the crypts of Lieberkühn, and give by mitosis rise to rapidly proliferating daughter cells that undergo 4-5 further rounds of mitosis. The daughter cells migrate out of the crypts and migrate further upwards to the villi (Fig. 1). During this upward migration these cells differentiate into three principal cell types, enterocytes, goblet cells and endoendocrine cells, which constitute 95%, 5%, and <1% of the epithelial cells. Occasionally, proliferating cells migrate downwards to the bottom of the crypts and differentiate into Paneth cells. These Paneth cells together with the goblet cells form the defensive cell types of the

intestine. The Paneth cells secrete bactericidal enzymes and anti-microbial peptides, whereas the goblet cells secrete mucins and trefoil peptides. The enterocytes have a folded apical membrane with many digestive enzymes and transporter proteins to degrade and transport nutrients, respectively. The enteroendocrine cells produce very diverse types of hormones, functioning to coordinate and optimize the digestive processes in the gut.

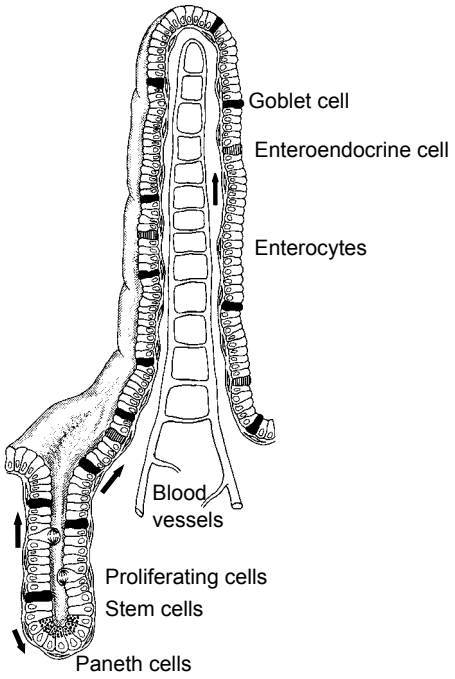

Figure 1. Schematic picture of duodenal crypt/villus epithelium. Daughter cells of the stem cells in the crypt migrate to the villus (arrows) and differentiate into enterocytes, goblet cells (indicated in black) and enteroendocrine cells (hatched). Some daughter cells migrate to the bottom of the crypt (arrow) and differentiate into Paneth cells.

The secretory proteins within the epithelium of the small intestine seem first and for all destined to serve protective purposes. Unique for the duodenum are the large folded structures of the mucosa, know as the Valves of Kerckring, which are thought to serve as brakes to temper the floods of acidic juices from the stomach. Within these Valves of Kerckring are large acinar glands, the Brunner's glands, opening into the crypts of Lieberkühn. These Brunner's glands secrete large amounts of bicarbonate to neutralize the acid from the stomach, and secretory proteins like mucins, trefoil peptide and growth factors. Mucins are known to form major secretory products, as the Brunner's glands stain intensely with periodic acid/ Schiff's reagent.

Pathology of the duodenum in relation to *H. pylori* and metaplasia

The pathology of the duodenum is relatively well studied as this part of the small intestine can relatively easily been reached by duodeno-gastroscopy. Many diseases are known to affect the

duodenum like celiac disease and Crohn's disease. One of the most frequent abnormalities is duodenal ulceration, which is almost exclusively associated with gastric *H. pylori*-infection (Tytgat et al., 1993). Far more common than the ulcerations are the cases of gastric metaplasia in the duodenum, which consists of patches of metaplastic cells on tops of villi, with a gastric secretory phenotype. These cells stain intensely pink in alcian blue (AB)/periodic acid-Schiff's (PAS) reagent staining, amidst the largely alcian blue-positive goblet cells of the crypts and villi.

Gastric metaplasia in the duodenal bulb (GMD) is characterized by replacement of intestinal epithelial cells with gastric-type mucous cells, and is frequently found in association with inflammation and *H. pylori* induced gastritis (Wyatt et al., 1990). GMD is defined as the occurrence of patches of gastric epithelial cells at the villus tip or side, which contain apically periodic acid-Schiff (PAS)-positive neutral mucin together with the absence of a brush-border. The formation of gastric-type epithelium in the duodenal bulb seems related with increased gastric acid output (Harris et al., 1996), which is the result of *H. pylori* induced antral gastritis. The high acid load in the duodenum can lead to a sequence of events including GMD, duodenitis, and ulceration. It is recognized that this is a continuum of events (Walker and Crabtree, 1998).

The low prevalence of GMD in children under the age of ten suggests that GM is not congenital in origin (Gormally et al., 1996). The origin of gastric metaplastic cells is still a matter of debate. Some researchers suggest that GMD might have been differentiated from Brunner's gland duct epithelium (Hanby et al., 1993; Liu and Wright, 1992), while Shaoul *et al.* found no evidence for this Brunner's duct epithelium differentiation, and suggested that goblet cells expressing both intestinal and gastric antigens may represent local precursors of GMD undergoing a transition to gastric-like cells of mixed phenotype at the site of early metaplastic patches. As GMD becomes more widespread, a more pure gastric phenotype emerges (Shaoul et al., 2000). Importantly, GMD can occur in areas of the intestine that lack Brunner's glands.

1.2. *Helicobacter pylori*

1.2.1. *H. pylori* is the most important gastric pathogen

Helicobacter pylori is a Gram-negative spiral-shaped bacterium (Fig. 2), specialized in colonization of the human stomach (Warren and Marshall, 1983). The bacteria are 2.5-5.0 μm long and 0.5-1.0 μm in diameter (Goodwin et al., 1989b). Initially, the bacterium was designated as *Campylobacter pyloridis*, as it appeared to have several similar features to the genus *Campylobacter* (Marshall et al., 1984). This specific denomination was grammatically incorrect and to comply to the rules of scientific nomenclature the name was revised to

Campylobacter pylori (Marshall and Goodwin, 1987). Studies on 5S and 16S ribosomal RNA sequencing revealed that *C. pylori* were very different from other campylobacters (Paster and Dewhirst, 1988; Romaniuk et al., 1987). Therefore, a new genus was established and was designated as *Helicobacter*. The new name of *Campylobacter pylori* became *Helicobacter pylori* (Goodwin et al., 1989a). Infection with *H. pylori* is very common, and can be detected in more than half of the global human population (Parsonnet, 1995). In developing countries, 70-90% of the population carries this bacterium, which is acquired before the age of ten and persists throughout life. In developed countries *H. pylori* is found in 25-50% of the population. In addition, in developed countries less than 10% of the children become infected (Dunn et al., 1997).

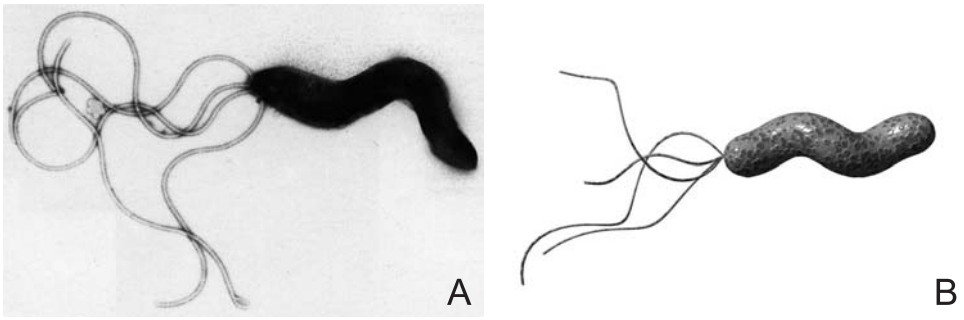

Figure 2. (A), electron microscopic picture of the characteristic S-shaped *H. pylori* with unipolar flagella. (B), 3-D computer image of *H. pylori*.

To colonize the stomach, *H. pylori* has to survive in the acidic environment of the gastric lumen. Its cytosolic urease enzyme hydrolyses urea into carbon dioxide and ammonia, which neutralizes gastric acidity (Marshall et al., 1990). Although well equipped to survive in strong acid, *H. pylori* is not an acidophile and needs to leave the lumen, also to avoid discharge in the intestine, to the more pH neutral environment near to the gastric epithelium. The bacterium needs to move through the viscous gastric mucus layer so that *H. pylori* can encounter the surface of the gastric epithelium. This movement is accomplished through the production of flagella. *H. pylori* has four to six unipolar flagella which are surrounded by a membranous sheath (Suerbaum et al., 1993). In addition, the movement is guided by chemotactic factors, which include urea and bicarbonate ions (Yoshiyama and Nakazawa, 2000). A final step in colonization of the stomach is the adhesion of *H. pylori* to the gastric surface epithelium. As a rule the bacterium does not enter the body, but seems to survive for length of years in the gastric mucus and in close association to the mucus-producing cells of the epithelium.

1.2.2. Mucosal immune response

The colonization of the stomach is not without consequences for the host. *H. pylori* induces a gastric inflammatory response, which consists of neutrophils, lymphocytes (T and B cells), plasma cells, and macrophages, along with varying degrees of epithelial cell degeneration and

injury (Goodwin et al., 1986). *H. pylori* produces a number of antigens, including lipopolysaccharide (LPS)-endotoxin. One of these endotoxins is *H. pylori* neutrophil-activating protein (HP-NAP)(Evans et al., 1995). HP-NAP is taken up by neutrophils or monocytes in the lamina propria, and stimulates the production of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6 (Crabtree et al., 1991). TNF-α promotes the adhesion of leucocytes to the site of infection. Processed LPS, together with IL-1 and IL-6, stimulates T helper cells ($CD4^+$) to produce IL-4, IL-5, IL-6, IL-8 and interferon-γ (INFγ) (Van Deuren et al., 1992). IL-8 is also produced by the injured gastric epithelial cells, and is an important chemotaxis for neutrophils and monocytes. IL-6 stimulates the differentiation of B cells into specific antibody producing cells. Production of IgM, secretory IgA, and IgG follow the B-cell response. While secretory IgA may play a role in minimizing bacterial adhesion, opsonisation and complement activation forms the main arm of the mucosal immune response. Other inflammatory mediators produced by activated neutrophils and monocytes include prostaglandin (PG), leukotrienes, proteases and reactive oxygen metabolites (ROMs). The release of proteases and ROMs is likely to be important in causing tissue damage, particularly when antioxidant defenses (e.g. vitamins C and E) are depleted (Sobala et al., 1991). Activated neutrophils and monocytes have the tendency to congregate around the proliferative region of the gastric pit and may cause lethal damage to the stem cells and result in glandular atrophy (Dixon, 1994), which may predispose to carcinogenesis by increasing likelihood of DNA mutagenesis. The accumulation of mutations may then lead to metaplasia, dysplasia and gastric cancer, the Correa's cascade, as mentioned above.

1.2.3. Epidemiology

H. pylori is recognized as the main cause of type B chronic gastritis and duodenitis, which could lead to gastric and duodenal ulcers. Of the infections with *H. pylori*, 6-20% result in peptic ulceration. Yet, less than 1% of these ulcerations will lead to gastric cancer. In addition, *H. pylori*-infection plays a role in 53% of gastric cancer cases in developing countries and 60% in developed countries. Infection with the bacterium has also been associated with gastric mucosa-associated lymphoid tissue (MALT) lymphomas, or MALTomas (Farthing, 1998; Parsonnet, 1998). Besides playing a prominent role in gastric pathology, gastric *H. pylori*-infection is most likely involved in the pathology of gastric metaplasia in the duodenum (GMD) (Wyatt et al., 1990) and possibly in the metaplasia of the esophagus (Barrett's esophagus, BE)(Richter et al., 1998).

1.2.4. Virulence factors

Several factors contribute to the outcome of disease, but the most important one are the virulence factors produced by any given strain. The major virulence factors are listed in table 1. Using the *cagA* and *vacA* genotypes, two types of *H. pylori* have been defined. Type I strains produce cytotoxin-associated gene A, CagA, and vacuolating cytoxin A, VacA, and are

associated with chronic gastritis, peptic ulcers, and gastric carcinoma. Type II strains do not contain the *cagA* gene, and produce no functional VacA cytotoxin. Type II strains are generally not associated with gastric pathology (Xiang et al., 1995)

Sequencing of the genome of two *H. pylori* strains: J99 (Alm et al., 1999) and TIGR26695 (Tomb et al., 1997) revealed a family of outer membrane proteins (Hops). Members of this family, BabA, AlpA, and AlpB likely play a role in *H. pylori* attachment to the gastric epithelium. BabA recognizes the Lewis B blood group antigen on human gastric epithelium (Boren et al., 1993). BabA is a blood group antigen-binding adhesin and encoded by the *babA2* gene in strain CCUG17875 and is composed of 721 amino acid residues. A second gene, *babA1* is identical to *babA2*, however, this gene lacks an insert of 10 base pairs, and was identified as a pseudogene, unable to infer Lewis B binding capacity to the bacterium (Ilver et al., 1998). No host receptor for AlpA and AlpB adhesins are known. However, the pattern of adherence through AlpA and AlpB of *H. pylori* to the gastric epithelium differs from BabA-mediated adherence, suggesting that a different receptor is involved (Odenbreit et al., 1999). Recently, a new *H. pylori* adhesin has been described; the sialic acid-binding adhesin (SabA). This SabA adhesin binds to the inflammation associated sialyl-dimeric-Lewis X antigen, which is expressed in human gastric epithelium during persistent infection (Mahdavi et al., 2002). SabA is encoded by gene JHP662 in strain J99 (Alm et al., 1999), and by gene HP0725 in strain TIGR26695 (Tomb et al., 1997). This gene encodes a 651-amino acid protein (70 kDa) and belongs, like BabA, AlpA and AlpB, to the Hop family of *H. pylori* outer membrane proteins.

Table 1. Major *H. pylori* virulence factors.

Factor	Function	Distribution
Urease	Buffers stomach acid	All strains
Flagella	Motility	All strains
HP-NAP	Neutrophil activation	All strains
BabA	Adherence to Lewis B blood group antigen	Prevalent on type I strains
VacA	Cytotoxity in vitro, role in vivo unknown	Most strains
PAI	31 genes encoding type IV secretion system	Type I strains
CagA	Immunodominant antigen (part of PAI), role unknown	Type I strains
PicB	Equivalent to CagE	Type I strains

Adapted from (Montecucco et al., 1999).

1.3. Mucins

1.3.1. Secretory mucins

Mucins are large glycoproteins that are often found on the surfaces of epithelial cells in the gastrointestinal tract. Several mucins can form a viscoelastic mucus gel layer that protects and lubricates the underlying epithelium. Other mucins form the glycocalyx on the apical cell membranes of cells, like on the intestinal enterocytes. To date 18 human mucins genes encoding epithelial mucin type proteins have been described and designated as MUC1, -2, -3A, -3B, -4, -5AC, -5B, -6, -7, -8, -9, -11, -12, -13, -15, -16, -17, and -18 (Gendler and Spicer, 1995; Gum et al., 2002; Pallesen et al., 2002; Pratt et al., 2000; Williams et al., 1999; Williams et al., 2001; Yin and Lloyd, 2001). The genes encoding MUC10 and MUC14 have thus far only been described in mice ((Melnick et al., 2001), and GenBank accession number NM_016885, respectively). An exhaustive overview of the structure and expression of MUC-type mucins in the human body has recently been published (Corfield et al, 2000).

The definition of mucins is a matter of debate for many years. However, all mucins are recognized to carry large amounts of O-linked carbohydrate structures, like the Lewis antigens that are implied as *H. pylori*-receptors (see 1.2.4.). Although all mucins are presently incorporated into one large gene family, this concept of just one mucin gene family may need to be abandoned. Among the present day MUC-type mucins several types can be distinguished (Dekker et al., 2002). The relationship between the MUC-type mucins is shown in figure 3 (Fig. 3, see *Appendix*). For the studies as presented here only the large gel-forming are of importance, i.e. MUC2, MUC5AC, MUC5B, and MUC6. These mucins have clearly evolved from one ancestral gene, as they are found clustered on one chromosomal location (11p15.5), and are most likely each involved in the formation of mucus gel-layers (Desseyn et al., 2000; Desseyn et al., 1998).

The secretory gel-forming mucins are very abundantly expressed in the various parts of the gastrointestinal tract. For long, workers in the field have assumed that these molecules form essential elements of the innate defense of the epithelium against sunstance and microorganisms in the gastrointestinal lumen. Only very recently this hypothesis was substantiated by the development of a MUC2 knockout mouse strain, in which the gene of one of the secretory gel-forming mucins (MUC2) was inactivated (Einerhand et al., 2002; Velcich et al., 2002).

MUC2 is the predominant secretory mucin in the intestine, and is particularly abundant in the colon (Van Klinken et al., 1995). It is specifically localized in the intestinal goblet cells of the normal intestinal epithelium, whereas it is absent from healthy esophagus and stomach epithelium. It appeared that the Muc2 knockout mice are very susceptible to develop inflammatory bowel syndrome (Einerhand et al., 2002). In Muc2 knockout mice the intestinal

goblet cells are seemingly absent. However, expression of TFF3, another intestinal goblet cell marker, was still present in a high number of epithelial cells in the colon of the Muc2 knockout mice. Apparently, goblet cells in absence of Muc2 loose their characteristic goblet-like shape in histology, indicating that Muc2 is the major phenotypic determinant of goblet cells. Muc2 knockout mice spontaneously develop mild colitis when colonized by normal enteric bacteria under specified-pathogen-free conditions (Van der Sluis et al., 2002). However, the Muc2 knockout mice are extremely susceptible to cytotoxic luminal agents like dextrane sulfate sodium. Treatment with dextrane sulfate sodium led to very fulminant colitis within days, which was much more severe in each aspect than in wild type mice treated with dextrane sulfate sodium (Van der Sluis et al., 2002), indicating that Muc2 plays an essential role in epithelial protection. Furthermore, the Muc2 knockout mouse spontaneously develop within 6-12 months adenocarcinomas of the small and large intestine under specified pathogen free conditions, indicating that MUC2 protects against adenocarcinoma formation (Velcich et al., 2002). Although further data on the other secretory mucins are lacking, the picture from the MUC2 knockout mice suggests that the concept of innate protection of the epithelium by the gel-forming mucins is viable.

In parallel with the studies as described in this thesis, work was performed to localize *H. pylori* and two secretory MUC-type mucins in gastric biopsies of infected patients. MUC5AC and MUC6 were localized in the human stomach by immunohistochemistry using anti-polypeptide antibodies. These mucins were co-localized with *H. pylori* bacteria as present *in situ* in tissue samples of infected patients through immunohistochemistry (Van den Brink et al., 2000b)(Fig. 4, see *Appendix*). MUC5AC was produced by the surface cells and the neck cells of the epithelium, and MUC5AC was also found in the mucus overlaying these cells. Strikingly, virtually all *H. pylori* bacteria present in each infected patient co-localized very specifically with the extracellular MUC5AC and with the apical cell membrane of MUC5AC-producing cells (Fig. 4, *Appendix*). MUC6 was produced in a subset of epithelial cells clearly distinct from the MUC5AC-producing cells, and *H. pylori* was hardly ever associated with these cells. This constitutes a thus far unprecedented example of the co-localization of *H. pylori* with a particular candidate receptor molecule for the bacterium.

1.3.2. Mucin-associated TFF peptides

Trefoil factor family peptides (TFF peptides) form a small family of bioactive peptides that are found in the secretory cell types of the gastrointestinal tract. They are almost exclusively expressed in cells that also produce the four major secretory MUC-type mucins as mentioned above. Three TTF peptides are known, TFF1 and TFF2 are predominantly expressed in the stomach, whereas TFF3 is expressed in the intestinal goblet cells (Table 2). Some publications indicate that physical interactions might exist among secretory MUC-type mucins and the TFF peptides (Newton et al., 2000; Tomasetto et al., 2000).

We have used the expression of the TFF peptides as additional markers to study the complex secretory phenotypes of MUC-type mucin-producing cells in the upper gastrointestinal tract. It was found that TFF1 was particularly expressed in cells that also produced MUC5AC, whereas TFF2 was often co-expressed with MUC6. TFF3 was usually found together with MUC2 (see review (Hoffmann et al., 2001). From our analyses in several organs and many cell types it appeared that this simple picture needed considerable modification. Particularly in metaplastic epithelia many different combination of secretory mucins and TTF peptides are found, indicating that the respective genes are apparently regulated independently, making the TFF peptides independent markers of the secretory cell types of the upper gastrointestinal tract.

TFF peptides are clearly involved in the innate protection of the gastrointestinal epithelium. Several independent lines of evidence, particularly from studies in cell-lines and in TFF knockout mice, it appeared that TFF protects the gastrointestinal epithelium by two basic mechanisms (reviewed by (Wong et al., 1999). First, TFF peptides inhibit the apoptosis in damaged epithelia, to spare the maximal number of cells, to be able to maintain epithelial integrity as much as possible and to maintain the necessary functions for a prolonged period of time, Secondly, TFF are motogens, and act to speed up the movement of cells over exposed extracellular matrix. Therefore, they are important for the epithelial restitution, which occurs when epithelial cells are damaged and are sloughed off into the lumen. This process implies rapid movement of cells that fill in the gaps that have fallen in the epithelial barrier upon damage, which is a notably fast process and occurs in absence of cellular division. Mice, in which the TFF1 or TFF3 gene has been inactivated, leading to absence of the respective TFF-peptide, show severe pathology. The TFF1 knockout mice develop severe gastritis and adenocarcinomas of the stomach, in accordance with the main site of production of TFF1; the stomach (Lefebvre et al., 1996). The TFF3 knockout mice were extremely prone to colitis and had an enhanced incidence of colonic cancer, both in conjunction with the fact that TFF3-peptide is produced in large amounts in the colon (Mashimo et al., 1996)

Table 2. Location of trefoil factor family genes in the human gastrointestinal tract.

TFF gene	Chromosome	Major expression in normal gastrointestinal tract
TFF1	21q22.3 (Tomasetto et al., 1992)	Gastric surface mucous cells (Rio et al., 1988)
TFF2	21q22.3 (Tomasetto et al., 1992)	Gastric mucous neck cells (Tomasetto et al., 1990)
TFF3	21q22.3 (Chinery et al., 1996)	Intestinal goblet cells (Podolsky et al., 1993)

H. pylori is known to affect the epithelial turnover and to damage epithelia, primarily through the induction of a inflammatory reaction in and close to the epithelium (see 1.2). These adverse effects include induction of apoptosis in the epithelium. As the TFF peptides are known to counteract the induction of apoptosis, the TFF peptides may be involved in the host response towards *H. pylori*.

1.4. Aims and outline of this thesis

Upper gastointestinal complaints in humans are closely associated with just one pathogen: the bacterium *Helicobacter pylori*. This bacterium is not only one of the few bacteria that is able to invade and colonize the stomach, but also gives rise to consistent and severe pathology, like gastritis and gastric ulcer disease. A vast body of research in the past decade has focused on the pathogenic mechanisms by which *H. pylori* is able to elicit its malicious effects on human health. Yet, still several elementary questions remain to be solved. For one, it was not known which host molecule functions as foothold for the adhesins of the bacterium. Secondly, it remained to be determined if this *H. pylori* receptor was uniquely expressed in the stomach, explaining the tropism of the bacterium towards the stomach. And thirdly, it was not determined if the possible presence of this receptor in other upper gastrointestinal organs could indicate if or how *H. pylori* is involved in pathogenic processes in these organs. In our attempts to find answers to these questions we focused on the primary target of *H. pylori*, the human patient. We collected tissue samples of human stomach and of the adjacent organs, the esophagus and the duodenum of individuals that were either or not infected with the bacterium. Our hypothesis was that mucins could fulfill an important role as receptors for the bacterium. *H. pylori* resides in the mucus-layer or attached to the mucus-producing cells of the stomach, making involvement of the secretory mucin molecules that build this mucus-layer in the attachment of the bacterium likely.

Our work involved a multi-step approach to try to identify one of the secretory mucins as a *H. pylori* receptor:

1. We identified the most abundant secretory mucin in the human stomach.
2. We determined the normal expression pattern of secretory mucins in the normal and *H. pylori*-infected stomach, to see if and how *H. pylori* affects the production of the gastric mucins.
3. We determined the mucin expression pattern in the normal esophagus and in metaplastic esophagus epithelium, i.e. Barrett's esophagus, to see if the gastric mucins were also expressed in the normal and metaplastic epithelium of the esophagus.
4. We determined if and/or how the presence of *H. pylori* in the stomach affects the mucin expression pattern in Barrett's esophagus
5. We determined the mucin expression pattern in the normal duodenum and in gastric metaplasia within the duodenum, to see if the gastric mucins were expressed in the normal and metaplastic epithelium of the duodenum.
6. We determined if and/or how the presence of *H. pylori* in the stomach affects the mucin expression pattern in the duodenal epithelium.
7. Having identified the most likely *H. pylori*-receptor candidate among the secretory mucins, we determined if *H. pylori* bacteria were able to adhere to tissue sections where this mucin was expressed, and thus if *H. pylori* co-localized with the potential receptor in various tissue samples wherein this particular mucin was expressed.

The most important candidate for a *H. pylori* receptor was the gastric mucin that forms the most important structural component of the mucus layer that lines the stomach. *H. pylori* is seen *in vivo* to reside either within the mucus-layer or closely associated with the cells producing the mucin. Therefore, we set out to identify the mucin responsible for the gastric mucus-layer, by gastric cDNA sequence analysis. The results of this analysis as laid out in Chapter 2 demonstrated that MUC5AC is the one large secretory, gel-forming mucin that is responsible for the gastric mucus-layer.

Gastric infection by the bacterium is known to give rise to inflammation of the gastric mucosa, and affect the different cell types in the gastric epithelium. Since *H. pylori* co-localizes *in vivo* with MUC5AC and MUC5AC-producing cells of the gastric epithelium, the question can be posed how *H. pylori* affects the MUC5AC-producing cells in the epithelium of the infected stomach. In Chapter 3, we have studied the major cell types in the human gastric antrum in tissue from healthy and infected individuals, by studying the major secretory products; mucins, trefoil factors and hormones. It appeared that the turnover of the gastric epithelium was much higher in the infected individuals and that all the secretory cell types of the antrum epithelium were affected. In particular, the surface epithelial cells, which are responsible for the production of MUC5AC, were diminished.

Another effect of gastric *H. pylori*-infection is its association with the development of gastric cancer. We sought to identify one of the forerunners of gastric adenocarcinoma, intestinal metaplasia, within the gastric mucosa and to study the MUC5AC expression in the intestinal metaplasia (Chapter 3). The expression of mucins and trefoil factors in the intestinal metaplasia showed that MUC5AC was largely absent from intestinal metaplasia.

From our work as described in Chapter 3, it appeared that gastric intestinal metaplasia loses it MUC5AC expression, and thereby a main *H. pylori* receptor. Another disease of the upper gastrointestinal tract is constituted by the Barrett's' esophagus, which is considered a pre-cancerous lesion in the esophagus. As the Barrett's esophagus is recognized by its very high mucin expression, we set out to determine if MUC5AC is expressed in normal and Barrett's esophagus. If MUC5AC, as a main *H. pylori* receptor, would be expressed in either normal or Barrett's esophagus it could be envisioned that *H. pylori* could be involved by the development of Barrett's esophagus and esophagus cancer.

We determined the expression of the four major secretory mucins and the trefoil factors in Barrett's esophagus tissue of individuals with and without gastric *H. pylori* infection. We studied the expression of these secretory products in various stages of Barrett's and tried to correlate the expression patterns of these markers with the presence of *H. pylori* in the stomach of these patients (Chapters 4 and 5). MUC5AC was expressed in the tissue of virtually all Barrett's esophagus patients, and its expression pattern was not different in Barrett's esophagus tissue of patients suffering from gastric *H. pylori*-infection. In fact, the

expression of none of the secretory mucins could be correlated to gastric *H.* pylori-infection. Also, the extent of MUC5AC expression pattern in Barrett's epithelium was not affected by the stage of the Barrett's esophagus. Instead, the expression of the intestinal mucin MUC2 in Barrett's esophagus was demonstrated to correlate with progression of the epithelium towards cancer (Chapter 4). A more in detail analysis of the expression patterns of the secretory mucins in Barrett's esophagus revealed that the expression patterns of the secretory mucins became more chaotic during increased expression of MUC2 (Chapter 5). In the initial stages of Barrett's esophagus (i.e. no MUC2 expression) the epithelial expression of MUC5AC resembled the gastric epithelium, but with the increased expression of the intestinal mucin MUC2, the expression of MUC5AC was increasingly disturbed and began to overlap with other secretory products in the epithelium (Chapter 5).

Downstream of the stomach lies the duodenum, an organ exposed to the adverse effects of gastric *H. pylori*-infections, as the occurrence duodenal ulcers are very closely correlated to gastric *H. pylori*-infection. We determined the expression patterns of the secretory mucins and trefoil factors in the epithelium of the duodenum of individuals who suffered or not from gastric *H. pylori*-infection (Chapter 6). The occurrence of gastric metaplasia in the duodenal bulb was closely correlated to gastric *H. pylori*-infection. MUC5AC expression was also closely correlated with *H. pylori*-infection of the stomach, as MUC5AC was unequivocally expressed in all metaplastic cells. However, MUC5AC was also expressed in a very small number of normal duodenal goblet cells, in *H. pylori*-infected as well as non-infected individuals. Our data on the MUC5AC expression in metaplasia of the duodenal bulb led us to propose a model that describes the growth of gastric metaplasia as a phenotypic switch in existing cells rather than a separate cell-lineage with a separate stem cell (Chapter 6).

Having analyzed the MUC5AC expression in the stomach, esophagus, and duodenum, it appeared that MUC5AC was expressed in very distinct expression patterns in each of these epithelia, amidst epithelial cells expressing the other three major secretory mucins, MUC2, MUC5B, and MUC6. Meanwhile, from the work of others (discussed in Chapter 7) it had appeared that a carbohydrate structure, the Lewis B blood group antigen, functioned as a *H. pylori* receptor. Since mucins are very rich in carbohydrate structures, we tested the hypothesis that Lewis B structures on the MUC5AC molecules functioned as *H. pylori* receptors. We used *H. pylori* bacteria that were fluorescently labeled and put these on sections of esophagus, stomach and duodenal tissues, which were known to express MUC5AC. MUC5AC and the Lewis B antigen co-localized well in these tissues, and *H. pylori* added to the sections co-localized to MUC5AC as well as to Lewis B carbohydrate structures. Only *H. pylori* strains that were able to bind to Lewis B bound to MUC5AC, whereas a strain that was unable to bind to Lewis B did not bind to MUC5AC, indicating that Lewis B on MUC5AC may be an important receptor for *H. pylori* (Chapter 7).

The possible roles of MUC5AC in the *H. pylori*-infection are discussed in Chapter 8. From our collective data it seems likely that MUC5AC is an important receptor for *H. pylori*, and that MUC5AC is the primary gene product that carries the receptor structure, the Lewis B blood group antigen. On the other hand, there are multiple indications that *H. pylori*-infection itself is able to affect the MUC5AC synthesis. The latter is discussed in the light of possible host-defense mechanisms against *H. pylori*, or conversely the possible inductive role of receptor-production (i.e. MUC5AC) by the bacterium.

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Chapter 2

Molecular cloning of human gastric mucin MUC5AC reveals conserved cysteine rich D-domains and a putative leucine zipper motif

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Biochem Bioph Res Commun 1998; 245(3): 853-859

Footnote
The nucleotide sequences presented in this chapter have been submitted to Genbank with accession number: AF043909.

Abstract

To further clone the human gastric mucin MUC5AC cDNA, we screened a human gastric cDNA library with previously identified MUC5AC sequences. We obtained 32 independent clones encoding newly identified sequences comprising the entire N-terminal sequence of MUC5AC, up to 3024 bp upstream of the previously identified MUC5AC sequences. The N-terminus of MUC5AC shows high homology (43% identity) with the N-terminus of MUC2 and contains three domains homologous to the D-domains found in the pro-von Willebrand factor. Furthermore, the N-terminus of MUC5AC contains a putative leucine zipper motif not found in any other mucin identified so far. Moreover, a large central repetitive sequence was identified encoding approximately 2500 amino acids (7.5 kb). We were able to establish that the MUC5AC cDNA together with the previously identified 6.1 kb of MUC5AC cDNA sequence is about 16.6 kb, encoding 5525 amino acids. A model of the domain structure of MUC5AC is presented.

Abbreviations: HGM, human gastric mucin; TRESPASSER, Two RESidue Pattern Analysis for Sequence-structurE Relationships.

Introduction

The gastric mucus layer protects the underlying epithelium against mechanical, chemical and enzymatical damage. The mucus in the stomach consists mainly of the gel-forming human gastric mucin (MUC5AC), which is expressed by the surface mucous cells of the gastric glands (Klomp et al., 1995; Nordman et al., 1995). The MUC5AC gene is a member of a family of at least nine genes designated MUC1-8 including MUC5B and MUC5AC (Van Klinken et al., 1995). The MUC5 subdivision into MUC5AC and MUC5B is due to the fact that Nguyen and co-workers initially cloned partial MUC5 cDNAs from tracheo-bronchial tissue, and designated these MUC5A, MUC5B and MUC5C (Nguyen et al., 1990). Although all were assigned to chromosome 11p15, it was subsequently shown by physical mapping that the partial MUC5A and MUC5C cDNAs were derived from the same gene, whereas MUC5B was not. Therefore, the genes are now defined as MUC5AC and MUC5B, respectively (Dufosse et al., 1993; Guyonnet Duperat et al., 1995).

Reported sequences of the mucin cDNAs are rarely full-length, because of the highly repetitive nature and the extremely large size of the mucin mRNAs. MUC5AC cDNA, that is thought to comprise about 17 kb (Debailleul et al., 1998), was thus far incompletely sequenced. The original MUC5AC cDNA clones encode parts of a Proline, Threonine, Serine (PTS)-rich repeat region, characteristically located in the central part of the members of this class of glycoproteins (Guyonnet Duperat et al., 1995; Van Klinken et al., 1995). Using a DNA-probe deduced from a tryptic peptide of isolated tracheo-bronchial mucin, a cDNA was cloned from a human nasal polyp cDNA library and designated MUC5 (clone NP3a) (Meerzaman et al., 1994). This MUC5 cDNA is 3.5 kb and codes for the C-terminal part of a mucin. Another cDNA of 2.5 kb, designated HGM-1, was isolated from a stomach cDNA library using a DNA-probe based on the sequence of a tryptic peptide from isolated human gastric mucin (HGM) (Klomp et al., 1995). The 5' part of the MUC5 (NP3a clone) and the 3' part of HGM-1 cDNAs include areas highly similar (> 90%) to MUC5AC cDNA sequences determined by Guyonnet-Duperat and co-workers (Guyonnet Duperat et al., 1995), and it is very likely that all these are part of the same gene, i.e. MUC5AC. To date the N-terminal sequence of MUC5AC is unidentified and the exact length of central tandemly repeated region is unknown.

To determine the structure and function of MUC5AC, we aimed to further clone and characterize the MUC5AC cDNA. By PCR on genomic DNA using unique primers in the 3' part of HGM-1 and in the 5' part of MUC5 (clone NP3a), we verified that HGM-1 and MUC5 cDNAs are derived from one and the same gene, and that the repetitive region located in between the primers is at the most 8.5 kb. We screened a human gastric cDNA library with a cDNA probe encompassing the 5'-end of MUC5AC clone HGM-1, and report here the identification of the N-terminus of MUC5AC, containing conserved cysteine-rich D-domains, and a putative leucine zipper motif not found in any other mucin identified so far.

Materials and Methods

All reagents were obtained from the following firms: Stratagene, La Jolla, CA, USA; Boehringer Mannheim, Germany; Schleicher and Schuell, Dassel, Germany; PE Applied Biosystems, Foster City, CA, USA; Qiagen, Hilden, Germany; Promega, Leiden, The Netherlands.

Screening of a human gastric cDNA library

Previously we have constructed a human gastric cDNA library in lambda ZAPII (Stratagene) (Klomp et al., 1995). This library contained $6.6x10^5$ independent plaque forming units (pfu) and was amplified once. In total $1.8x10^6$ pfu were plated onto *E. coli* XL1-Blue. Filters were screened with a ^{32}P-labeled SalI-PvuII fragment of the published HGM-1 cDNA clone (Klomp et al., 1995) according to the manufacturers protocol (Stratagene). In a subsequent screening a ^{32}P-labeled SalI-PstI fragment of one of the resulting positive cDNA clones, pSK5, was used as probe. In the final screening of the library, a ^{32}P-labeled PstI-EcoRI fragment of one of the resulting clones, pSK17, of the second screening was used as probe. Filters (Schleicher and Schuell) were hybridized in duplicate at 65°C in 0.5 M NaH_2PO_4 pH 7.2, 7% SDS and 1 mM EDTA, according to the method of Church and Gilbert (Church and Gilbert, 1984). Positive plaques were rescreened and the corresponding plasmids were excised with the help of Exassist helper phage according the manufacturers protocol (Stratagene). Plasmids were isolated using the Wizard Plus kit (Promega) and subsequently sequenced.

Sequence analysis of cDNA clones

MUC5AC cDNA containing plasmids were double-strandedly sequenced using Taq dye-nucleotide cycle sequencing kit with fluorescently labeled nucleotides (PE Applied Biosystems) and T3 and T7 primers in a Perkin Elmer 9600 thermocycler according to the manufacturers protocol. Sequence reactions were analyzed on an Applied Biosystems model 377 sequencer. Sequences were analyzed using Macintosh Sequence Navigator and Autoassembler software. The derived amino acid sequence of MUC5AC is compared to known amino acid sequences using the Clustal W program (Thompson et al., 1994).

PCR and Southern blot analysis

Genomic DNA was derived from human blood cells. For the PCR reaction 500 ng genomic template DNA was used in combination with primers P82: 5'-ATCAACATCATCCATGTCGACCAC-3' and P83: 5'-ACCATGGAAGTAGACAGGGAAGGATAC-3' based on a non-repetitive sequence within the 3' part of HGM-1 (nt 1633-1656) and 5' part of MUC5 (nt 409-429) (clone NP3a), respectively (Klomp et al., 1995; Meerzaman et al., 1994). The PCR in a total volume of 25 µl was largely based on the expand long PCR protocol (Boehringer). Final PCR reaction conditions were: 10 mM Tris-HCl pH 9.2, 2.8 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 0,06% (v/v) Tween-20, 0.4% (v/v) DMSO, 1.4 U Taq/Pwo DNA polymerase mix, 0.3 µM of each primer, 500 ng genomic DNA, 275 µM of each dATP, dCTP, dGTP, and dTTP. The PCR reaction

was carried out as follows: 1 min and 15 sec 93 °C, 10 cycles of 15 sec 93 °C, 30 sec 67 °C, and 6 min 68 °C. These 10 cycles were followed by another 10 cycles of 15 sec 93 °C, 30 sec 67°C, 6 min 68 °C with a 20 sec extension for each cycle. After these cycles a 10 min extension step at 68 °C followed. The PCR-product was analyzed on a 0.8% agarose gel followed by Southern blot analysis according to standard procedures (Sambrook et al., 1989). The Southern blot was hybridized at 65 °C in 0.5 M NaH_2PO_4 pH 7.2, 7% SDS and 1 mM EDTA according to the method of Church and Gilbert (Church and Gilbert, 1984) with a ^{32}P-labeled 277 bp EcoRI-NcoI fragment comprising the 3' part of the HGM-1 cDNA clone (Klomp et al., 1995).

Results

Polymerase chain reaction and Southern blot analysis

To ensure that the HGM-1 and the MUC5 (clone NP3a) DNA sequences are closely linked on the genome and to determine the length in between, a PCR was performed using human genomic DNA and primers based on non-repetitive sequences within the 3' part of HGM-1 and 5' part of MUC5 (clone NP3a) sequences (Klomp et al., 1995; Meerzaman et al., 1994). Analysis of the PCR reaction on a 0.8% agarose gel resulted in a PCR product of 8.5 kb (Fig. 1A). To verify that the 8.5 kb band consisted of MUC5AC sequences this band was further characterized by Southern blot analysis. This blot was incubated with a radioactively labeled probe consisting of 277 bp EcoRI-NcoI fragment of the 3' part of HGM-1 (Fig. 1B). This probe reacted with the 8.5 kb band and not with the two other faint bands of 2.9 and 3.1 kb, that were also visible on agarose gel. This indicates that the 8.5 kb band contains HGM-1 sequences and that HGM-1 and MUC5 sequences are closely linked. The region in between HGM-1 and NP3a is 7.5 kb. Since the 5' part of the MUC5 (NP3a clone) and the 3' part of HGM-1 cDNAs are closely linked and include areas highly similar (> 90%) to MUC5AC cDNA sequences determined by Guyonnet Duperat and co-workers (Guyonnet Duperat et al., 1995), it is very likely that all these are part of the same gene, i.e. MUC5AC. Based on the lengths of HGM-1 (2.6 kb), MUC5 (clone NP3a, 3.5 kb) and the region in between (7.5 kb), the MUC5AC cDNA must at least be 13.5 kb. However, thus far the N-terminal MUC5AC sequence is unknown and therefore the full length cDNA is most likely larger than 13.5 kb.

Isolation of MUC5AC cDNA clones

In order to further study MUC5AC, we have sought to clone the N-terminus of human MUC5AC. Therefore, we screened a human gastric cDNA library containing $6.6x10^5$ independent plaque forming units. Approximately $1.8x10^6$ recombinant plaques were screened with a 5' fragment of HGM-1 as probe. Sixteen independent plaques were positive and sequence analysis revealed that 8 of these contained inserts with sequences overlapping with the probe. To obtain additional 5' MUC5AC sequences, two subsequent screenings of this library were carried out, using the most upstream part of one of the MUC5AC resulting positive clones as probe. These three screenings resulted in the isolation of 32 independent

clones with inserts of at least 1 kb containing overlapping MUC5AC sequences. Four independent clones located at the extreme 5' end revealed the presence of one in frame ATG starting at nucleotide position 33 (data not shown).

Combined double-stranded sequence analysis of all 32 positive clones revealed a novel sequence, designated HGM-2, and the 5' part of HGM-1 (1092 bp). The newly identified sequence HGM-2 encompasses 3024 bp upstream of HGM-1. This HGM-2 sequence together with HGM-1 encompasses 4116 bp and encodes the 1373 residues of the N-terminal sequence of MUC5AC as illustrated in figure 2.

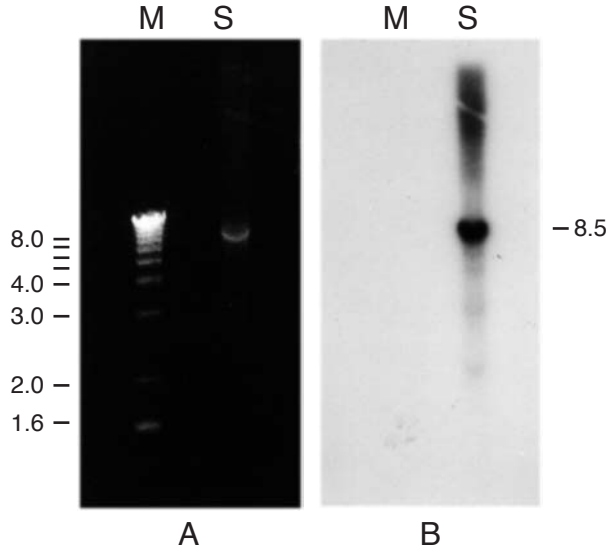

Figure 1. PCR analysis of the region between HGM-1 and MUC5 (NP3a). (A) Analysis on a 0.8% agarose gel of a PCR on genomic DNA using primers based on non-repetitive sequences within the 3' part of HGM-1 and 5' part of MUC5 (clone NP3a) sequences (Klomp et al., 1995; Meerzaman et al., 1994). M: Marker DNA of which the sizes are indicated. S: Sample of PCR reaction with a product of 8.5 kb. (B) Southern blot of agarose gel presented in panel A, hybridized with the labeled 3' end of HGM-1.

Analysis of MUC5AC amino acid sequences

The N-terminal MUC5AC sequence, as shown in figure 2, revealed a potential signal sequence of 19 amino acids (von Heijne, 1986). Furthermore, the sequence contains a high amount of cysteine residues (8.4 %) and three potential *N*-glycosylation sites. Sequence alignment of the N-terminus of MUC5AC using the Clustal W program (Thompson et al., 1994) showed high homology with the amino-terminal sequences of MUC2 (43% identity) and the pre-pro von Willebrand factor (pp-vWF) (29% identity) (Fig. 2). The N-terminus of MUC5AC, just like MUC2 and pp-vWF, contains several D-domains: D1 (residues 80 and 437); D2 (residues 438 and 801) and D3 (residues 904 and 1290). In the D-domains especially the cysteine residues are conserved. Interestingly, we also compared the MUC5AC sequence with known sequence motifs reported in the PROSITE database and this revealed a leucine zipper heptad repeat pattern between amino acids 273 and 300: X_3-Leu-X_6-Leu-X_6-Leu-X_6-Leu-X_3 (where X may be any residue, Fig. 2) (Bairoch, 1993). Essential features of leucine

zippers are that they form a coiled coil over a distance covering eight helical turns in such a way that the leucines align along one face of the helix, and that interdigitation of the leucine side chains facilitate dimerization (O'Shea et al., 1989).

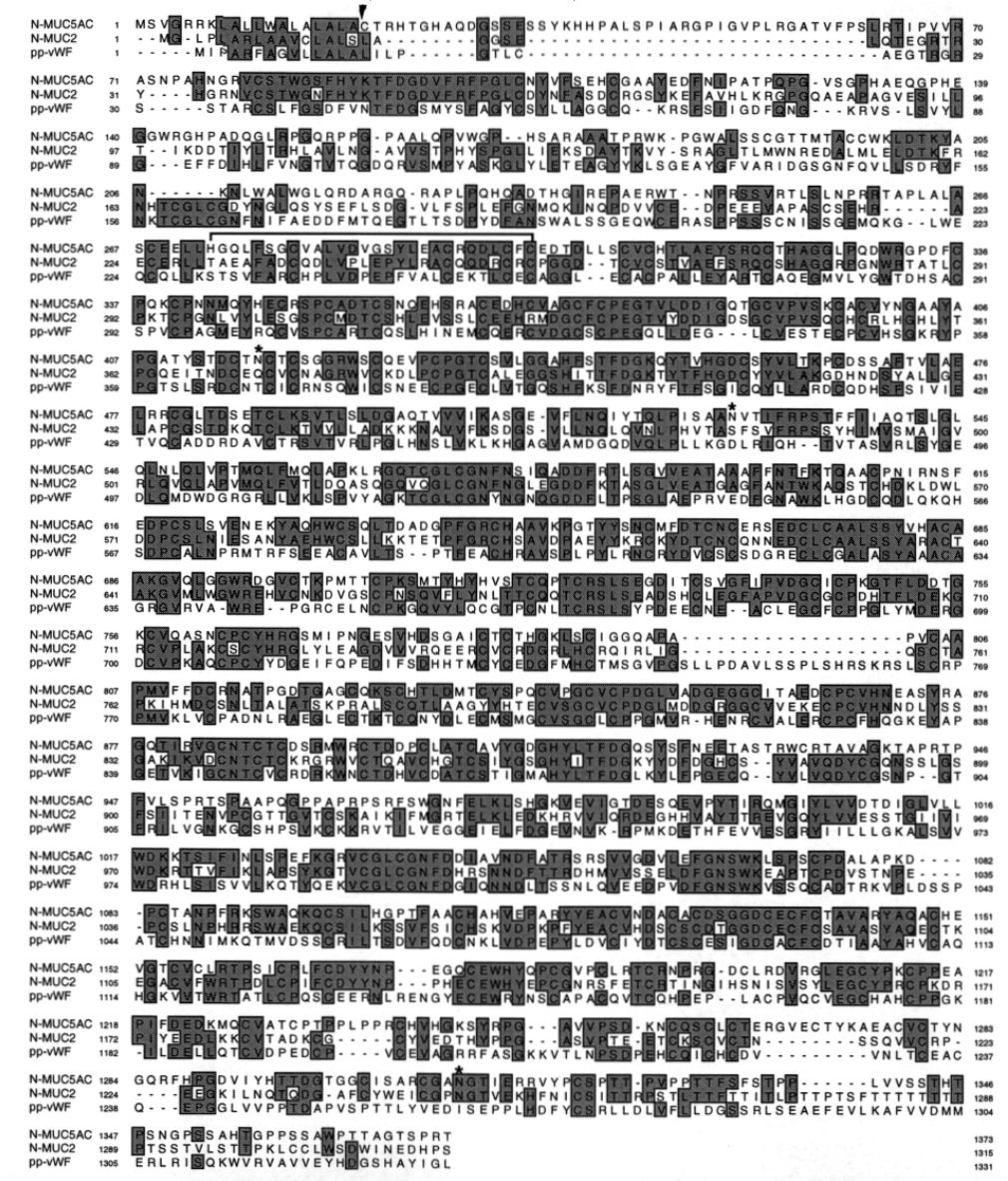

Figure 2. Comparison of N-terminal amino acid sequence of MUC5AC, MUC2 and the pre-pro von Willebrand factor (pp-vWF). The N-terminal sequence of MUC5AC is derived from the translated sequences of the newly identified HGM-2 (1008 residues) and the N-terminal part of HGM-1 (375 residues). The cleavage site of the potential signal sequence of 19 amino acids is indicated by an arrowhead (von Heijne, 1986). The leucine zipper motif in MUC5AC (residues 273-300) is indicated by a bracket. Asterisks mark potential *N*-glycosylation sites. The N-terminal sequence of MUC5AC is compared with the N-termini of MUC2 (Gum et al., 1994) and pp-vWF (Bonthron et al., 1986). Identical amino acids are boxed.

Since there are a number of proteins with the leucine heptad repeat sequence that do not form a leucine zipper structure, we wanted to elucidate if the leucine heptad repeat sequence in the amino-terminus of MUC5AC is a real leucine zipper with a coiled coil structure. Therefore, we used the method TRESPASSER: Two RESidue Pattern Analysis for Sequence-structurE Relationships (Hirst et al., 1996). This method for discriminating between zippers and non-zippers is based on the analysis of "disfavored" patterns, patterns of two residues that occur commonly in non-zippers, but rarely in leucine zippers. This program predicts if a sequence is a leucine zipper, based on the analysis of 525 non-zipper sequences and 61 zipper sequences. The TRESPASSER score on the MUC5AC sequence HGQ**L**FSGCVA**L**VDVGSY**L**EACRQD**L**CFC was 27. This means that there is only a 2-3% probability that the sequence is a non-zipper. Thus the TRESPASSER predicts is that the leucine repeat sequence within the N-terminus of MUC5AC is a leucine zipper (with a probability >95%). A leucine zipper heptad repeat is not found in vWF or MUC2 (Fig. 2).

In figure 3 the leucine zipper of a MUC5AC homodimer is drawn in the standard coiled-coil notation. The positions in the heptad repeat are labeled a-g; the leucines occur at the fourth position of a heptad, position d. Leucine is the most stabilizing amino acid in position d in the coiled-coil structure (Moitra et al., 1997). The dimer interface ("inside residues") is made up of positions a, d, e and g. Residues at position b, c, and f ("outside residues") are not in the position to make interhelical contacts.

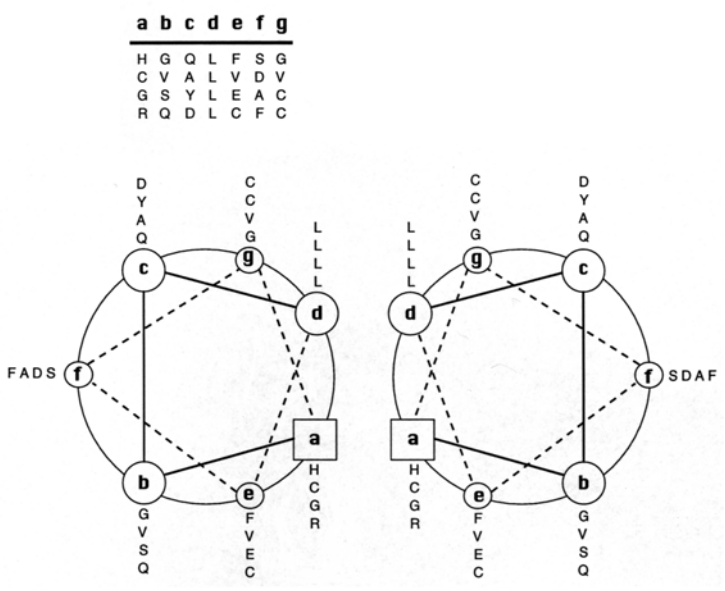

Figure 3. Schematic representation of the proposed amphipatic leucine zipper helix corresponding to amino acids His273 to Cys300 of MUC5AC (HGM-2). Helical wheel representation of a MUC5AC homodimer. View is from the N-terminus. Residues from the first two helical turns are boxed or circled. Helical positions are labeled a-g. The dimer interface ("inside residues") is made up of positions a, d, e and g. Residues at position b, c, and f ("outside residues") are not in the position to make interhelical contacts. Adapted from O'Shea et al., 1989.

Discussion

As many other repetitive and/or large cDNA sequences, mucin cDNAs are difficult to clone full length. This is also true for human mucin, MUC5AC, that is highly expressed in the stomach. Thus far only parts of its cDNA have been cloned (Guyonnet Duperat et al., 1995; Klomp et al., 1995; Meerzaman et al., 1994). However, the N-terminus and a large part of the PTS-rich central region were unidentified. Here we report the further cloning and characterization of MUC5AC cDNA. To determine the length of the central MUC5AC region, we performed a PCR using human genomic DNA and primers based on non-repetitive sequences within the 3' part of HGM-1 and 5' part of MUC5 (clone NP3a) sequences (Klomp et al., 1995; Meerzaman et al., 1994) (Fig. 4). The PCR revealed a fragment of 8.5 kb, which on Southern blot was recognized by a probe encompassing the 3'-end of the HGM-1. This indicates that HGM-1 and MUC5 (NP3a) are closely linked within the genome and that the unidentified region in between both sequences is 7.5 kb. Because the 3' part of HGM-1 and the 5' part of MUC5 (NP3a) contain sequences that are highly similar (>90%) to the PTS-rich repeats alternated with cysteines-rich domains as found in the original MUC5AC cDNA clones (Guyonnet Duperat et al., 1995), we speculate that the 7.5 kb region most likely encodes PTS-rich repeats alternated with cysteines-rich domains, similar to the central region of MUC5B (Desseyn et al., 1997). It is of note, that we cannot exclude the possibility that the 7.5 kb fragment contains intron sequences, because the PCR was performed on genomic DNA. However, this is not likely, because in all other cloned human mucins genes, MUC1, MUC2, MUC5B, and MUC7 (Bobek et al., 1993; Desseyn et al., 1997; Gum et al., 1994; Ligtenberg et al., 1990), the central repeat region is derived from one exon. Therefore, the central region of MUC5AC is probably also derived from one exon.

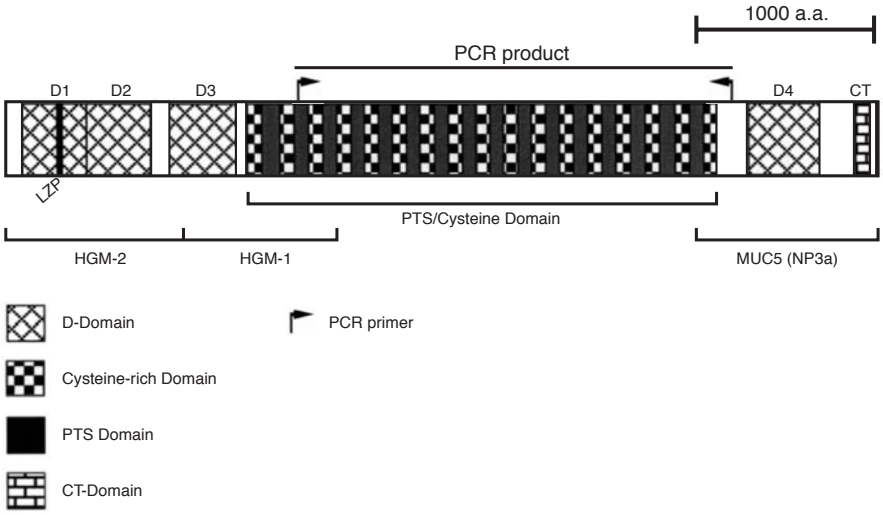

Figure 4. Domain model of the MUC5AC protein. The 4 D-domains (D1-4), the leucine zipper (LZP) and the C-terminal domain (CT) are indicated. The length of the PCR-product (shown in figure 1) is marked with a line and the primers used in the PCR are indicated by arrows. The brackets under the box model demarcate sequences of HGM-1 and 2 and MUC5 (NP3a) as well as the repetitive region containing PTS-rich repeats alternated with cysteine-rich repeats.

In order to clone the N-terminus of human MUC5AC, a human gastric cDNA library was screened with a fragment of HGM-1. In total we obtained 32 recombinant clones containing overlapping MUC5AC sequences. The last screening of the human stomach cDNA library revealed four independent clones, which contained the most upstream sequences and an in frame ATG. Together the cloned MUC5AC sequences comprise 1373 amino acids (Fig. 2). An overlap was observed with HGM-1 (850 amino acids) from amino acid 1009 to 1373. The sequence comprising the 1008 newly identified amino acids was designated as HGM-2. Together, HGM-1 and HGM-2 (in total 1858 amino acids) are called the N-terminus of MUC5AC. Since the N-and "C-termini" of MUC5AC comprises 1858 and 1167 amino acids, respectively, and the central region comprises about 2500 (about 7.5 kb), MUC5AC is about 5525 amino acids in length. This means that the mRNA of MUC5AC must be at least 16.6 kb, which is in agreement with the estimated size of about 17 kb on Northern blot (Debailleul et al., 1998). A schematic representation of the complete coding region of MUC5AC is shown in figure 4, which is based on the data presented in this paper combined with the other published MUC5AC data (Guyonnet Duperat et al., 1995; Klomp et al., 1995; Meerzaman et al., 1994). Several different sequence motifs found in MUC5AC are indicated and discussed below.

Sequence alignment with the here reported 1373 N-terminal amino acids of MUC5AC showed high homology with the N-termini of MUC2 and the pre-pro von Willebrand factor (pp-vWF) (Fig. 2). The N-terminus of MUC5AC contains D-domains D1, D2, and D3, originally identified in the pp-vWF (Bonthron et al., 1986; Ruggeri and Ware, 1993), whereas the C-terminus of MUC5AC contains the D4 domain (Meerzaman et al., 1994). These D-domains in pp-vWF are involved in multimerization (Ruggeri and Ware, 1993). Within these D-domains the cysteine residues are especially conserved. Cysteine residues within the D3 domain of pp-vWF (residues 1046-1458, Fig. 2) play an important role in the intermolecular disulfide bond formation necessary for multimer formation, whereas the cysteines within D1, D2 and D4 are involved in intramolecular bonds formation (Ruggeri and Ware, 1993). One might expect that the D-domains, as found in MUC5AC, fulfill the same function.

That the oligomerization process of MUC5AC proceeds by a similar mechanism as the vWF is substantiated by the finding that the cysteine-rich carboxyl terminal (CT-) region of MUC5AC displays high homology to the cysteine-rich CT-region of vWF (Meerzaman et al., 1994). Disulfide-linked dimerization resides within the CT-region of vWF and occurs in the endoplasmic reticulum (Marti et al., 1987; Voorberg et al., 1991). Recently, Van Klinken et al.(Van Klinken et al., 1998) showed in human colonic cell line LS174T that MUC5AC dimerization occurs in the RER. Therefore, it is likely that MUC5AC dimerization proceeds similar to the vWF.

Interestingly, within domain D1 of MUC5AC a leucine zipper pattern (between residues 273 and 300) is located. Based on the TRESPASSER score this leucine zipper pattern forms a coiled coil with a calculated probability of >95%. The leucine zipper pattern is present in many different dimeric proteins such as several transcription factors (Busch and Sassone-Corsi, 1990), membrane proteins of viruses with putative roles in viral infectivity (Li et al., 1996; Ramsdale et al., 1996; Wilson et al., 1981), and the membrane-associated α1,3-

fucosyltransferase of *Helicobacter pylori* (Ge et al., 1997). By forming a coiled coil structure leucine zippers facilitate dimerization via non-covalent interactions (O'Shea et al., 1989). However, dimerization of MUC5AC most likely occurs in the endoplasmic reticulum via disulfide-linkage of the CT-region. Therefore, it is tempting to speculate that the leucine zipper in the N-terminus of MUC5AC is involved in non-covalent homo-oligomerization via the N-termini of MUC5AC dimers. The leucine zipper motif located within domain D1 may be important in stabilizing the non-covalent interactions between two D1 domains of different MUC5AC molecules and therefore participate in the multimerization process of MUC5AC. The leucine zipper pattern is not found in vWF, MUC2 or any other mucin, indicating that the interactions of domain D1 of MUC5AC might be stronger than the interaction between D1 domains of adjacent MUC2 or vWF molecules. However, this has to be further investigated. In conclusion, we have cloned the N-terminus of MUC5AC, which reveals conserved cysteine rich D-domains and a putative leucine zipper motif**.**

Acknowledgements

This research was supported by a grant from the Dutch Digestive Diseases Foundation (to JHBvdB). Additional grants were obtained from the following foundations: “De Drie Lichten”, “Gastrostart”, “Jan Dekker en dr Ludgardine Bouwman”, “Irene Kinderziekenhuis”.

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Chapter 3

Infection with *Helicobacter pylori* changes the coordinate expression of the major secretory proteins in the human antrum

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Submitted

Abstract

Aim: Gastric *Helicobacter pylori* infection induces inflammation in gastric mucosa, and is associated with gastric cancer. We investigated expression of the secretory proteins in antrum epithelium in order to establish how *H. pylor*i affects antrum functions and which specific alterations are associated with intestinal metaplasia (IM), a pre-cancerous lesion. Methods: Antrum biopsy specimens were collected from 102 individuals (49 *H. pylori*-infected). Immunohistochemistry was performed for secretory mucins (MUC2, MUC5AC, MUC5B, and MUC6), TFF-peptides (TFF1, TFF2, and TFF3), endocrine peptides (gastrin, chromogranin A) and for proliferating cells (Ki-67). Protein expression was quantified morphometrically. Results: *H. pylori*-infection was correlated to inflammation, atrophy and proliferation, but not to occurrence of IM. IM was unequivocally characterized by MUC2 expression, and was correlated to atrophy. Infection was correlated to decreased MUC5AC, TFF1, and TFF2 expression and increased MUC6 and MUC5B expression. Remarkably, infection led to a coordinated shift of different cell types expressing MUC5AC, MUC6, and MUC5B, gastrin, CGA, or Ki-67 towards the upper regions of the glands. Conclusions: Aberrant epithelial proliferation, induced by *H. pylori*-infection and inflammation, triggers coordinate deregulation of protein expression in the antrum. As IM was correlated with atrophy, but not with *H. pylori*-infection, *H. pylori* is only indirectly involved in the development of this pre-cancerous lesion.

Abbreviations: AB, Alcian blue; CGA, Chromogranin A; IM, Intestinal metaplasia; PAS, Periodic acid/Schiff's reagent; TFF, trefoil factor family; ECL, enterochromaffin-like.

Introduction

Since the discovery of *Helicobacter pylori* (Marshall, 1983; Warren and Marshall, 1983), chronic infection with this bacterium is identified as the major etiologic factor of gastritis, gastric ulcers, gastric atrophy, and gastric carcinoma (Marshall and Warren, 1984; Parsonnet et al., 1991). In 1994, the international Agency for Research on Cancer (IARC, Lyon, France), classified *H. pylori*-infection as a carcinogenic agent class I. Concomitant with the infection by *H. pylori* both the proliferation and the apoptosis in the tissue increases (Moss et al., 2001) and references therein), altering the turnover of the epithelium. Gastric epithelial turnover is a dynamic process, which is characterized by continuous cell apoptosis, which is normally balanced by cell proliferation. Disturbance of the balance between apoptosis and proliferation could interfere with the normal mucosal homeostasis. A consequence of enhanced apoptosis in the gastric mucosa could lead to gastric mucosal atrophy (Genta, 1997). In gastric mucosal atrophy, defined as "loss of appropriate glands", the glands that are normally present in an area of the stomach disappear, and are either replaced by extracellular matrix (usually fibrous tissue), or by other types of glands (e.g., intestinal or pyloric metaplasia)(Smith and Genta, 2000). Another possible consequence of increased apoptosis could be increased cell proliferation to maintain mucosal homeostasis. Yamaguchi and coworkers observed in BALB/C mice, which were infected with *H. pylori*, increased proliferation as respond to increased apoptosis (Yamaguchi et al., 2000).

Longstanding infection is further associated with an increased risk of gastric cancer, in particular adenocarcinoma of the gastric epithelium. One of the early changes in the epithelium, which is generally acknowledged as an early marker in the development of cancer, is the occurrence of metaplasia in the gastric epithelium. Generally this metaplasia is of the intestinal-type (Correa, 1992). Next to profound histological changes, in particular the occurrence of intestinal type goblet cells, a number of genes are expressed that are more or less specific for the intestine, like MUC2 and TFF3 (Chang et al., 1994; Podolsky et al., 1993).

In this study we have investigated three different groups of secretory proteins of the antrum in relation to gastric *H. pylori*-infection. The first group consists of the secretory mucins (MUC2, MUC5AC, MUC5B, and MUC6). Secretory, gel-forming mucin is the most important structural component of the mucus gel-layer and plays an important role in the protection of the underlying epithelium (Van Klinken et al., 1995). The second group of secretory proteins consists of TFF-peptides (formerly P-domain peptides or trefoil factor (Wright et al., 1997)), which are often expressed in close association with secretory mucins (Wong et al., 1999). In humans three TFF-peptides have been identified: TFF1 (formerly pS2), TFF2 (formerly hSP), and TFF3 (formerly hP1.B/hITF). TFF-peptides very likely play a role in gastrointestinal defense and repair (Hoffmann et al., 2001). The third group of secretory proteins comprises endocrine cell markers, gastrin and chromogranin A (CGA). Gastrin is produced as a prohormone by G-cells located within the gastric antrum. The most important role of gastrin is the regulation of gastric acid secretion. In the human stomach, enterochromaffin-like (ECL) cells of the corpus have been identified as the main source for chromogranin A (CGA) expression (Dockray et al., 1996). Besides ECL cells, CGA expression has been demonstrated in antral G-cells

(Wiedenmann and Huttner, 1989). CGA is thought to influence granule stability, prohormone processing and peptide sorting into the regulated secretory pathway (Hocker et al., 2001 and references therein). To investigate the possible effect of *H. pylori*-infection on the proliferation, we used a specific antibody to Ki-67. This nuclear protein is expressed in proliferating cells, (G1, S, G2, and M phase), but not in resting cells (G0 phase) (Gerdes et al., 1984).

Thus far, it has remained uncertain how the *H. pylori*-induced changes in expression of the secretory proteins of the antrum correlate. As most studies just concentrated on one or a few of the many products of the antrum, the underlying mechanism was thus far not revealed. In this study we demonstrated the close correlations of *H. pylori*-infection with the expression of the various secretory proteins of the antrum, and with proliferation. As it appeared *H. pylori* induced a coordinate rearrangement of all secretory cell types in the infected antrum. We also studied the expression of secretory proteins in the intestinal metaplasia (IM) in the antrum, and also showed that the presence of intestinal metaplasia influenced expression of secretory mucins in antral epithelium distant from the metaplasia.

Materials and Methods

Patients and Tissue

Biopsy specimens of the antrum of 102 patients were collected per endoscopy, as part of a prospective study on the occurrence of *H. pylori* in adult patients with upper abdominal complaints. Patients were asked to abstain from anti-acids drugs at least one week prior to the endoscopy. The biopsy specimens were collected in the Academic Medical Center in Amsterdam, with permission of the Medical Ethics Committee. Two biopsy specimens were taken from the antrum per patient, and immediately fixed in PBS-buffered 4% (wt/vol.) paraformaldehyde solution for 4 h and then processed into paraffin blocks according to standard procedures. Clinical data were recovered from the pathologist's records, based on at least two separately collected biopsy specimens from closely adjacent gastric mucosa, using the criteria of the Sydney classification (Dixon et al., 1996). *H. pylori*-infection was recorded as based on standard histological staining and by microbiology. Active inflammation was recorded semi-quantitatively: 0, no active inflammation; 1, mild active inflammation; 2, moderately active inflammation; and 3, marked active inflammation. Atrophy was also recorded semi-quantitatively: 0, absent; 1, mild; 2, moderate; and 3, marked.

Histochemistry

Serial tissue sections were stained in a standard procedure with hematoxylin and eosin, and by alcian blue/periodic acid Schiff's reagent (AB/PAS) at pH 2.5 to distinguish neutral and acid mucins.

Immunohistochemistry

Polyclonal antibodies raised in rabbits were used: anti-MUC5B, anti-MUC6, anti-TFF1, anti-TFF2, anti-TFF3, anti-gastrin, and anti-CGA (Table1). Mouse monoclonal antibodies were also used: anti-MUC2, anti-MUC5AC, and anti-Ki-67 (Table 1). Tissue sections were deparaffinized through three changes of xylene and then rehydrated through a series of decreasing concentrations of ethanol solution to distilled water. Endogenous peroxidase activity was inactivated in 3% (vol./vol.) hydrogen peroxide in phosphate buffered saline (PBS) for 30 min and washed in PBS for 5 min. Antigen retrieval was performed by heating the sections for 10 min at 100°C in 10 mM citrate buffer, pH 6.0, and then left to cool to room temperature for 20 min. Sections were washed three times for 5 min in PBS and incubated with 1% (wt/vol.) blocking agent (Boehringer, Mannheim, Germany) in PBS for 30 min. Primary antibodies were diluted in PBS (Table 1), and incubated with the tissues for 16 h at 4°C. Slides were washed three times for 5 min in PBS, followed by incubation with biotinylated secondary antibodies, and avidin-biotin peroxidase complex (Vectastain ABCkit, Vector laboratories, Burlingame, UK) according to the manufacturer's protocol. Staining was performed using 0.5 mg/ml 3,3'-diaminobenzidine/0.03% (vol./vol.) hydrogen peroxide in imidazole (30 mM)/EDTA (1 mM) pH 7.0. Sections were rinsed in water, dehydrated through a series of increasing concentrations of ethanol solutions, and mounted under cover slips. Control stainings were performed, leaving each of the primary antibodies out off the procedure, resulting in absence of staining.

Table 1. Characteristics of antibodies and dilutions used for immunohistochemical methods.

Name in this study	Name in reference	Epitope	Dilution	Reference
Anti-MUC2	WE9	Peptide domain	1:50	(Tytgat et al., 1995)
Anti-MUC5AC	45M1	Peptide domain	1:50	(Bara et al., 1998)
Anti-MUC5B	anti-BGBM	Deglycosylated polypeptide	1:3000	(Van Klinken et al., 1998)
Anti-MUC6	M6.1	Peptide in VNTR* domain	1:200	(De Bolos et al., 1995)
Anti-TFF1	anti-pS2	C-terminal peptide	1:4000	(Rio et al., 1991)
Anti-TFF2	anti-hSP	C-terminal peptide	1:2000	(Srivatsa et al., 2002)
Anti-TFF3	HM:169	C-terminal peptide	1:3000	(Podolsky et al., 1993)
Anti-Gastrin	A0568	Gastrin-17	1:2000	DAKO, Glostrup, DK)
Anti-CGA	A0430	20 kD C-terminal peptide	1:1000	DAKO, Glostrup, DK)
Anti-Ki-67	MiB-1	Peptide	1:1000	(Gerdes et al., 1984)

*VNTR, variable number of tandem repeats.

Semi-quantitative scoring of Histological staining

The extent of expression of each protein was determined semi-quantitatively on serial sections (Fig. 1). Two independent observers, who were blinded to the clinical status of the patients, determined the expression. The surface-to-gland axis and the expression patterns of the respective proteins were determined on at least 2 separate locations within each tissue section. Upon disagreement among the observers the average scores were taken. Biopsy specimens were

cut perpendicular to the mucosal surface, and only those sections were assessed where the whole pit-gland axis was fully visible. As some biopsy specimens were only cut near ideal, along the surface-to-gland axis, we chose to use a relative scale for the protein expression along this axis rather that an absolute scale. Moreover, due to either hyper-proliferation or atrophy in particular specimens the surface-to-gland axis varied in absolute length. To visualize and correlate coordinate protein expression in the antrum epithelium in this study the relative measurements, as explained in figure 1, appeared very instructive.

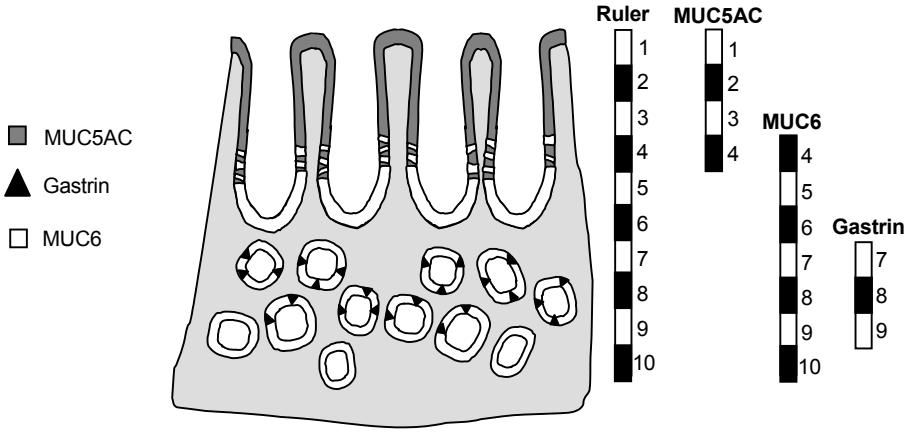

Figure 1. Schematic representation of the quantitation of secretory protein expression in the antrum. The scheme represents an antrum biopsy specimen, and indicated are the typical expression patterns of three secretory proteins, MUC5AC (dark gray), MUC6 (white) and gastrin (black triangles). The microscopic images of the sections of these biopsy specimens were projected through a CCD video camera onto a monitor, and the total surface-to-gland axis was assessed using a ruler, dividing this axis in 10 regions of equal length. Notice that this scoring system measures the protein expression relatively; the length of the axis of the individual sections was taken as 100%. The expression of the individual proteins was assessed by identifying the regions where the protein was detected. Further note that the intensity of staining was not accounted for in these measurements; the absence or presence of a protein was scored per defined region. The number of regions staining positively along the pit-gland axis was taken as measure of the extent of expression, in this example: MUC5AC, MUC6, and gastrin; score 4, 7, and 3, respectively. To assess coordinated protein expression along the pit-gland axis we also determined either the limits of protein expression, like for MUC5AC and MUC6 in this example (scoring both 4 in this example), or the peak of protein expression, like for gastrin in this example, scoring 8.

Intestinal Metaplasia

Intestinal metaplasia (IM) was detected histochemically in each biopsy specimen by its characteristic morphology using hematoxylin/eosin and AB/PAS staining. Protein expression of the above mentioned proteins were determined within the metaplastic epithelial structures. Distinction was made between expression of the respective proteins in the characteristic goblet cells and the other epithelial cells within the IM. To assess effects on protein expression distant from the IM, the expression of the marker proteins in the epithelium was determined at a distance of at least 0.5 mm from the metaplasia.

Statistics

The χ^2 test or Fisher's exact test was used to analyze differences in frequencies. Spearman's correlation test was used to analyze correlations between parameters. Statistical significance was defined at $P < 0.05$.

Results

Pathology

Antrum biopsies were prospectively collected from 102 patients, of whom 49 suffered from *H. pylori*-infection at the time of endoscopy. We analyzed the correlations between *H. pylori*-infection and the extent of inflammation, atrophy, and proliferation (Figs 2 and 3). Proliferation was detected using an anti-Ki-67 antibody (Fig. 4, see *Appendix*), and expression of Ki-67 was quantified as explained in figure 1. There was a strong and highly significant correlation between *H. pylori*-infection and the active inflammation of the mucosa (Fig. 2A). Both *H. pylori*-infection and active inflammation also correlated independently and statistically significant with mucosal atrophy, and proliferation (Figs 2 and 3).

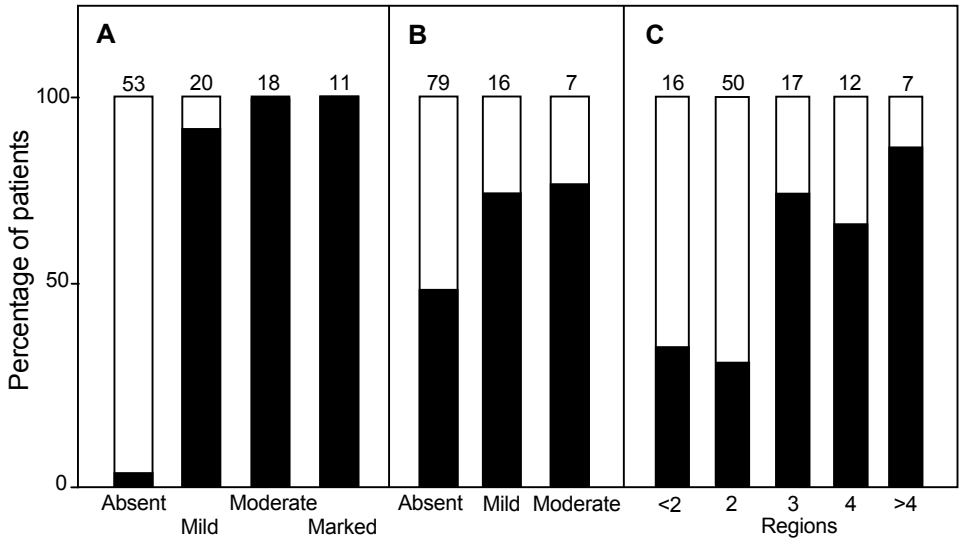

Figure 2. *H. pylori*-infection is related to increased mucosal inflammation, atrophy, and proliferation. Acute mucosal inflammation (Panel A) and mucosal atrophy (Panel B) were scored according to Sydney classifications. The presence of *H. pylori* was scored as present or absent. The epithelial proliferation was determined by immunohistochemical staining for Ki-67 (Panel C), where after the number of regions containing Ki-67-positive cells, indicated along the X-axis, were taken as a measure of proliferation (see Materials and Methods, and Fig. 1). The percentage of *H. pylori*-infected patients is indicated in black and the percentage of *H. pylori*-negative individuals is indicated in white. The number of patients within each group is indicated above each bar. Spearman's correlation test was used to determine significant correlations. *H. pylori*-infection was positively correlated to inflammation ($\rho=0.907$, $P < 0.0005$), atrophy ($\rho=0.189$, $P < 0.05$), as well as proliferation ($\rho=0.358$, $P < 0.0005$).

Expression of secretory proteins and KI-67 during *H. pylori*-infection

The biopsy specimens of the 102 patients were stained for the presence of MUC5AC, MUC5B, MUC6, TFF1, TFF2, TFF3, gastrin, and CGA. Expression of each protein could be identified in each patient. Figure 4 (see *Appendix*) shows representative examples of an *H. pylori*-infected and a healthy individual. MUC5AC was expressed in the surface epithelium, in the epithelium of the gastric pits, and to a variable extent into the glandular structures. In contrast, both MUC5B and MUC6 were only expressed in the glands, and never at the surface, in a manner complementary to the MUC5AC expression pattern. TFF1 was expressed in the surface and pit epithelium, and to a variable extent deeper into the glands. TFF2 was expressed in the entire epithelium in nearly all patients. TFF3 was characteristically expressed in the surface epithelium and in the lower part of the glands, but usually not in the pit region of the surface-to-glands axis. Gastrin and CGA were expressed in quite a number of cells in the glands. Analysis of the number of cells and their morphology in consecutive sections makes it likely that these two polypeptides were expressed at least partly within the same cells. The cells expressing gastrin or CGA were usually found in the same regions, as defined by the method depicted in figure 1. However, CGA was in general expressed in fewer cells compared to gastrin. These cell types will be described separately in this study, as gastrin- and CGA-expressing cells, respectively.

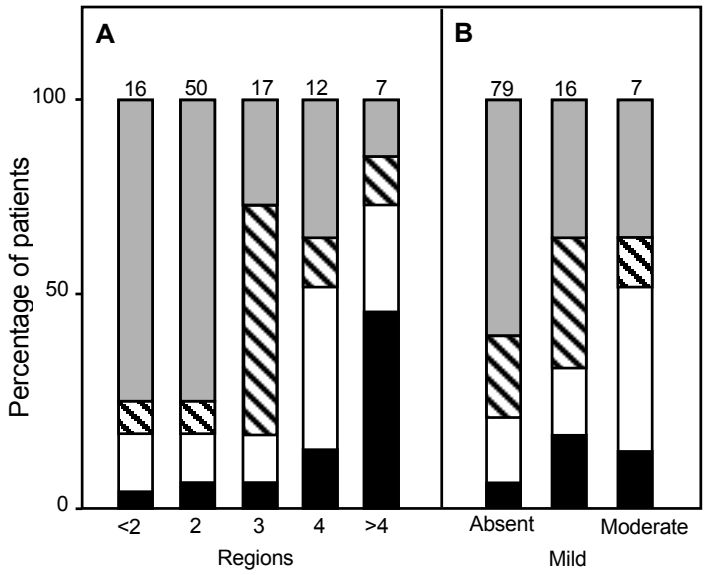

Figure 3. Acute mucosal inflammation correlates to increased epithelial proliferation and atrophy. Acute mucosal inflammation and mucosal atrophy were scored according to Sydney classifications. The epithelial proliferation was determined by immunohistochemical staining for Ki-67 (Panel A), where after the number of regions containing Ki-67-positive cells were taken as a measure of proliferation (see Materials and Methods, and Fig. 1). Atrophy (Panel B) was scored as absent, mild, or moderate. The extent of inflammation is indicated: gray, no inflammation; cross-hatched, mild inflammation; white, moderate inflammation; and black, marked inflammation. The number of patients within each group is indicated above each bar. Spearman's correlation test was used to determine significant correlations. Inflammation was positively and statistically significantly correlated to proliferation (Panel A, ρ=0.332, $P < 0.001$) and atrophy (Panel B, ρ=0.235, P <0.02).

When reviewing the sections of all patients it became highly probable that the expression patterns of the secretory proteins shifted in a coordinate manner along the surface-to-gland axis. Also the position of the proliferating cells, as detected by Ki-67 staining, seemed to follow this pattern (Fig. 4, *Appendix*). Therefore, we localized and quantified the extent of expression of each protein along the surface-to-gland axis, as explained in figure 1.

In the lower regions of the surface-to-gland axis (regions 4-9), there was less frequent expression of MUC5AC in the *H. pylori*-infected individuals than in the non-infected patients (Fig. 5). Conversely, the expression of both MUC6 and MUC5B was more frequently found in these regions (i.e. regions 4-8 for both MUCs) of the surface-to-gland axis in the *H. pylori*-infected individuals (Fig. 5).

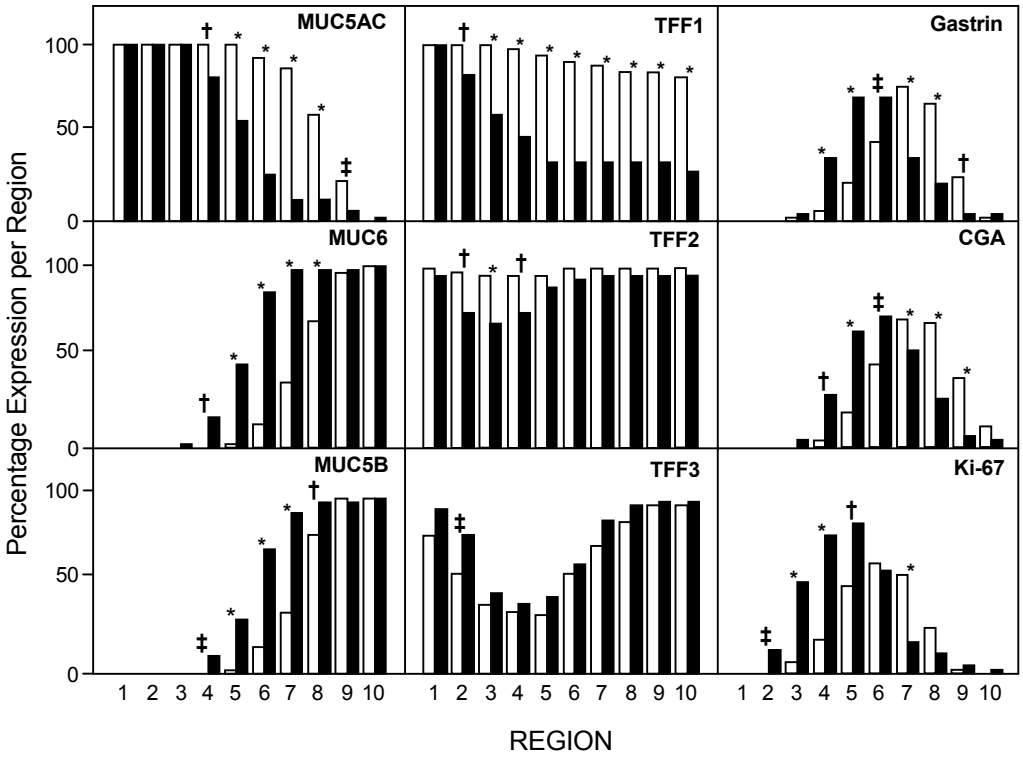

Figure 5. Expression of secretory proteins and KI-67 along the surface-to-gland-axis of the antrum. As exemplified in figure 1, the surface-to-gland axis was divided into ten equal regions. The expression of the indicated secretory proteins and of Ki-67, visualized as in figure 4, was assessed per region. Region 1 denotes by definition the most superficial region along the axis, whereas region 10 corresponds to the deepest parts of the glands. Distinction was made between healthy biopsy specimens (n=53, white bars), and *H. pylori*-infected specimens (n=49, black bars). Presence of a protein per region was expressed as the percentage of patients showing positive staining in a particular region. For example in region 5, 100% of healthy patients showed expression of MUC5AC, whereas only 58% of the *H. pylori*-infected patients expressed MUC5AC. Statistically significant differences between protein expression per region were assessed by χ^2 test. (‡, P <0.05; †, P <0.005; *, P <0.0005).

TFF1 was found along all regions of the surface-to-gland axis in more that 90% of the non-infected group (Fig. 5). However, TFF1 was significantly less frequently found in all regions, except region 1, in the *H. pylori*-infected group. TFF2 showed the most widespread expression, and was only significantly less frequently found in regions 2-4 of the *H. pylori*-infected group, but even in these regions the expression was still found in at least 80% of the patients (Fig. 5). TFF3 expression was most often found in the surface regions of the antrum and in the deeper glandular regions, whereas the lower pit region (regions 3-5) was often free of TFF3 expression (Fig. 5). There were hardly any differences in TFF3 expression between normal and *H. pylori*-infected individuals.

Gastrin, CGA, and Ki-67 showed peaks of expression along the surface-to-gland axis. As noted, gastrin and CGA showed overlapping expression patterns. In healthy individuals, the mean peaks of both gastrin and CGA expression were found in region 7 (Fig. 5). The mean expression of Ki-67 was found just above the peak in gastrin and CGA expression at region 6 in non-infected individuals (Fig. 5). Although the regions of expression of these three markers usually overlapped in individual patents, this characteristic distribution of gastrin, CGA, and Ki-67 along the surface-to-gland axis was very consistently found. Remarkably, each of these proteins showed a mean expression peak more closer to the surface in the *H. pylori*-infected patients. Thus, gastrin, CGA, and Ki-67 in the infected tissue showed mean peaks of expression in regions 6; 6; and 5, respectively (Fig. 5). Interestingly, the relative positions of the expression of these three markers in the individual infected patients remained very similar to the non-infected individuals: from surface to glands: Ki-67, CGA, and gastrin in overlapping regions.

Table 2. Correlations between extent of expression of secretory proteins and *H. pylori*-related pathology.

	MUC2	MUC5AC	MUC5B	MUC6	TFF1	TFF2
H. pylori		-0.667**	0.619**	0.691**	-0.572**	-0.392**
Inflammation		-0.688**	0.602**	0.714**	-0.594**	-0.447**
Atrophy	0.198*				-0.246*	

NOTE: The presence of *H. pylori* was scored as either absent or present, whereas inflammation was scored semi-quantitatively based on the Sydney classifications (see Materials and Methods). Expression of the secretory proteins was determined as the number of regions along the surface-to-gland axis in which expression was found (see Figs 1 and 4). Of the proteins studied, only MUC2 expression was scored as either absent or present in the biopsy specimens. The extent of expression of TFF3, gastrin and CGA did not correlate significantly to *H. pylori*-infection, inflammation, or atrophy, and were therefore omitted from the table.
* P <0.05; ** P <0.0005

Coordinated protein expression

From the analysis shown in figure 5 it seemed evident that the expression of secretory proteins within the antrum epithelium shifted coordinately, depending on the *H. pylori*-related pathology. We analyzed the extent of expression of the individual secretory proteins within the antrum epithelium and related these to the degrees of *H. pylori*-infection and inflammation within the individual biopsy specimens (Table 2). It appeared that the extent of expression of MUC5AC, TFF1, and TFF2 was negatively affected both with increasing infection as well as inflammation, whereas the areas within the epithelium showing MUC5B and MUC6 expression were positively

correlated to both increasing infection and inflammation (Table 2). Either infection or inflammation did not affect the extent of expression of TFF3, gastrin, or CGA. Interestingly, the correlations of the individual secretory proteins were equally strong with *H. pylori*-infection as with active inflammation.

Coinciding with *H. pylori*-infection we observed a shift of the average lower limit of MUC5AC-expression in the epithelium towards the gastric surface (Fig. 5). At the same time, the average upper limits of both MUC6 and MUC5B expression, and the average peaks in the expression of gastrin, CGA, and Ki-67 also shifted towards the surface. Using the data from the individual patients we determined the correlations between the limits of expression (i.e. for MUC5AC, MUC6, and MUC5B) and the peak expression (i.e. for gastrin, CGA, and Ki-67). We found that each of these six limits or peaks correlated strongly and statistical highly significantly with all other limits or peaks of expression, when analyzed pair wise (ρ=0.376 to 0.917, P <0.0005 for each pair wise correlation). We also used the data of the individual patients to analyze the correlation of these limits and peaks in protein expression using multivariate analysis (ANOVA). Figure 6 shows a schematic summary of the major changes in the epithelium of the antrum due to *H. pylori*-infection, indicating that the coordinated shift in MUC5AC, MUC6, MUC5B, gastrin, CGA, and Ki-67 expression was statistically highly significant. Changes in the extent of expression of the TFFs, or in the limits of expression of the TFFs along the surface-to-gland axis, did not correlate to the shifts in limits or peaks of protein expression of the six above mentioned proteins, as analyzed by pair wise correlation analysis.

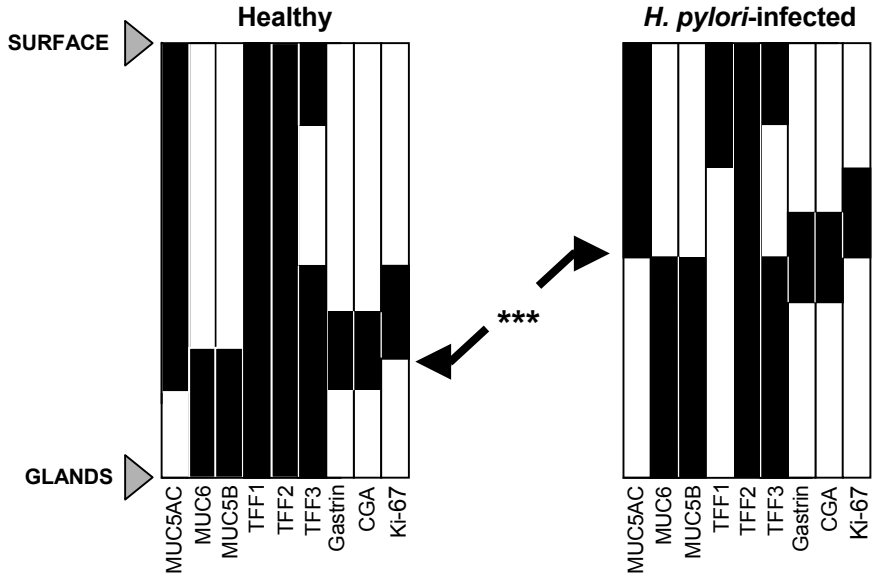

Figure 6. Coordinately changed protein expression in antrum during *H. pylori*-infection. The black boxes represent areas along the surface-to-gland axis where the expression of the respective proteins was found in more than 50% of the patients (see Fig. 5). Six parameters were analyzed in conjunction by multivariate analysis: the lower limit of MUC5AC expression, both the upper limits of MUC6 and MUC5B expression, and the peaks of expression of gastrin, CGA and Ki-67. These parameters appeared to co-vary in a highly coordinate manner, as tested by ANOVA; *** P <0.0005.

Expression of secretory proteins and KI-67 in Intestinal Metaplasia

IM was identified in sections of each biopsy specimen by histochemical hematoxylin/eosin and AB/PAS staining by characteristic morphological features, in particular the occurrence of alcian blue-positive goblet cells. In 25 patients IM was demonstrated using histochemical staining. The smallest metaplastic structures constituted of only one transected tubular structure, and only seldom the entire surface-to-gland axis was replaced by metaplastic tissue. We analyzed the correlations between the presence of IM and *H. pylori*-infection, inflammation, atrophy, and proliferation. IM correlated only significantly with mucosal atrophy (Fig. 7), but not with *H. pylori*-infection, inflammation, or proliferation (not shown).

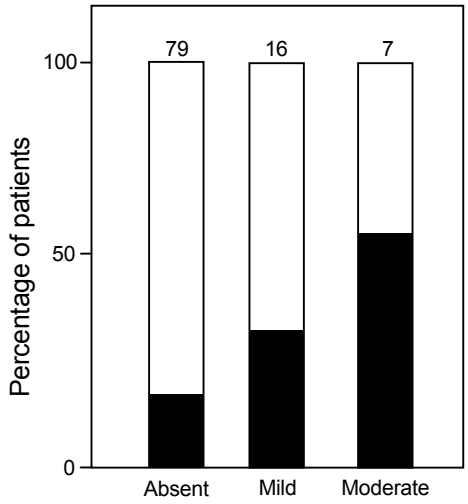

Figure 7. Mucosal atrophy and intestinal metaplasia are positively correlated. The presence of atrophy and intestinal metaplasia were scored according to the Sydney classifications. Metaplasia is scored as either absent or present, whereas atrophy was scored as absent, mild, or moderate. Marked atrophy was not observed in our patient group. Indicated are the percentage of patients without metaplasia (white), and the percentage of patients with metaplasia (black). The number of patients within each group is shown above each bar. The correlation between atrophy and intestinal metaplasia was statistically significant (ρ=0.198, P <0.05; Spearman's test).

We identified the expression patterns of the secretory proteins and Ki-67 in IM (Fig. 8, see *Appendix*). Sections of all biopsy specimens were stained by anti-MUC2 antibodies, and all goblet cells within each IM were found to be selectively stained for MUC2 (Fig. 8, *Appendix*, and Table 3). Of the other proteins, MUC5AC, TFF1, TFF2, and TFF3 were often found in the metaplastic epithelium, in particular in the goblet cells of the IM (Table 3). Yet, these proteins were not unequivocally found in all goblet cells of each IM, and were present in variable combinations. In the example shown in figure 8 (*Appendix*), only MUC2, TFF2, and TFF3 were expressed in goblet cells, while gastrin, CGA, TFF1, TFF3, and Ki-67 were expressed in the non-goblet cells of the IM. Whereas MUC2 was never observed in the other, non-goblet cells of the IM epithelium, all other proteins including Ki-67 were found in at least some IM specimens (Table 3).

Table 3. Protein expression in Intestinal Metaplasia.

	Cell type (% of patients with positive staining)	
	Goblet cells	Epithelium
MUC2	100	0
MUC5AC	62	33
MUC6	0	20
MUC5B	0	29
TFF1	71	71
TFF2	71	43
TFF3	63	25
Gastrin	0	35
CGA	0	58
Ki-67	0	60

NOTE: Intestinal metaplasia (IM) was observed in 25 of the 102 patients. The IM varied in size, but alcian blue-positive goblet cells were always identified. We determined qualitatively if proteins were expressed in the IM, thereby distinguishing expression within the goblet cells and the other epithelial cells of the IM.

We analyzed if the presence of MUC2-expression, i.e. the presence of IM, affected the gene expression in the non-metaplastic epithelium. The expression patterns like displayed in figure 4 were measured for each protein at a distance of at least 0.5 mm from the nearest IM in each tissue section. When the correlations between MUC2 and the other proteins were considered pair wise, we found that the average limits of the expression of the other three MUCs correlated significantly with MUC2 expression. The ρ for the correlations of MUC2 with MUC5AC, MUC6, and MUC5B were 0.208, 0.228, and 0.213, respectively (all, $P < 0.05$). Thus, the average MUC5AC expression diminished, whereas MUC6 and MUC5B expression was increased along the surface-to-gland axis, when IM was present at a considerable distance (Fig. 9). The positions of the average peaks in the expression of gastrin, CGA, and Ki-67 in the distant mucosa did not correlated with the presence of MUC2 or IM (not shown). Also the extent of expression of the TFFs, measured as the number of positive regions along the surface-to-gland axis, was not correlated to the presence of MUC2 or IM (not shown).

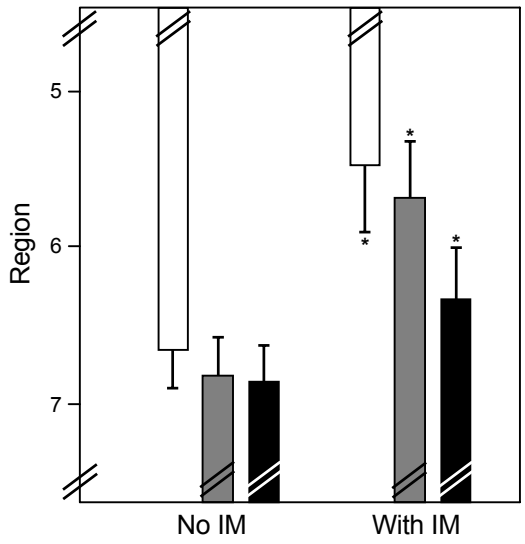

Figure 9. Expression of MUC5AC, MUC6, and MUC5B in non-metaplastic mucosa distant from intestinal metaplasia. Expression of the proteins as displayed in figure 4 were localized at a distance from the intestinal metaplasia in the manner displayed in figure 1. Measurements were performed by two independent observers in duplicate at a distance of at least 0.5 mm from the IM. Of the 102 patients, 25 were found to have IM. Only mucin expression was affected by the presence of IM, the other proteins expression patterns were not significantly affected. White bars, MUC5AC; gray bars, MUC6; and black bars, MUC5B. Statistical differences were determined (χ^2 test; * P <0.05).

Discussion

We found positive correlations between *H. pylori*-infection and active inflammation, mucosal atrophy, and proliferation. Previous studies have also shown increased epithelial proliferation in *H. pylori*-infected individuals, whereas after eradication therapy proliferation returns to normal levels (Moss et al., 2001 and references therein). Increased proliferation in response to *H. pylori*-infection may be the result of increased gastrin production (Johnson, 1988). Our study does not indicate an increase in number of gastrin-producing cells, notwithstanding a possible higher output of gastrin per cell. An enhanced gastric cell proliferation could also be the result of induced inflammatory response, leading to type B gastritis (Genta, 1997; Smith and Genta, 2000). *H. pylori* has been associated with increased gastric epithelial apoptosis in several studies (Moss et al., 2001and references therein). To maintain mucosal homeostasis, increased rates of apoptosis may also trigger increased proliferation. The mechanism by which *H. pylori* induces apoptosis in gastric epithelial cells remains unclear. Taken our and the published results together, it seems evident that *H. pylori*-infection plays a direct causal role in gastric inflammation, whereas changes in proliferation and occurrence of atrophy are most likely the effects of the inflammatory processes within the gastric mucosa, which are induced by *H. pylori*.

One of our primary aims was to investigate the effects of *H. pylori*-infection on the expression of secretory proteins in the antrum epithelium. MUC5AC was expressed in a manner complementary to MUC6 both in *H. pylori*-infected and healthy individuals. MUC5AC expression was localized to the surface and gastric pit epithelium, whereas MUC6 expression was localized to the glandular structures, and never found in the surface epithelium. With respect to the localization of MUC5AC and MUC6, this reciprocal mucin expression was described in one of our earlier studies (Van den Brink et al., 2000b), and is in accordance with the results of Ho et al (Ho et al., 1995a). In agreement with one recent study, we demonstrated that MUC5B expression was also found in the antrum of *H. pylori*-infected and healthy individuals (Longman et al., 2000). In *H. pylori*-infected patients there was less MUC5AC expression in the lower regions of the surface-to-gland axis, while MUC5B and MUC6 expression was more pronounced in these regions compared to non-infected individuals. The decreased MUC5AC expression in *H. pylori*-infected patients is consistent with the findings of Byrd et al. who studied gastric-type mucin expression in 29 *H. pylori*-positive patients (Byrd et al., 1997). Although, we found a similar shift of MUC5AC expression towards the surface epithelium and an increased number of MUC6-expressing cells, we never detected MUC6-positive cells in the surface epithelium of *H. pylori*-infected patients.

TFF-peptides were expressed in the antrum of all individuals. TFF1 expression was generally localized to the surface and pit epithelium and to some extent into the deeper glands, while TFF2 expression was found throughout the entire epithelium. Within the gastrointestinal tract, the stomach is considered the major source of TFF1 and TFF2 expression (Hanby et al., 1993a; Hanby et al., 1993b). We also showed TFF3 expression in the surface epithelium and the lower parts of the glands. In parallel with our study, TFF3 expression was found in normal and pre-neoplastic gastric epithelium (Taupin et al., 2001). It is known that TFF-peptides are commonly expressed in association with secretory mucins (Sands and Podolsky, 1996). The major source of TFF3 expression in the gastrointestinal tract are intestinal goblet cells, where TFF3 is co-expressed with MUC2 (Podolsky et al., 1993). In antrum, TFF3 was expressed in the same regions as MUC5AC and MUC5B. MUC5AC/TFF3 and MUC5B/TFF3 co-expression patterns were previously observed in the respiratory tract (Hovenberg et al., 1996; Wiede et al., 1999). The major difference we observed between *H. pylori*-infected and non-infected patients, with respect to the extent of TFF-peptide expression, was that TFF1 expression was significantly less frequently found in regions deeper into the glands in the *H. pylori*-infected group. TFF1 is thought to be expressed in close association with MUC5AC expression in the antrum (Ho et al., 1995a; Rio et al., 1988). MUC5AC and TFF1 expression are part of the same phenotype, and consequently, as these cell types are diminished in *H. pylori*-infected individuals, these two important secretory proteins may decrease coordinately.

The endocrine cell markers, gastrin and CGA, were primarily co-localized to the same cells. Gastrin is exclusively expressed by antral G-cells (McColl et al., 2000), and virtually all of these cells were also stained for CGA, as has also been demonstrated by others (Norlen et al., 2001; Van den Brink et al., 2000a; Wiedenmann and Huttner, 1989). Occasionally, CGA expression was found in cells not stained for gastrin, but the identity of this small subset of

CGA-positive cells was not investigated further. In non-infected individuals, gastrin and CGA showed peaks of expression, and were expressed in the lower part of the surface-to-gland axis, whereas in *H. pylori*-infected patients the peaks of gastrin and CGA expression was shifted more closely towards the surface epithelium. The same was true for the proliferating marker Ki-67. Yet, in *H. pylori*-infected patients the Ki-67-expression was significantly increased compared to non-infected individuals. Increased proliferation with an upward shift of proliferating cells towards the surface of the gastric epithelium during *H. pylori*-infection was also described by Anti et al (Anti et al., 1998).

Taken the expression profiles of MUC5AC, MUC5B, MUC6, Gastrin, CGA, and Ki-67 together, we demonstrated a highly coordinated change of protein expression in the *H. pylori*-infected antrum. Given the shift in the position of the proliferating cells it seems evident that changes in the relative size of the different populations of the predominant cell types underlie these observed changes in gene expression. The possible factors that could induce such shifts in the size of the cell populations are not known, but a clue regarding a possible mechanism comes from the work of Van den Brink et al (Van den Brink et al., 2001). They demonstrated in the gastric corpus that Sonic hedgehog (Shh), which acts as a morphostat, regulates gastric gland morphogenesis. Shh controls the expression of at least 3 other factors important for epithelial differentiation. In mice, inhibition of Shh markedly enhanced gastric epithelial proliferation and changed the gastric pit-gland asymmetry. Suggested is that Shh is a candidate polarizing signal in the maintenance of this pit-gland asymmetry in the adult stomach. In the antrum, a similar morphostat has not been identified: Shh is absent in the antrum (Van den Brink et al., 2001). But when present, the expression of such a morphogen could be changed in *H. pylori*-infected epithelium, and consequently resulted in coordinate redistribution of cell types along the surface-to-gland axis.

IM of the stomach is part of the now widely accepted hypothesis of a multi-step sequence that starts with gastritis and, through atrophy, metaplasia, and dysplasia could lead to gastric cancer (Correa, 1992). The genetic background of IM is not known. Suggested is that IM may be the result of mutations of the genome in gastric epithelial stem cells (Tahara et al., 1994). Of the 102 patients we studied, 25 patients showed IM. We found a statistically significant correlation of IM with gastric atrophy, suggesting that *H. pylori*-infection may only be indirectly responsible for this pre-neoplastic disease. We investigated the expression patterns of the secretory proteins in IM. All goblet cells within IM were positive for MUC2, in agreement with other reports (Ho et al., 1995b; Reis et al., 1999), making it a very reliable marker for IM. Co-expression of MUC2 and TFF3 is typical for intestinal goblet cells (Chang et al., 1994; Podolsky et al., 1993). In our study, TFF3 expression was often found in goblet cells of IM. However, TFF3 expression was also demonstrated in non-goblet cells of IM, as well as in the normal antral epithelium of all individuals. TFF3, unlike MUC2, can therefore not be considered a true "intestinal marker", and is not a reliable marker of IM in the gastric mucosa.

Interestingly, we found that the mucin expression in non-IM epithelium at some distance from the site of IM was changed, compared to mucin expression in patients without IM. MUC5AC expression at distance from IM was diminished, whereas MUC6 and MUC5B

expression were increased. As the presence of IM was not correlated to *H. pylori*-infection or inflammation, this effect seems independent form the *H. pylori*-/inflammation-induced shift in mucin expression. This unprecedented finding suggests the existence of an independent mechanism influencing the mucin expression, which acts at a distance from IM.

In conclusion, we have demonstrated that *H. pylori*-infection of the antrum resulted in a coordinated deregulation of secretory protein expression and proliferation. The changes in expression of the secretory proteins suggest that *H. pylori*-infection is responsible for changes in: 1. The sizes of cell populations within the antral epithelium, and 2. Antral functions that are related to these secretory (glyco-) proteins. The first, in conjunction with the increased epithelial proliferation, is most likely indicative of the changed epithelial dynamics, and may be mediated by the inflammation within the *H. pylori*-infected mucosa. Changes in function can be inferred from the large differences in the numbers of cells exerting those functions. In particular, defensive modalities may be changed. The production of secretory mucins seems altered, which may result in a mucus gel-layer of different composition and properties. As the mucus gel-layer is thinner in *H. pylori*-infected individuals (Newton et al., 1998; Newton et al., 2000), an increased penetration of potentially damaging substances towards the mucosa may be implicated to increase gastritis. Although less salient, changes in TFF-peptide expression may change the capacity for epithelial repair. In particular, the decrease in the number of cells expressing TFF1 may indicate a potential deficit in epithelial response towards the inflamed conditions in *H. pylori*-infected tissue. Most interestingly, we recently found indications that MUC5AC is a major receptor for *H. pylori*, as the bacteria were shown to co-localize in situ with MUC5AC and with MUC5AC-producing cells in the human stomach (Van den Brink et al., 2000b). Thus, *H. pylori* induces a reduction in the number of its main target cells within the epithelium, and thereby possibly reduces one of its main receptors. This could indicate that the reduction in the number of MUC5AC-producing cells is a possible mechanism of the gastric epithelium to try to dispel the bacterium.

Acknowledgements

Dr Gijs van den Brink and dr Kristien Tytgat are gratefully acknowledged for their efforts in collecting the biopsies for this study. This research was financially supported by the Dutch Digestive Diseases Foundation, Irene Foundation, Jan Dekker/Ludgardine Bouman Foundation and Foundation “De Drie Lichten”. The authors thank the providers of the antibodies: dr G. Offner (anti-BGBM), dr C. de Bolós (anti-M6.1), dr A. Giraud (anti-pS2 and anti-hSP) and prof D.K. Podolsky (HM:169 and WE9).

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Chapter 4

Barrett's esophagus is characterized by expression of gastric-type mucins (MUC5AC, MUC6) and TFF peptides (TFF1 and TFF2), but the risk of carcinoma development may be indicated by the intestinal-type mucin, MUC2

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Hum Pathol 2002; 33(6): 660-668

Abstract

Barrett's esophagus (BE) consists of metaplastic epithelium of the esophagus, generally diagnosed by mucin histochemistry. We aimed to determine which mucins were expressed in BE, and to relate their expression to BE pathology. Archival biopsies of 4 patient groups were selected, based on standard histochemistry: BE without inflammation, BE with inflammation, ulcerating BE, and BE with dysplasia. Sections were stained by immunohistochemistry for secretory mucins (MUC2, MUC5AC, MUC5B, and MUC6), the proliferation marker Ki-67, and mucin-associated trefoil factor family (TFF) peptides (TFF1, TFF2, and TFF3). MUC5AC and TFF2 were expressed at similar high levels in each clinical group. Intestinal metaplasia (IM), detected both histochemically and by the intestinal mucin MUC2, was lowest in inflamed BE. The expression of the intestinal-type TFF3 did not differ among the groups. Ulcerating BE was distinguished by very low expression of MUC6 and MUC5B, but very high expression of TFF1. Proliferation was not different among the groups. In the total group of BE patients, *H. pylori*-infection of the stomach correlated with a decreased TFF2 expression in the BE epithelium. We conclude that BE is best characterized by the specific expression of the gastric-type markers, MUC5AC, MUC6, TFF1, and TFF2. Ulcerating BE constitutes the most distinguished group with respect to mucin and TFF expression. Of the intestinal markers, MUC2 is very specific for IM in BE, whereas TFF3 is not a marker for IM. The low occurrence of IM in inflamed BE suggests that these patients may have the lowest risk of developing carcinoma.

Abbreviations: AB, alcian blue; BE, Barrett's esophagus; GERD, gastro-esophageal reflux disease; GM, gastric metaplasia; HID, high iron diamine; IM, Intestinal metaplasia PAS, periodic acid-Schiff; PBS, phosphate-buffered saline; TFF, trefoil factor family.

Introduction

Barrett's esophagus (BE) is a precancerous lesion that is generally thought to progress into adenocarcinoma (Jankowski et al., 1999). Classification of the risk of cancer development is usually determined by mucin histochemistry. Three dyes - alcian blue (AB), high iron-diamine (HID), and periodic acid-Schiff reagent (PAS) - are used to histochemically distinguish the mucins produced. These dyes are specific for carbohydrates and their modifications, but do not reveal the underlying molecular identity of the mucins expressed. Consequently, which mucins were expressed in BE was not known until very recently (Arul et al., 2000; Guillem et al., 2000).

We have investigated the relationship between the occurrence of complete and incomplete intestinal metaplasia (IM) in BE, as assessed by histochemical methods, and the expression of secretory mucins by the BE epithelium. We chose to study the 4 major secretory mucins, clustered on chromosome 11p15: MUC2, MUC5AC, MUC5B, and MUC6. These mucins are produced in large amounts in the normal healthy gastrointestinal tract, where they are responsible for the formation of mucus-gel layers (Van Klinken et al., 1997), and thus it seemed likely that these mucins would be responsible for the mucin-secreting phenotype of the epithelium in early stages of BE. Also, membrane-bound mucins (i.e., MUC1, MUC3, and MUC4) were previously demonstrated in BE epithelium by in situ hybridization (Arul et al., 2000; Guillem et al., 2000). Yet the expression of the latter mucins is in general not related to secretory phenotypes per se. In addition, we have investigated the expression of TFF peptides (formerly called P-domain peptides or trefoil factors), which are expressed in association with the above-mentioned secretory mucins (Podolsky et al., 1993; Sands and Podolsky, 1996). We used specific antipeptide antibodies to detect each of these markers. Thus we were able to study the production of the secretory mucins at the polypeptide level rather than by the confusing heterogeneity of the sugar constituents of the mature mucus glycoproteins.

We have studied 4 groups BE patients: without inflammation, with inflammation, with ulcerations, with dysplasia. These groups were chosen because they seemed to represent a natural sequence of early events in the possible progression of BE towards cancer (Mueller et al., 2000). We sought to determine if our gastric- and intestinal-type markers (i.e., secretory mucins and TFF peptides) could help improve our understanding of this sequence of events. Our data revealed that the morphology and staining characteristics of the mucin-producing cells appeared to be informative in this respect, and that therefore the expression of the underlying mucin and TFF gene products may reveal a much more detailed image of the differences between the diverse clinical manifestations of BE.

Helicobacter pylori infection of the stomach is the major cause of gastritis, gastric ulcers, and ultimately gastric cancer (Dooley et al., 1989; Parsonnet et al., 1991). The role of this bacterium in BE is controversial and not very well understood (Richter et al., 1998). The relation between the BE pathology and persisting *H. pylori*-infection in the stomach has been long debated. The general thoughts are that BE is positively correlated to gastroesophageal reflux disease (GERD), whereas *H. pylori*-infection of the antrum often leads to gastric atrophy and decreased acid output of the stomach. Because *H. pylori*-infection is correlated

with decreased acid reflux to the esophagus, it seems likely that *H. pylori*-infection of the stomach could be beneficial to BE pathology. As part of this retrospective study, we assessed the effects of gastric *H. pylori*-infection on the BE pathology. Half of the patients in each group had gastric *H. pylori*-infection at the time of examination, allowing us to independently determine the effects of gastric infection with *H. pylori* on the expression of the mucins and TFF peptides in BE epithelium.

Materials and Methods

Patients

Tissue samples were retrieved from archives of patients with endoscopic evidence of BE. The biopsy specimens were collected between January 1993 and November 1999 at the University Hospital Leuven, Belgium. BE pathology and *H pylori* status in stomach and gastric pathology (based on at least 2 stomach biopsies) were retrieved from clinical records. Based on hematoxylin and eosin staining of sections of the biopsies specimens, tissue samples of 71 patients were divided into four groups: non-actively inflamed BE (n=20), actively inflamed BE (n=16), ulcerating BE (n=18), and BE with dysplasia (n=17). These 4 groups were further subdivided according to the presence or absence of gastric *H pylori*-infection at the time of endoscopy.

Histochemistry

This retrospective study was performed on Carnoy's fixed, paraffin-embedded tissue. Serial tissue sections were stained in a standard fashion with hematoxylin and eosin, alcian blue/periodic acid Schiff (AB/PAS) at pH 2.5 for neutral and acid mucins, and high-iron diamin/alcian blue (HID/AB) at pH 2.5 to distinguish sialomucins and sulfomucins.

Immunohistochemistry

Polyclonal antibodies raised in rabbits were used: anti-MUC5B (BGBM; a gift from dr G. Offner), anti-MUC6 (6.1; a gift from dr C. de Bolos), anti-TFF1 (pS2; a gift from dr A. Giraud), anti-TFF2 (hSP; a gift from dr A. Giraud), and anti-TFF3 (HM:169; a gift from prof. D.K. Podolsky). Mouse monoclonal antibodies were also used: anti-MUC2 (WE9; a gift from prof. D.K. Podolsky), anti-MUC5AC (45M1; Novocastra, Newcastle, UK), and anti-Ki-67 (MiB-1; Dianova, Hamburg, Germany). Tissue sections were deparaffinized through 3 changes of xylene and then rehydrated through a series of decreasing concentrations of ethanol solution to distilled water. Endogenous peroxidase activity was blocked in 3% (vol./vol.) hydrogen peroxide in phosphate-buffered saline (PBS) for 30 minutes and washed in PBS for 5 minutes. Antigen retrieval was performed by heating the sections for 10 minutes at 100°C in 10 mM citrate buffer, pH 6.0, and then left to cool to room temperature for 20 minutes. Sections were washed 3 times for 5 minutes in PBS and incubated with 1% (wt/vol.) blocking agent (Boehringer, Mannheim, Germany) in PBS for 30 minutes. Primary antibodies were diluted in PBS (Table 1), and were incubated with the tissues for 16 hours at 4°C. Slides were

washed 3 times for 5 minutes in PBS, followed by incubation with biotinylated secondary antibodies and avidin-biotin peroxidase complex (Vectastain ABCkit; Vector laboratories, Burlingame, UK) according to the manufacturer's protocol. Staining was performed using 0.5 mg/ml 3,3'-diaminobenzidine/0.03% (vol./vol.) hydrogen peroxide in imidazole (30 mM)/EDTA (1 mM) pH 7.0. Sections were rinsed in water, dehydrated through a series of increasing concentrations of ethanol solutions, and mounted under cover slips. Control stainings were performed, leaving each of the primary antibodies out of the procedure, resulting in absence of staining.

Semi-quantitative scoring of histological staining

Intestinal metaplasia (IM) was scored according to 'classical' methods (Filipe and Jass, 1986): 0, contains no goblet cells and only gastric-type epithelium; 1, complete IM with AB-staining of goblet cells; 2, incomplete IM with neutral and/or AB staining of intermediate cells; 3, intermediate cells stained with HID. This scoring method was only qualitative and non-linear.

The expression of proteins was scored as follows for MUC2, MUC5AC, MUC5B, MUC6, TFF1, TFF2, and TFF3: 0, staining was absent; 1, staining in 0 to 25% of the BE epithelium; 2, staining in 25% to 50% of the BE epithelium; 3, staining of 50% to 75% of the BE epithelium. Staining of >75% of the epithelium was never observed for a single marker. This scoring system was linear and semi-quantitative.

The staining of Ki-67 was scored as follows: 0, absence of staining; 1, 1 to 10 positive BE epithelium cells per section; 2, 11 to 30 positive BE epithelium cells per section; and 3, >30 positive BE epithelium cells per section. This scoring method was nonlinear, but it was semiquantitative. Staining intensity was not accounted for in any of the methods.

Table 1. Characteristics of antibodies used for immunohistochemical methods.

Name in this study	Name in reference	Epitope	Dilution	Reference
anti-MUC2	WE9	Peptide domain	1:50	(Tytgat et al., 1995)
anti-MUC5AC	45M1	Peptide domain	1:50	(Bara et al., 1998)
anti-MUC5B	BGBM	Deglycosylated polypeptide	1:3000	(Van Klinken et al., 1998)
anti-MUC6	6.1	Peptide in VNTR* domain	1:200	(De Bolos et al., 1995)
anti-TFF1	anti-pS2	C-terminal peptide	1:4000	(Rio et al., 1991)
anti-TFF2	anti-hSP	C-terminal peptide	1:2000	(Srivatsa et al., 2002)
anti-TFF3	HM:169	C-terminal peptide	1:3000	(Podolsky et al., 1993)
anti-Ki-67	MiB-1	Peptide	1:1000	(Gerdes et al., 1984)

*VNTR, variable number of tandem repeats.

Table 2. Patient numbers of the clinical groups (n=71).

H. pylori	Negative	Positive
Non-inflamed BE	10 (7/3)	10 (6/4)
Actively inflamed BE	8 (7/1)	8 (7/1)
BE with ulcer	10 (6/4)	8 (7/1)
BE with dysplasia	8 (6/2)	9 (8/1)

NOTE: Sex ratio is indicated (m/f). *H. pylori* status refers to the presence of the bacterium in antrum biopsy specimens of the patients.

Statistics

The χ^2 test or Fisher's exact test was used to analyze differences in frequencies. Spearman's correlation was used to analyze correlations between parameters. The student *t* test was used to analyze the age distribution of the various clinical groups. Statistical significance was defined at $P < 0.05$ in each test.

Results

Patients

The mean age of the patients was 64.5 years (n=71; range, 22 to 91 years) (Table 2). The mean age of the clinical groups did not differ significantly (*t* test). The age of the individual patients did not correlate significantly with any of the other parameters in this study. As noted in other studies (Cameron and Lomboy, 1992), significantly more male (n=53) patients than female (n=18) patients were identified with BE ($P < 0.0005$). The sex ratio did not differ statistically among groups. Moreover, patient gender did not correlate with any of the other parameters in this study.

Histological characteristics of BE

Normal esophagus epithelium was usually seen in the sections, often continuous with the BE epithelium. None of the mucins (MUC2, 5AC, 5B, or 6) or of the TFF peptides (TFF1, 2, or 3) was expressed in the normal stratified epithelium of the esophagus (not shown). Of these markers, only MUC5B was detected in the submucosal glands of the esophagus (not shown), in agreement with previous findings (Arul et al., 2000). However, in the staining for Ki-67 expression, a discrete layer of cells within the stratified epithelium showed selective and intense staining for this proliferation marker (not shown). This Ki-67 staining of the normal non-BE epithelium served as an internal control for the detection of Ki-67 in the BE epithelium, which was often found to contain only small amounts of Ki-67 positive cells.

The IM, as characterized by the presence of AB positive goblet cells, was usually found at the mucosal surface and was seldom found associated with the deeper glandular epithelium (Fig. 1A, see *Appendix*). HID-positive cells were found everywhere in the BE epithelium, but most often as part of the surface epithelium (Fig. 1B, *Appendix*). Besides goblet cells, non-goblet cells were often HID-positive (Fig. 1B, *Appendix*).

Of the mucins, MUC2 was associated specifically with goblet cells in IM and was found usually at the mucosal surface (Figs 1C and 2A, see *Appendix*). MUC5AC was the most extensively expressed mucin in BE epithelium, localizing to the surface epithelium and extending to a variable degree into the glandular structures (Fig. 2B, *Appendix*). MUC5AC and MUC6 expression overlap slightly, but are generally located in separate regions of the glandular structures; MUC5AC was found in the superficial epithelium, and MUC6 was found in the deeper glands (Fig. 2C, *Appendix*). Like MUC6, MUC5B localized to the deeper glands (Fig. 2D, *Appendix*).

TFF peptide expression was very prominent in BE epithelium. TFF1 was found primarily in the surface epithelium of BE (Fig. 2E, *Appendix*). TFF2 appeared to be the most widely expressed TFF peptide, found in the whole continuum of the surface epithelium and the connecting deeper glandular structures (Fig. 2F, *Appendix*). TFF3 was less extensively expressed than the other TFFs, primarily localized deeper in the glandular structures and absent from the goblet cells of IM (Fig. 2G, *Appendix*).

Proliferation, as determined by staining of Ki-67 (Fig. 2H, *Appendix*), was not localized to particular regions within the BE epithelium. In general, small numbers of cells were stained when compared to other tissues, such as normal stomach epithelium (data not shown). This suggest that the BE epithelium generally does not turn over rapidly.

Metaplasia and the expression of secretory proteins in BE

'Classical' classification of BE, using AB/PAS and HID/AB staining, revealed that 20 of the 71 patients were negative for IM and demonstrated only gastric-type epithelium. Dysplastic BE has a significantly different distribution of IM classes than the other groups (Fig. 3). In particular, the dysplastic BE group has a very low number of IM-negative patients, whereas IM score 3 is overrepresented (Fig. 3). The actively inflamed BE group showed a significantly higher number of IM-negative patients than the other groups. Based on the criterion that presence of IM is closely associated with the progression towards adenocarcinoma (Ho et al., 1995), it seems that inflamed BE is the least-progressed form of BE with respect to cancer development.

In quite a number of patients, MUC2 staining was absent (Fig. 4A). The highest percentage of MUC2-negative patients was found in the group with inflamed BE. In general, the expression of MUC2 was significantly lower in this group than in any other groups. Also the expression of MUC2 was significantly lower in the noninflamed group than in the group with dysplasia (Fig. 4A).

MUC5AC was the most characteristic mucin in BE, because it was the most widely expressed in the epithelium. In 49 of the 71 patients, up to 75% of the BE epithelium expressed MUC5AC (i.e., score 3), and in only 3 patients was MUC5AC absent (Fig. 4B). MUC5AC expression was not significantly different in any of the clinical groups.

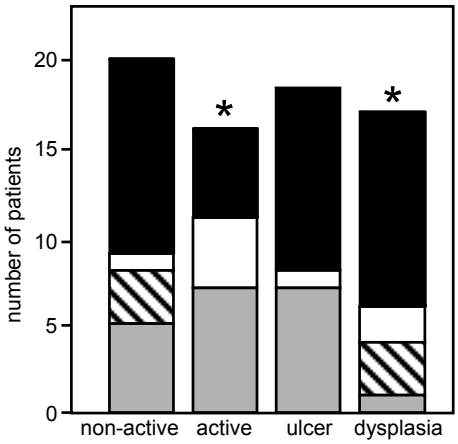

Figure 3. Intestinal metaplasia was scored in the BE epithelium of each clinical group. Intestinal metaplasia (IM) was scored according to the 'classical' methods (Filipe and Jass, 1986): IM0 (gray), contains no goblet cells and only gastric-type epithelium; IM1 (crosshatched), complete IM with AB-staining of goblet cells; IM2 (white), incomplete IM with neutral and/or AB staining of intermediate cells; IM3 (black), intermediate cells stained with HID. This scoring method was qualitative and nonlinear. *Indicates that the frequency of the IM score differed statistically significant from all other groups (P <0.05). Clinical groups as depicted on the x-axis are non-active, not actively inflamed BE; active, actively inflamed BE; ulcer, ulcerating BE; dysplasia, BE with dysplasia.

The extent of MUC5B expression differed most dramatically among the clinical groups. MUC5B expression was very low in the ulcerated BE group compared to the other groups (Fig. 4C). Expression levels were higher in the inflamed group than in the ulcerated group, and this level of expression differed significantly from each other group. MUC5B was most widely expressed in the noninflamed and the dysplasia groups, and the expression levels between these 2 groups did not differ.

MUC6 levels were significantly lower in the ulcerated BE group than in any other group (Fig. 4D). The levels of MUC6 expression among the other 3 groups did not differ significantly.

TFFs were extensively expressed in the BE epithelium. TFF1 expression was significantly higher in the ulcerated BE group than in the other 3 groups (Fig. 4E). The other 3 clinical groups showed less-extensive expression of TFF1, and the levels were not significantly different. TFF2 is the most extensively expressed marker in this study, expressed in all patients with no significant differences in expression levels among the groups (Fig. 4F). TFF3 was the least extensively expressed marker; expression was generally limited and was completely absent in 20 of the 71 patients. The extent of TFF3 expression did not differ significantly among the groups (Fig. 4G). Because both TFF3 and MUC2 are considered intestinal markers (Chang et al., 1994; Podolsky et al., 1993), the correlation between these markers was analyzed. It appeared that the extent of MUC2 and TFF3 expression in individual patients correlated (ρ=0.240, P =0.045; Spearman's test), but from the immunohistochemical data, (shown in Fig. 2), TFF3 was never observed in goblet cells, in contrast to MUC2. In addition, TFF3 expression was most often found in other structures of the BE epithelium than where the MUC2-expressing goblet cells were found. Likewise, TFF3 expression in the individual patients was not significantly correlated to the presence of IM in the BE epithelium.

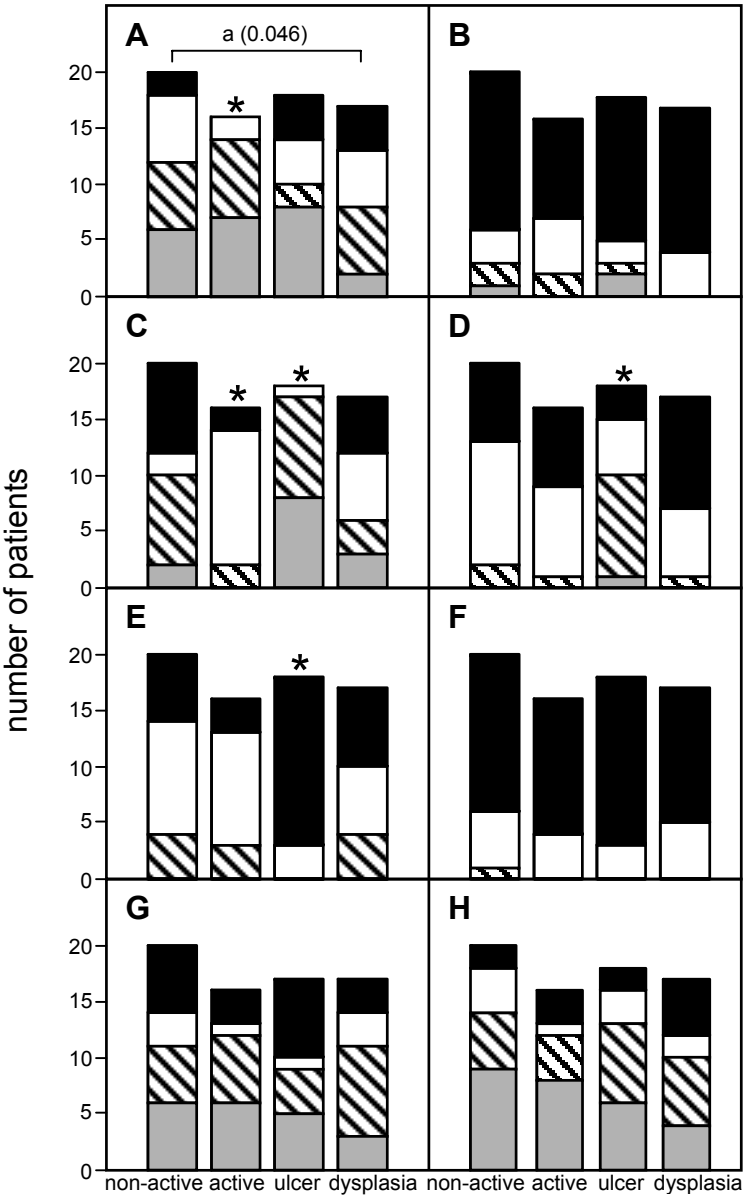

Figure 4. Mucin, TFF-peptide, and Ki-67 expression in BE. The expression of proteins was scored as described in materials and methods. (A) MUC2; (B) MUC5AC; (C) MUC5B; (D) MUC6; (E) TFF1; (F) TFF2; (G) TFF3. Increasing levels of expression are indicated in classes as 0 (gray), 1 (crosshatched), 2 (white), and 3 (black). This scoring system (A through G) was linear and semiquantitative in BE epithelium of the various clinical groups. (H) Proliferative cells were detected for their expression of Ki-67, using the MiB-1 monoclonal antibody. The staining of Ki-67 was scored as follows: 0, absence of staining (gray); 1, 1 to 10 BE epithelium cells positive per section (crosshatched); 2, 11 to 30 BE epithelium cells positive per section (white); 3, >30 BE epithelium cells positive per section (black). This scoring method was nonlinear, but semiquantitative. *Indicates that frequency of the protein score differed statistically significant from each of the other three groups (P <0.05); “a” indicates that one group differed specifically from another (P value in brackets). Clinical groups on the x-axes are as in figure 3.

Table 3. Correlations between the extent of MUC2 and MUC5AC expression and the presence of IM and sulfomucins.

MUC 2 expression	**0**	**1**	**2**	**3**	FET/ Spearman's rho, *P*
IM absent	18	2	0	0	*P* <0.0005/ 0.693, *P* <0.0005
IM present	5	19	17	10	
HID-staining negative	19	3	3	0	*P* <0.0005/ 0.615, *P* <0.0005
HID-staining positive	4	18	14	10	
MUC5AC expression	**0**	**1**	**2**	**3**	
IM absent	1	4	7	8	*P* =0.002/ 0.393, *P* =0.001
IM present	2	1	7	41	
HID-staining negative	1	4	9	11	*P* =0.002/ 0.384, *P* =0.001
HID-staining positive	2	1	5	38	

NOTE: 'IM absent' represents the IM0 score, whereas 'IM present' represents the total of IM1, IM2, and IM3 scores. Likewise, the presence of sulfomucins, as detected through the HID-staining, was scored either present or absent. Statistical analyses were performed by Fisher's exact test (FET) and Spearman's correlation analysis. The extent of expression of MUC5B and MUC6 did not correlate with the presence of either IM or sulfomucins in the BE epithelium, and are therefore not included in this table.

Correlations between mucin expression and the presence of IM and sulfomucins

Because the classical assessment of the stage of IM is based on histochemical staining of secretory mucins, it is essential to determine whether this can be related to the expression of the 4 secretory mucins in the present study. The correlations are summarized in Table 3. The occurrence of IM and sulfomucins were scored as either absent or present. MUC2 expression was significantly correlated to the presence of IM in the tissue (Table 3). The presence of sulfomucins is generally seen as an "advanced" stage in progression toward adenocarcinoma (Jass, 1981; Torrado et al., 1997). HID-staining of the BE epithelium was also significantly correlated with the extent of the MUC2 expression (Table 3). Yet MUC2 was confined to goblet cells in the BE epithelium (Figs 1 and 2) and was not expressed in most of the cells scored as HID positive in the classical IM stage assessment (Fig. 1). The cells characterized as sulfomucin-producing most likely produced MUC5AC, as is evident from Figures 1 and 2. Indeed, the extent of MUC5AC expression was positively correlated both with the presence of IM and with positive HID staining of the epithelium (Table 3). Taken together, these findings indicate that the expression of sulfated structures on MUC5AC was significantly correlated to the presence of MUC2-expressing goblet cells.

Proliferation in BE epithelium

BE is generally considered a precancerous lesion. Therefore, proliferation seems a logical parameter to assess to determine which stage of BE is more advanced in possible cancer progression. We detected Ki-67 as a measure of proliferation in the various clinical groups (Fig. 4H). We were not able to find any statistically significant differences in the extent of Ki-67 expression among the clinical groups (Fig. 4H). This suggested that proliferation and cell turnover are not different among these groups. Because proliferation was generally low in BE

compared to in adjacent native stratified esophageal epithelium (not shown), the turnover of the BE epithelium seems relatively low.

Relations of BE and expression of immunohistochemical markers in BE with *H. pylori*-infection of the stomach

Atrophic gastritis was closely correlated with the presence of *H. pylori* in the stomach; 11 of the 13 patients with atrophic gastritis were infected with *H. pylori* (χ^2 test, P =0.006, Spearman's test, ρ=0.334). We analyzed the presence of *H. pylori* in the stomach and the occurrence of gastric atrophy in relation to the (immuno-) histochemical parameters for all patients (n=71). *H. pylori*-infection and gastric atrophy were not correlated significantly with the extent of expression of MUC2, MUC5AC, MUC5B, MUC6, TFF1, TFF3, or Ki-67 (not shown). Moreover, no correlations were identified between *H. pylori*-infection of the stomach and the presence of IM or the identification of sulfomucins in the BE epithelium. Yet a highly significant negative correlation was found between the extent of TFF2 expression in the BE epithelium and *H. pylori*-infection of the stomach (Table 4). Also, the occurrence of gastric atrophy correlated negatively and significantly with the TFF2 expression (see Table 4).

Table 4. Effect of *H. pylori*-infection in the stomach and atrophic gastritis on epithelial TFF2 expression in BE tissue (all patients, n=71).

TFF2 expression	**0**	**1**	**2**	**3**	FET/Spearman's rho, *P*
H. pylori-infected	0	1	14	20	*P* =0.001/ -0.399, *P* =0.001
Non-*H. pylori*-infected	0	0	3	33	
Atrophic gastritis	0	1	5	7	*P* <0.05/ -0.240, *P* =0.046
No atrophy	0	0	12	46	

NOTE: Statistical analyses were performed by Fisher's exact test (FET) and Spearman's correlation analysis.

Discussion

Our study was the first to investigate 7 protein markers for gastrointestinal secretory phenotypes against a well-defined clinical background representing the early, premalignant stages of BE. When compared to other regions of the gastrointestinal tract, the BE epithelium showed a MUC-type mucin and TFF expression resembling normal human stomach epithelium, in which the expression of these MUCs and TFFs has been demonstrated previously (De Bolos et al., 1995; Hanby et al., 1993; Ho et al., 1995; Reis et al., 1999; Rio et al., 1988; Taupin et al., 2001; Van den Brink et al., 2000; Van Klinken et al., 1997). In BE, MUC5AC expression was characteristically localized to the surface epithelium. MUC6 expression was found in the deeper glandular structures and usually overlapped only slightly with MUC5AC. MUC5B expression was localized to the deeper glands and often co-localized with MUC6. TFF1 and TFF2 expression was found in the surface epithelium, whereas TFF2 expression extended into the deeper glandular structures. TFF3 expression was much less extensive than for the other TFF peptides and was localized primarily in the deeper glandular

structures. Our findings regarding the expression of MUC2, MUC5AC, MUC6, TFF1, and TFF2 in BE have been corroborated by work of others (Hanby et al., 1994; Labouvie et al., 1999; Podolsky et al., 1993). However, Arul and co-workers were unable to demonstrate immunohistochemically MUC5B expression in BE epithelium (Arul et al., 2000). This discrepancy is probably the result of the use of different antibodies against MUC5B. We have demonstrated earlier that the anti-MUC5B antibody used in this and our previous studies (Van Klinken et al., 1998) specifically recognizes human MUC5B in several different tissues both in immunohistochemical and biochemical techniques.

Patches of IM within BE were characterized by expression of MUC2 within goblet cells, which is very characteristic for normal intestinal epithelium and for the goblet cells within IM of the stomach (Chang et al., 1994; Reis et al., 1999; Van Klinken et al., 1997; Van Klinken et al., 1998). The other MUCs and the TFF peptides that were studied were also expressed in IM, but were not characteristic for IM. In the normal human intestine, TFF3 is most often co-expressed with MUC2 (Podolsky et al., 1993; Wong et al., 1999), but to the best of our knowledge TFF3 was not previously detected in BE. In contrast to the intestinal situation, however, no TFF3 expression was shown in goblet cells of the IM, and TFF3 was not associated specifically with IM. MUC2 expression was significantly lower in patients with inflamed BE and significantly higher in those with dysplastic BE. Because MUC2 is an exclusive marker for IM, MUC2 expression in BE is associated with an increased risk of progression to malignancy (Mueller et al., 2000; Sampliner, 1998). This suggests that patients in these groups have the lowest and highest risk, respectively, of developing carcinoma.

The most distinguished clinical group is ulcerating BE with significantly lower MUC5B and MUC6 expression but significantly higher TFF1 expression. The decreased expression of these protective mucus-forming mucins may be related to the increased vulnerability of the BE epithelium towards ulceration. TFF peptide production is often up-regulated at sites of gastrointestinal damage in conditions as ulceration (Playford, 1995; Wong et al., 1999). Because TFFs in general play a role in gastrointestinal mucosal defense and repair (Wong et al., 1999), the up-regulation of TFF1 could signify the continuous urge to repair the BE epithelium with ulcers.

It has been demonstrated that in dysplasia and adenocarcinoma of the esophagus, the apoptotic labeling index was decreased, and the Ki-67 labeling index is increased, indicating increased cell turnover relative to BE epithelium (Halm et al., 2000; Hong et al., 1995). In general, Ki-67 expression was lower in BE than in normal stratified esophagus epithelium. Thus the turnover of epithelial cells in BE in each of the clinical groups is most likely low. We found no statistically significant difference in Ki-67 expression among the clinical groups in this study.

BE is often thought to arise from reflux of the acidic stomach contents into to esophagus, as in GERD (Blaser, 1992). *H. pylori*-infection in the stomach is related to gastric atrophy, which leads to decreased acidic output of the stomach, which is potentially protective against the progression of BE toward adenocarcinoma (Blaser, 1992). In our study we sought to determine whether the presence of *H. pylori* or gastric atrophy in the stomach effected the

expression of the MUCs and TFF peptides in BE epithelium. We found a negative and statistically significant correlation between *H. pylori*-infection and gastric atrophy with the extent of TFF2 expression in BE. This decrease in TFF2 expression may be due to the fact that the gastric juice in the reflux of *H. pylori*-infected patients is less acidic, which may have a direct effect on the TFF2 gene expression. However, the decrease of TFF2 expression in BE epithelium could also be the result of a non-*H. pylori*-related biological mechanism.

In conclusion, BE is best characterized by expression of the gastric-type mucins MUC5AC and MUC6 and gastric-type TFF peptides TFF1 and TFF2, whereas MUC2 was specifically expressed only in IM. The low occurrence of IM and MUC2 in inflamed BE suggests that these patients may have the lowest risk of developing adenocarcinoma. With respect to expression of secretory mucus-forming mucins and TFFs, the group with ulcerating BE was the most distinguished patient group. The present study indicates that MUC2, 5AC, 5B, and 6 and TFF1, 2, and 3 gene expression patterns are reliable markers for metaplastic BE in the preneoplastic progression to adenocarcinoma of the esophagus.

Acknowledgement

The authors acknowledge the pilot work on BE done by drs Jan-Willem Van Klinken and Jos Boshuizen, which guided our thoughts in the setup of this study. This research was supported by grants from the Dutch Digestive Diseases Foundation, Irene Foundation, Jan Dekker/Ludgardine Bouman Foundation, and Foundation “De Drie Lichten”. They thank their international colleagues who provided antibodies: dr G. Offner (BGBM), dr C. de Bolós (anti-M6.1), dr A. Giraud (pS2 and hSP) and prof. D.K. Podolsky (HM:169 and WE9).

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Chapter 5

Gastric-type mucin and TFF-peptide expression in Barrett's esophagus is disturbed during increased expression of MUC2

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Abstract

Aim: Barrett's esophagus constitutes metaplastic epithelium of the esophagus, often diagnosed by mucin histochemistry. We determined the mucins and trefoil factor family (TFF)-peptides that were expressed in Barrett's esophagus, in order to study changes in protein expression in early stages of Barrett's esophagus development. Methods: Biopsy specimens of 71 Barrett's esophagus patients were collected, and sections were stained for secretory mucins by histochemistry. Immunohistochemistry was performed for secretory mucins (MUC2, MUC5AC, MUC5B, MUC6), TFFs (TFF1, TFF2, TFF3), and proliferation (Ki-67). Protein expression in the tissue was measured semi-quantitatively. Results: MUC5AC and TFF1 showed high levels and strong co-localization in the surface epithelium, whereas MUC6, MUC5B and TFF3 were found in the deeper glandular structures. TFF2 was found in both surface and glandular epithelium. The coordinate expression patterns of these six markers were similar to gastric antrum epithelium. MUC2 expression was ubiquitously associated with goblet cells within intestinal metaplasia, occurring in 68% of patients, and was correlated to increasing proliferation in the epithelium. Conclusions: Virtually all cells in Barrett's esophagus epithelium displayed a secretory phenotype, demonstrating a coordinate gastric-type MUC and TFF expression. When MUC2 expression was more pronounced, the expression patterns of the other MUCs and the TFFs was increasingly disturbed. MUC2 expression may constitute a marker for an early change in the phenotype of Barrett's esophagus as a pre-cancerous lesion.

Abbreviations: AB, alcian blue; PAS, periodic acid-Schiff; TFF, trefoil factor family peptide.

Introduction

Barrett's esophagus is generally considered to constitute a precancerous lesion, with a high risk of progressing into adenocarcinoma of the esophagus, via the metaplasia-dysplasia-adenocarcinoma sequence (Jankowski et al., 1999). Classification of the risk of cancer development is classically determined based on mucin histochemistry. Three staining methods (alcian blue, periodic acid-Schiff reagent, and high iron diamine) are used to distinguish the types of mucins produced. These dyes are specific for carbohydrates and their modifications, but do not reveal the underlying mucin genes that are expressed. As a consequence, it was not until very recently that some light was shed on the expression of mucins in Barrett's esophagus (Arul et al., 2000; Guillem et al., 2000).

We have investigated the relation between the occurrence of gastric-type and intestinal-type metaplasia in Barrett's esophagus, as assessed by histochemical methods, and the expression of secretory mucins by the Barrett's esophagus epithelium. We chose to study the four major secretory mucins, clustered on chromosome 11p15, MUC2, MUC5AC, MUC5B, and MUC6. These mucins are produced in large amounts in the normal healthy gastrointestinal tract where they are responsible for the formation of mucus-gel layers (Van Klinken et al., 1997), and therefore is seemed likely that these mucins would be responsible for the mucin-secreting phenotype of the epithelium in early stages of Barrett's esophagus. Also membrane-bound mucins were previously demonstrated in Barrett's esophagus epithelium by in-situ hybridization, i.e. MUC1, MUC3 and MUC4 (Arul et al., 2000; Guillem et al., 2000). Yet the expression of the latter mucins is in general not related to secretory phenotypes *per se*. In addition, we have investigated the expression of trefoil factor family (TFF)-peptides (formerly P-domain peptides or trefoil factors), which are expressed in association with the above mentioned secretory mucins (Podolsky et al., 1993; Sands and Podolsky, 1996). We used specific anti-peptide antibodies to detect each of these markers. Thus, we were able to study the production of the secretory mucins at the polypeptide level rather than by the confusing heterogeneity of the sugar constituents of the mature mucus glycoproteins.

Intestinal metaplasia is often seen as a more advanced stage of metaplasia within the esophagus and its occurrence is described to correlate to adenocarcinoma development (Jankowski et al., 1999). Yet it remains unclear how the development of intestinal metaplasia progresses and how this development relates to other early changes within the Barrett's esophagus epithelium. Besides the biomarkers we used in our study, other biomarkers of Barrett's esophagus metaplasia have been studied. Increased expression was demonstrated within the intestinal type metaplasia of Barrett's esophagus for the following markers: proliferating cell nuclear antigen, epidermal growth factor receptor, transforming growth factor β, and sucrase-isomaltase. However, the level of expression of these markers is also elevated in dysplastic lesions or esophageal adenocarcinoma (reviewed by Krishnadath et al., 2001). Therefore, these are not suitable as markers for changes in gene expression during early stages of Barrett's esophagus metaplasia.

Barrett's esophagus is thought to arise from the normal multi-layered esophagus epithelium, and to appear first as gastric-type metaplasia. Then, from the gastric-type epithelium,

epithelium seems to arise that is described as intestinal metaplasia. We studied the expression of mucins and TFF-peptides during this hypothetical sequence, first, to closely study the sequence of events during these changes in the phenotype of the Barrett's esophagus epithelium, and second, to identify markers to fine-tune the diagnostic possibilities for the early stages of disease.

Materials and Methods

Patients and tissue

Tissue samples of Barrett's esophagus epithelium were retrieved from the archive of the Gastrointestinal Pathology Unit, Catholic University Leuven, Belgium (n=71). Barrett's esophagus tissue was carefully selected based on both endoscopic and pathological records. A broad selection of Barrett's esophagus pathology was made, including Barrett's esophagus with and without inflammation, ulcerating Barrett's esophagus, and Barrett's esophagus with occasional patches of hyperplastic epithelium. Four to nine endoscopic biopsies were taken per patient, and none of the patients showed abnormalities to the esophagus other than the columnar Barrett's esophagus epithelium, replacing the normal stratified esophageal epithelium. The tissue was fixed routinely in Carnoy's fixative and paraffin embedded.

Histochemistry

Serial tissue sections (5 μm) were stained in a standard fashion with hematoxylin and eosin, or with alcian blue/periodic acid Schiff's reagent (AB/PAS) at pH 2.5 for neutral and acid mucins, or by high iron diamine/alcian blue at pH 2.5 to distinguish sialomucins and sulfomucins.

Antibodies

The following polyclonal antibodies raised in rabbits were used: anti-MUC5B (anti-BGBM (Van Klinken et al., 1998), kindly provided by Dr. G. Offner), anti-MUC6 (M6.1 (De Bolos et al., 1995), kindly provided by Dr. C. de Bolos), anti-TFF1 (anti-pS2 (Rio et al., 1991), kindly provided by Dr. A. Giraud), anti-TFF2 (anti-hSP (Srivatsa et al., 2002), kindly provided by Dr. A. Giraud) and anti-TFF3 (HM:169 (Podolsky et al., 1993), kindly provided by Prof D.K. Podolsky). Also mouse monoclonal antibodies were used: anti-MUC2 (WE9 (Tytgat et al., 1995), kindly provided by Prof D.K. Podolsky), anti-MUC5AC (45M1 (Bara et al., 1998), Novocastra, Newcastle, UK) and anti-Ki-67 (MIB-1 (Gerdes et al., 1984), Dianova, Hamburg, Germany). All antibodies were directed against peptide epitopes of the respective (glyco-) proteins.

Immunohistochemistry

Tissue sections were deparaffinized through three changes of xylene and then re-hydrated through a series of decreasing concentrations of ethanol solution to distilled water. Endogenous peroxidase activity was blocked in 3% (vol./vol.) hydrogen peroxide in phosphate buffered saline (PBS) for 30 min and washed in PBS for 5 min. Antigen retrieval was performed by heating the sections for 10 min at 100°C in 10 mM citrate buffer, pH 6.0, and then left to cool to

room temperature for 20 min. Sections were washed three times for 5 min in PBS and incubated with 1% (wt/vol.) blocking agent (Boehringer, Mannheim, Germany) in PBS for 30 min. Primary antibodies diluted in PBS were incubated with the tissues for 16 h at 4°C. Slides were washed three times for 5 min in PBS, followed by incubation with biotinylated secondary antibodies, and avidin-biotin peroxidase complex (Vectastain ABCkit, Vector lab., Burlingame, UK) according to the manufacturers protocol. Staining was performed using 0.5 mg/ml 3,3'-diaminobenzidine/ 0.03% (vol./vol.) hydrogen peroxide in imidazole (30 mM)/EDTA (1 mM) pH 7.0. Sections were rinsed in water, dehydrated through a series of increasing concentrations of ethanol solutions, and mounted under cover slips. Control staining was performed leaving out the primary antibodies. Co-localization was assessed using standard diaminobenzidine-immunohistochemistry on serial sections.

Semi-quantitative scoring of histological staining

Intestinal metaplasia was determined 'classically' by AB/PAS staining (Filipe and Jass, 1986). The presence of AB/PAS-positive goblet cells was taken as evidence for the presence of intestinal metaplasia. The expression of proteins was scored as follows for MUC2, MUC5AC, MUC5B, MUC6, TFF1, TFF2, and TFF3: 0, staining was absent; 1, staining in 0-25% of the Barrett's esophagus epithelium; 2, staining in 25-50% of the Barrett's esophagus epithelium; 3, staining of 50-75% of the Barrett's esophagus epithelium. Staining >75% of the epithelium was never observed for a single marker. This scoring system is both linear and semiquantitative. The staining of Ki-67 was scored as follows: 0, absence of staining; 1, 1-10 Barrett's esophagus epithelial cells positive per section; 2, 11-30 Barrett's esophagus epithelial cells positive per section; 3, >30 Barrett's esophagus epithelial cells positive per section. This scoring method is nonlinear, but it is semiquantitative. Note that the staining intensity is not accounted for in these methods.

Co-localization was scored semiquantitatively, as follows: The area of epithelial structures expressing a particular protein within the Barrett's esophagus epithelium was set at 100%. Then, the area expressing the co-localizing protein was determined within the area of expression of this first protein. Score: 0, no co-localization; 1, co-localization in 0-25% of the structures; 2, co-localization in 25-50% of the structures; 3, co-localization in 50-75% of the structures; 4, 100% co-localization.

Statistics

The χ^2 test or Fisher's exact test was used to analyze differences in frequencies. Spearman's correlation test was used to analyze correlations between parameters. Statistical significance was defined at $P < 0.05$.

Results

Patients

The mean age and gender of the Barrett's esophagus patients was as expected from earlier studies (Cameron and Lomboy, 1992). The mean age of the patients was 64.5 years (n=71, range 22-91 years). The age of the individual patients did not correlate significantly with any of the parameters as measured in this study. More male (n=53) patients were identified than female (n=18) patients. The gender of the patients did also not correlate with any of the other parameters in this study.

AB/PAS staining was routinely performed to distinguish gastric-type metaplasia from intestinal-type metaplasia within the Barrett's esophagus epithelium. The gastric metaplasia epithelium of Barrett's esophagus was characteristically stained intensely by PAS, in sharp contrast to normal stratified epithelium of the esophagus, which was PAS-negative (Fig. 1, see *Appendix*). The intestinal-type metaplasia within Barrett's esophagus, as characterized by the presence of AB/PAS-positive goblet cells (Fig. 1, *Appendix*), was usually observed embedded within gastric-type metaplasia epithelium. Intestinal metaplasia was typically found continuous with the mucosal surface, and was more rarely found to extend into the deeper glandular epithelium. Intestinal metaplasia was observed in 68% of the patients (48/71), always in combination with gastric metaplasia, which was found in all patients (71/71).

Table 1. Extent of expression of secretory proteins in Barrett's esophagus.

Marker	Mean expression (SEM)*	n
TFF2	2.73 (0.06)^{A}	71
MUC5AC	2.54 (0.10)^{A}	68
TFF1	2.28 (0.09)^{B}	71
MUC6	2.17 (0.09)^{B}	70
MUC5B	1.54 (0.12)^{C}	58
TFF3	1.37 (0.14)^{C}	50
MUC2	1.20 (0.12)^{C}	48

NOTE: The expression of each protein was measured immunohistochemically in the Barrett's esophagus tissue, and then the scores were averaged per marker (n=71). n, The number of patients where expression was found. *Identical symbols indicate values that differ not statistically; values with different symbols were significantly different, P <0.05.

Mucin and TFF-peptide expression in Barrett's esophagus

Normal stratified esophagus epithelium was usually seen in sections of the biopsies, often continuous with the Barrett's esophagus epithelium. None of the mucins (MUC2, -5AC, -5B, or -6) or TFF-peptides (TFF1, -2, or -3) was expressed in the normal stratified epithelium of the esophagus (not shown). Of these markers, MUC5B was detected in the submucosal glands of the esophagus (not shown).

As expected from the intense PAS-staining of Barrett's esophagus epithelium most cells appeared to produce abundant secretory mucins. MUC2 was uniquely associated with

goblet cells in intestinal metaplasia (Fig. 2A, see *Appendix*), and was found usually at the mucosal surface and less often deeper into the mucosa. MUC5AC was extensively expressed the surface epithelium of Barrett's esophagus, extending to variable degrees into the deeper parts of glandular structures (Fig. 2B, *Appendix*). MUC6 was abundantly expressed in the deeper glandular structures (Fig. 2C, *Appendix*). Like MUC6, MUC5B localized to the deeper glands (Fig. 2D, *Appendix*), but was expressed in much less epithelial cells than MUC6.

TFF-peptide expression was also very widespread in Barrett's esophagus epithelium. TFF1 was found primarily in the surface epithelium of Barrett's esophagus (Fig. 2E, *Appendix*). TFF2 appeared to be the most widely expressed TFF and was often found in the whole continuum of the surface epithelium and the connecting deeper glandular structures (Fig. 2F, *Appendix*). TFF3 was frequently detected in virtually all Barrett's esophagus epithelial cells (as in Fig. 2G, *Appendix*), but was also recurrently found only in the glandular structures.

Following immunohistochemistry, we determined semi-quantitatively the degree of expression of these secretory proteins within the Barrett's esophagus epithelium (Table 1). A characteristic combination of very extensive expression of MUC5AC, MUC6, TFF1, and TFF2 expression was found in almost all patients. Of these, MUC5AC and TFF2 were most extensively expressed within the Barrett's esophagus epithelium, closely followed by TFF1 and MUC6. MUC5B, TFF3, and MUC2 expression were less widespread within Barrett's esophagus epithelium, and were also not found in all patients (Table 1). The expression patterns of MUC5AC, MUC5B, MUC6, TFF1, TFF2, and TFF3 in Barrett's esophagus were very similar to the expression patterns as detected in the glands of the normal human antrum (not shown).

Based on the semi-quantitative measurements of the expression of each MUC and TFF, we analyzed the correlations between the respective expression levels within Barrett's esophagus epithelium (Fig. 3). The extent of MUC2 expression correlates positively with the extent of expression of both MUC5AC and TFF3 (Figs 3A and B). The expression levels of MUC5B and MUC6 were positively correlated (Fig. 3C). The extent of expression of TFF1 correlates negatively with both MUC5B and MUC6, but positively with TFF3 (Figs 3D-F). TFF2 and TFF3 expression levels correlated positively in Barrett's esophagus epithelium (Fig. 3G).

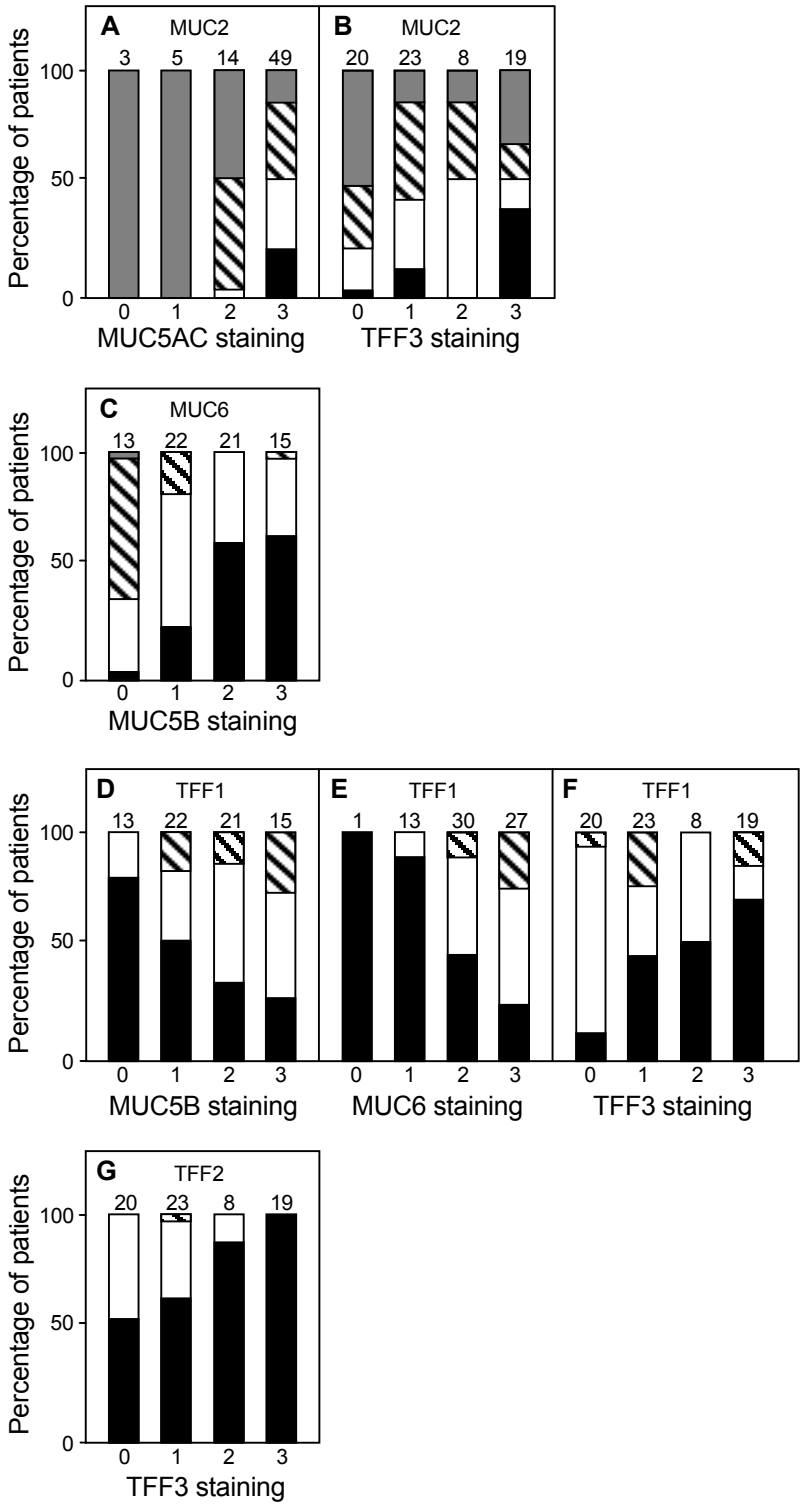
Co-localization of MUC and TFF expression

Co-localization of the MUCs was assessed semi-quantitatively by immunohistochemistry of the individual proteins on adjacent tissue sections (Table 2). MUC2 was very strongly co-localized with MUC5AC, i.e. nearly all structures that contained MUC2-positive goblet cells also contained MUC5AC-positive cells. Some co-localization of MUC2 with MUC5B and MUC6 was observed, but the extent of overlapping expression was much less than for MUC5AC (Table 2). In contrast, MUC5AC did not show very strong co-localization to any other MUC. Strongest co-localization of MUC5AC was found with MUC2, but co-localization with MUC5B and MUC6 was low (Table 2). MUC5AC was found to co-localize with MUC2 within goblet cells in most (35/48) patients. The extent of MUC5B expression was relatively low (Table 1), yet most of the MUC5B was expressed in epithelial structures that also expressed MUC6. Co-localization of MUC5B with MUC2 or MUC5AC was low. Like MUC5AC, MUC6 was not strongly

localized to any other MUC, however it overlapped equally with MUC5AC as with MUC5B expression (Table 2). From the combined data of figures 2 and 3 and table 2 it appears that (i) MUC5AC and MUC2 were most often found in similar structures at the surface of the epithelium, and showed coordinated expression levels, and (ii) the glandular structures of Barrett's esophagus expressed both MUC6 and MUC5B, and also these MUCs show coordinated expression levels.

TFF1 co-localized with MUC5AC in all patients (66/66), but hardly ever with MUC6 (4/69), whereas TFF1 co-localized rarely with MUC2-positive structures (13/49). In contrast, TFF2 was found to co-localize in most patients with MUC5AC (60/66) as well as with MUC6 (64/69). TFF2 was however very rarely found in MUC2-positive structures (4/49). TFF3 was expressed in only 70% of the patients (Table 1), but when it was found it co-localized usually with MUC6 (37/49), and less frequently with MUC5AC (20/47). TFF3 was seldom found in MUC2-positive structures (14/40). In summary, TFF1 was primarily found in MUC5AC-positive epithelium, TFF2 was found in association with both MUC5AC as well as MUC6-positive epithelium, and TFF3 was primarily found to co-localize with MUC6.

Figure 3. (right page) Correlations between the expression levels of the individual MUCs and TFFs in Barrett's esophagus epithelium. The extent of expression of each of the secretory proteins, MUC2, MUC5AC, MUC5B, MUC6, TFF1, TFF2, and TFF3, was determined by immunohistochemistry, and was scored in four categories. Increasing extent of expression was indicated from 0 to 3 (see Materials and Methods), and is indicated along the X-axis. The correlations between the individual proteins were determined using Spearman's test. Only statistically significant correlations were depicted: Other correlations appeared not significant. Above each bar the number of patients within each category is given. MUC2 staining is indicated (A, B): gray bars, staining was absent; crosshatched bars, staining in 0-25% of the Barrett's esophagus epithelium; white bars, staining in 25-50% of the Barrett's esophagus epithelium; black bars, staining of 50-75% of the Barrett's esophagus epithelium. The extent of MUC2 expression was positively correlated to both the level of MUC5AC (A) and TFF3 (B) expression, $\rho = 0.570$ ($P < 0.0005$) and 0.240 ($P < 0.05$), respectively. The extent of MUC6 expression is indicated by the differently colored bars (C) as specified above for MUC2. The extent of MUC6 expression was positively correlated to the extent of MUC5B expression, $\rho = 0.537$ ($P < 0.0005$). TFF1 staining is indicated by the differently colored bars (D-F) as specified above for MUC2. The extent of TFF1 expression was negatively correlated to both the extent of MUC5B (D) and MUC6 (E) expression, $\rho = -0.350$ and -0.415, respectively (both $P < 0.005$), but correlated positively to TFF3 expression (F, $\rho = 0.282$, $P < 0.05$). TFF2 staining is indicated by the differently colored bars (G) as specified above for MUC2. The extent of TFF2 expression was positively correlated to the extent of TFF3 expression, $\rho = 0.401$ ($P < 0.005$).


A
MUC2
3 5 14 49
B
MUC2
20 23 8 19
Percentage of patients
100
50
0
0 1 2 3
MUC5AC staining
0 1 2 3
TFF3 staining
C
MUC6
13 22 21 15
Percentage of patients
100
50
0
0 1 2 3
MUC5B staining
D
TFF1
13 22 21 15
E
TFF1
1 13 30 27
F
TFF1
20 23 8 19
Percentage of patients
100
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0
0 1 2 3
MUC5B staining
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TFF2
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Percentage of patients
100
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TFF3 staining

Proliferation and the occurrence of intestinal metaplasia in relation to expression of MUCs and TFFs

Increased proliferation and the occurrence of intestinal metaplasia are expected to be hallmarks of tumor development within Barrett's esophagus epithelium (Jankowski et al., 2000). Therefore, we compared proliferation and intestinal metaplasia in Barrett's esophagus epithelium with the expression patterns of the MUCs and TFFs. Within the stratified epithelium of the normal esophagus, staining for Ki-67 expression displayed a discrete layer of proliferating cells (Fig. 2H). This Ki-67 staining of the normal epithelium served as an internal control for detection of Ki-67 in the Barrett's esophagus epithelium, which was often found to contain only very small numbers of positive cells (Fig. 2H). This indicated that Barrett's esophagus epithelium in general does not proliferate rapidly. The proliferating cells were not specifically localized to particular regions within the Barrett's esophagus epithelium. The level of proliferation in the Barrett's esophagus epithelium was positively correlated with the presence of intestinal metaplasia (as scored by the AB/PAS staining, Fig. 4A), and also with the expression levels of MUC2 and TFF3 (Figs 4B and C). The occurrence of intestinal metaplasia further correlated positively with more extensive expression of MUC2, MUC5AC, and TFF2 (Figs 4D-F).

There were some striking correlations between the extent of proliferation, as measured by Ki-67 expression, and the co-expression of epithelial markers in Barrett's esophagus. Normally, the extent of co-localization between MUC5AC and MUC5B and between MUC5AC and MUC6, respectively, was low (Table 2). Yet, the extent of Ki-67 expression was positively correlated to increased overlap in the expression of MUC5AC with MUC5B (ρ=0.266, P =0.041), and to overlapping expression of MUC5AC with MUC6 (ρ=0.274, P =0.025). Whereas expression of TFF3 in MUC2-expressing structures was only found in 14/40 patients, the extent of overlap was positively correlated to increased Ki-67 expression (ρ=0.522, P =0.001). In contrast, the extent of overlap between markers that showed very strong overall co-localization was in general negatively effected by high proliferation in the Barrett's esophagus epithelium. In particular, Ki-67 expression was negatively correlated with the co-localization of MUC6 and MUC5B (ρ= - 0.360, P =0.004), and of TFF3 and MUC6 (ρ= - 0.369, P =0.009). Thus, it seems that the "organization" within the Barrett's esophagus epithelium was progressively lost when the degree of proliferation increased.

The notion that the "organization" within the Barrett's esophagus epithelium was progressively lost with increasing proliferation, increasing extent of intestinal metaplasia, and MUC2 expression, was further corroborated by the quantitation of co-localization of MUC2 and the other MUCs. The extent of expression of MUC2 was highly variable, yet closely correlated to the proliferation as well as to the extent of intestinal metaplasia in the Barrett's esophagus epithelium (Figs 4B and D). There were also significantly increased overlaps of MUC2 expression in the epithelium with each of the other MUCs with increasing MUC2 expression. The overlap of MUC2 expression with MUC5AC expression increased considerably (ρ=0.821, P <0.0005), followed by an increasing overlap with MUC6 (ρ=0.628, P <0.0005), but also the overlap with MUC5B increased significantly (ρ=0.355, P =0.02). It seems that MUC2

expression "grows" quite independently "into" all regions of the Barrett's esophagus epithelium, and thereby increasing overlaps with expression each of the other MUCs.

As noted, the extent of TFF3 expression was positively correlated with increasing proliferation in the Barrett's esophagus epithelium (Fig. 4C). TFF3 was most commonly co-localized with MUC6-expressing epithelium. However, when increasing TFF3 expression was considered, as occurred with increased proliferation, TFF3 expression increasingly overlapped with MUC5AC ($\rho = 0.569$, $P < 0.0005$).

Table 2. The extent of co-expression of MUCs within Barrett's esophagus epithelium.

In epithelium expressing:	There was co-localization with:			
	MUC2 (SEM)	MUC5AC (SEM)	MUC5B (SEM)	MUC6 (SEM)
MUC2	---	3.79 (0.13)	1.12 (0.23)	1.67 (0.19)
MUC5AC	1.55 (0.18)	---	0.38 (0.12)	1.12 (0.10)
MUC5B	0.86 (0.21)	1.22 (0.20)	---	3.58 (0.13)
MUC6	0.96 (0.18)	1.43 (0.13)	1.56 (0.16)	---

NOTE: The area of epithelial structures expressing a particular MUC was set at 100% (listed in the vertical column). Then the area expressing the co-localizing MUC (in the horizontal row) was estimated within the area of expression of this first MUC. Increasing co-localization was scored as 0 to 4, as explained in methods. Co-expression was only scored if the respective mucins were expressed in the Barrett's esophagus tissue of a patient. Thus, the numbers reflect mean degree of co-expression in those Barrett's esophagus tissues where both MUCs were expressed within Barrett's esophagus epithelium. All values were significantly different at $P < 0.05$, apart for the co-localization of MUC6 with MUC5AC and of MUC6 with MUC5B, which differed not significantly.

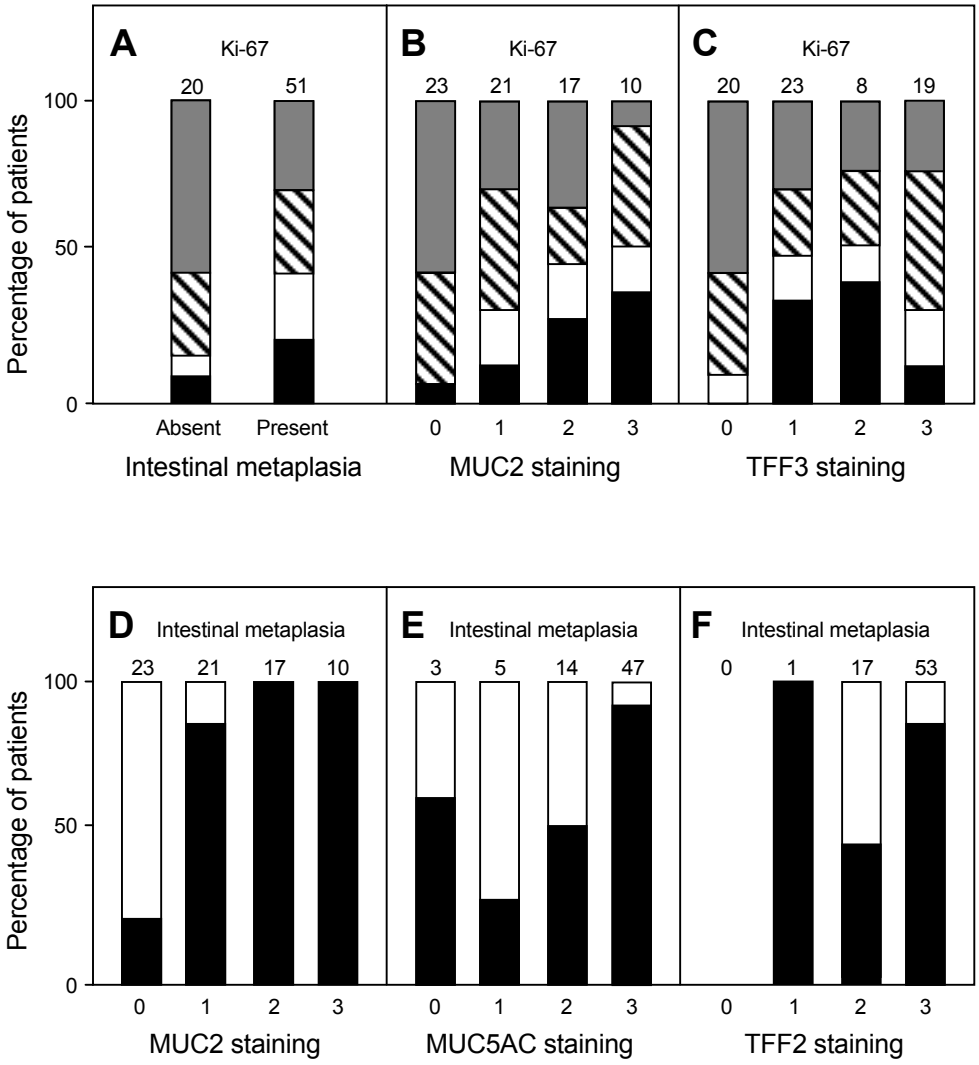

Figure 4. Correlations between proliferation, the presence of intestinal metaplasia, and the levels of MUC and TFF expression in Barrett's esophagus epithelium. The extent of expression of each of the secretory proteins, MUC2, MUC5AC, MUC5B, MUC6, TFF1, TFF2, and TFF3, was determined by immunohistochemistry, and was scored in four categories, 0-3 (see Materials and Methods), as indicated along the X-axis. Intestinal metaplasia was scored as either present or absent, based on AB/PAS staining. Proliferation was determined by the extent of Ki-67 expression, scored as 0-3 (see Materials and Methods). The correlations between the individual parameters were determined using Spearman's test. Only statistically significant correlations were depicted. Other correlations appeared not significant. Above each bar the number of patients within each category is depicted. Ki-67 staining is indicated by the differently colored bars (**A-C**): gray bars, staining was absent; crosshatched bars, staining in 1-10 cells per tissue section; white bars, staining in 11-30 cells per tissue section; black bars, staining >30 cells per section. The extent of Ki-67 expression was positively correlated to the presence of intestinal metaplasia (**A**, ρ=0.240, P <0.05), the level of MUC2 (**B**, ρ=0.369, P <0.005), and the level of TFF3 (**C**, ρ=0.256, P <0.05). Intestinal metaplasia was scored (**D-F**) as absent (white bars) or present (black bars). The presence of intestinal metaplasia was positively correlated to the level of MUC2 (**D**, ρ=0.693, P <0.0005), the level of MUC5AC (**E**, ρ=0.393, P <0.005), and the level of TFF2 (**F**, ρ=0.240, P <0.05).

Discussion

Generally it is thought that Barrett's esophagus signifies a risk of progression into adenocarcinoma of the esophagus. Early steps in the development of adenocarcinoma are that first normal stratified epithelium is triggered to transform into Barrett's esophagus epithelium consisting of gastric-type metaplasia, which is then transformed into Barrett's esophagus with intestinal metaplasia. Main arguments for this sequence are based on the respective frequencies of the pathological abnormalities. Small patches of Barrett's esophagus epithelium are usually of the gastric-type metaplasia, whereas intestinal metaplasia is less frequent and most often part of gastric metaplasia, and thus seems to arise from gastric metaplasia.

Our primary finding was that virtually all Barrett's esophagus epithelial cells produce at least one secretory MUC-type mucin in combination with one or more TFFs. None of the studied mucins is expressed in normal stratified squamous esophagus epithelium. Only MUC5B was found in the submucosal glands of the normal esophagus, corroborating earlier findings (Arul et al., 2000). Thus, MUCs and TFFs form excellent markers for Barrett's esophagus epithelium, whereas changes in their expression patterns may hint to changes in phenotype that could indicate very early progression in tumor development.

In general, we found that MUC and TFF expression in Barrett's esophagus epithelium without intestinal metaplasia resembled normal human gastric antrum epithelium. The expression patterns of these MUCs and TFFs in the human stomach were previously established (Hanby et al., 1993; Ho et al., 1995; Playford et al., 1995; Rio et al., 1988; Taupin et al., 2001; Van de Bovenkamp et al., 2001; Van den Brink et al., 2000; Wong et al., 1999). In short, both in gastric antrum and Barrett's esophagus epithelium, high levels of MUC5AC and TFF1 were found in the surface epithelium, high levels of MUC5B, MUC6 and TFF3 were present in glandular structures, and high levels of TFF2 were found throughout the epithelium. In intestinal metaplasia within Barrett's esophagus, as characterized by AB/PAS staining, MUC2 was specifically expressed in goblet cells of the intestinal metaplastic structures. A similar profile of mucin expression, as found in intestinal metaplasia of Barrett's esophagus, is demonstrated in intestinal metaplasia of human stomach (Reis et al., 1999). This suggests that intestinal metaplasia within Barrett's esophagus and gastric epithelium arise via similar mechanisms.

As it is assumed that intestinal-type metaplasia arises within gastric-type metaplasia of Barrett's esophagus, we sought to determine phenotypic changes within the Barrett's esophagus epithelium that coincided with the occurrence of MUC2 expression. We performed careful analysis of the overlapping expression patterns of the MUCs and the TFFs in conjunction with the occurrence of intestinal metaplasia and proliferation. We found that the gastric-type expression pattern of each of these markers was increasingly lost with increased extent of proliferation and intestinal metaplasia. This led to a model, summarizing our data, which is delineated below (Fig. 5).

Ki-67 is a nuclear antigen expressed in proliferating cells (Gerdes et al., 1984), which proved useful to differentiate between low- and high-grade dysplasia (Halm et al., 2000; Hong et al., 1995). In our study, Ki-67 staining occurred in the normal stratified esophagus epithelium as a discrete layer of proliferating cells (Fig. 5A). Compared to normal stratified esophagus

epithelium, Barrett's esophagus epithelium contained very small amounts of Ki-67 positive cells, which were mostly scattered in the epithelium (Fig. 5B and C).

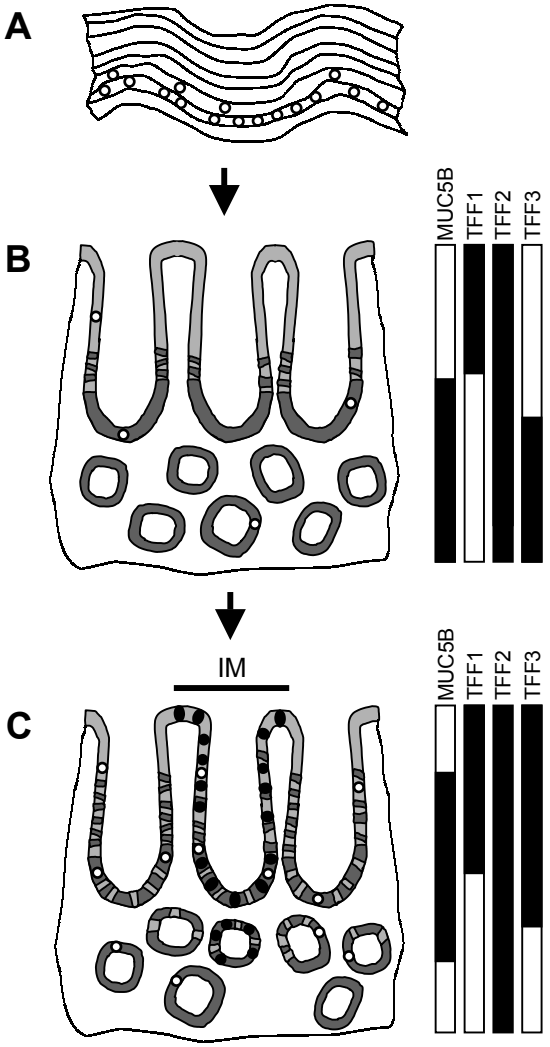

Figure 5. Model of early development of Barrett's esophagus showing increasing "loss of organization" in the Barrett's esophagus epithelium with increasing intestinal metaplasia. The first step is the conversion of the normal stratified epithelium of the esophagus (**A**) into a single-layered epithelium forming intricate glandular structures (**B**). Virtually all of these cells produce secretory mucins and associated TFF-peptides, mimicking gastric-type expression patterns. Yet, the degree of proliferation within this epithelium is low. In the next phase, patches of intestinal metaplasia (IM) arise (**C**). Together with the increasing expression of MUC2, in goblet cells within these patches of intestinal metaplasia, the expression of MUC5AC, -5B, and -6 and TFF1, -2, and -3 changes profoundly. The proliferation in the Barrett's esophagus epithelium is increased, yet remains below the level of the original stratified esophagus epithelium. With increasing proliferation, extent of intestinal metaplasia, and expression of MUC2, the co-ordinate gastric-type protein expression is increasingly lost, and more and more markers show significantly increasing overlaps in expression patterns. The expression of MUC2 (black circles), MUC5AC (light gray) and of MUC6 (dark gray) is schematically depicted, together with Ki-67-positive, proliferating cells (white circles). The boxes to the right represent the mean positions of MUC5B and TFF1, -2, and -3 along the glandular structures of Barrett's esophagus epithelium.

In the first step in Barrett's esophagus development a single layer of columnar epithelium, forming glandular structures, replaces the normal stratified epithelium that expresses no secretory mucins or TFF-peptides. Virtually all cells of the Barrett's esophagus epithelium produce secretory mucins and associated TFF-peptides, and the phenotype of this epithelium mimic the gastric-type (antrum) expression pattern (Fig. 5B). TFF1 co-localized in all patients with MUC5AC-positive epithelial structures in Barrett's esophagus epithelium. This TFF1/MUC5AC combination was also demonstrated in human gastric surface cells (Ho et al., 1995; Rio et al., 1988). TFF2 co-localizes in 91% and 93% of the patients with MUC5AC- and MUC6-positive structures, respectively. The TFF2/MUC6 combination was also found in the human gastric mucous neck cells (Hanby et al., 1993; Ho et al., 1995). MUC5B co-localized with MUC6 in nearly all patients, as in human gastric epithelium (Van de Bovenkamp et al., 2001). TFF3 primarily co-localizes with MUC6-positive glandular structures (76% of the patients), which was also observed in the human stomach (Taupin et al., 2001). In the intestine, TFF3 is strongly associated with MUC2 in goblet cells (Chang et al., 1994; Podolsky et al., 1993). Yet in Barrett's esophagus, TFF3 expression was not co-localized in MUC2-positive goblet cells. TFF3 expression in the absence of MUC2 expression is also recently described in the ulcer-associated cell lineage (Longman et al., 2000).

In the next phase, intestinal metaplasia and MUC2 expression appear and proliferation was increased. Concomitantly, the coordinated protein expression of the other MUCs and the TFFs is dramatically disturbed (Fig. 5C). We found that the level of proliferation was positively correlated with the presence of intestinal metaplasia, as assessed by AB/PAS staining, and the extent of MUC2 and TFF3 expression. The expression of MUC2 co-localized with MUC5AC within the intestinal metaplastic epithelium, whereas also the extent of expression of both MUCs correlated positively. Also the extent of TFF3 expression was positively correlated with the extent of MUC2 expression. Interestingly, TFF3 was expressed preferentially in MUC2-containing epithelial structures (but not within MUC-positive goblet cells), implying that TFF3 was found in patches of intestinal metaplasia. The extent of TFF2 expression was increased with increasing intestinal metaplasia, but showed no differences in co-localization. Significantly increased overlaps were demonstrated between the following markers: (i) MUC5AC and MUC6, (ii) TFF3 with MUC2, (iii) TFF3 with MUC5AC, and (iv) MUC5AC with MUC5B. In contrast, overlapping expression patterns of (i) MUC5B with MUC6 and (ii) TFF3 with MUC6 were decreased. Thus, the markers that normally showed strongly overlapping protein expression in gastric-type Barrett's esophagus epithelium showed diminished overlap in intestinal metaplasia. Conversely, the proteins that showed only marginally overlapping expression patterns showed increased overlaps with increasing intestinal metaplasia.

We realize that our study was based on tissue samples from a relatively small number of Barrett's esophagus patients and that we did not have any temporal information about the development of the gene expression over time in a given patient. It is worthwhile to validate these results in a larger set of patients. In particular prospective studies, collecting biopsy specimens from the same patients over time, will be necessary to confirm the sequence of events as suggested by us and summarized in Figure 5.

In conclusion, we demonstrated that virtually all cells in Barrett's esophagus have a secretory phenotype, i.e. each cell seems to produce at least one secretory MUC-type mucin and one TFF-peptide, and that Barrett's esophagus epithelium displayed primarily a gastric-type secretory phenotype, resembling the antrum. Intestinal metaplasia showed the unequivocal expression of the intestinal-type MUC2 in goblet cells. The coordinate gastric-type regulation of MUCs and TFFs was disturbed when intestinal metaplasia, MUC2 expression, and proliferation became more pronounced. With these findings we have indicated that the combination of MUC and TFF protein expression may be reliable markers to investigate the sequence of events in the very early development of pre-neoplastic Barrett's esophagus towards adenocarcinoma. In particular, MUC2 expression may constitute a marker for an early change in the phenotype of Barrett's esophagus as a pre-cancerous lesion.

Acknowledgements

This research was financially supported by the Dutch Digestive Diseases Foundation (Nieuwegein), Irene Children's Hospital Foundation (Arnhem), Jan Dekker/Ludgardine Bouman Foundation (Amsterdam), and Foundation "De Drie Lichten" (Leiden), each in the Netherlands.

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Chapter 6

Metaplasia of the duodenum shows a *H. pylori*-correlated differentiation into gastric-type protein expression

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Hum Pathol 2003; 34(2): 156-165

Abstract

The origin of gastric metaplasia of the duodenum (GMD) remains enigmatic. We studied expression of mucins and trefoil peptides in GMD to gain insight into its phenotype and origin. We examined duodenal tissue of 95 patients (0 to 83 years old, 26 with gastric *H. pylori*-infection) for presence of GMD. Expression was examined immunohistochemically of secretory mucins (MUC2, MUC5AC, MUC5B, and MUC6), trefoil peptides (TFF1, TFF2, and TFF3), and sucrase-isomaltase (SI). GMD, found in 37 patients, correlated positively to gastric *H. pylori*-infection, age, and villus atrophy. MUC2 and TFF3, expressed in normal goblet cells, were absent from 100% and 87% of GMD, respectively. GMD ubiquitously expressed MUC5AC, whereas MUC5AC expression in adjacent goblet cells was closely correlated with the extent of GMD. TFF1, TFF2, and MUC6 were found in 84%, 92%, and 65% of GMD, respectively. MUC5B was absent from epithelium and GMD. SI, expressed by villus enterocytes, was absent from GMD. Brunner's glands ubiquitously expressed MUC5B, MUC6, and TFF2. GMD was characterized by expression of gastric-type proteins MUC5AC, MUC6, TFF1, and TFF2 and absence of intestinal markers MUC2, TFF3, and SI. In terms of the location of metaplastic cells, our results suggest that epithelial cells migrating towards villus tips switch to gastric-type secretory cells. Positive correlation with infection suggests an inductive role of *H. pylori* in development of GMD.

Abbreviations: AB, Alcian blue; GMD, gastric metaplasia of the duodenum; PAS, periodic acid-Schiff reagent; SI, sucrase-isomaltase; TFF, trefoil factor family; UACL, ulcer associated cell lineage.

Introduction

Gastric metaplasia in the duodenal bulb (GMD) is characterized by replacement of intestinal epithelial cells with gastric-type mucous cells. GMD is frequently found in association with inflammation and *Helicobacter pylori* gastritis (Wyatt et al., 1990). GMD is defined as the occurrence of patches of gastric epithelial cells at the villus tip or side containing apical periodic acid-Schiff (PAS)-positive neutral mucin together with the absence of a brush border. The formation of gastric-type epithelium in the duodenal bulb seems to be related to increased gastric acid output (Harris et al., 1996). *H. pylori*-infection of the antrum leads to gastritis. In antral gastritis involves is hypergastrinemia and (due to intact body mucosal function) increased acid secretion resulting from the loss of regulatory feedback by somatostatin (McColl et al., 2000). The high acid load in the duodenum leads to a sequence of events including GMD, duodenitis, and ulceration. This sequence is recognized to be a continuum of events (Walker and Crabtree, 1998). In the literature there are conflicting findings concerning the relationship between *H. pylori* density in the antrum and the extent of GMD in patients suffering from duodenal ulcer. Harris et al., (1996) and Noach et al., (1993) found no decrease of GMD for at least 6 and 12 months after *H. pylori* eradication, respectively. However, Khulusi *et al.* demonstrated that the extent of GMD was correlated with antral *H. pylori* density; 6 months after the eradication of *H. pylori*, the extent of GMD was decreased (Khulusi et al., 1996; Khulusi et al., 1995b). The reason for this discordance appears to be due to sampling error at biopsy sites, the variable quality of methods used for *H. pylori* detection, and differences in the numbers of patients included for follow-up studies.

The low prevalence of GMD in children under the age of 10 suggests that GMD is not of congenital origin (Gormally et al., 1996). Nonetheless, the origin of gastric metaplastic cells remains controversial. Liu and Wright described tight cohorts or migration streams of periodic acid-Schiff (PAS)-positive cells that appeared to be derived from either Brunner's gland duct epithelium or basal buds growing out of the crypts of Lieberkühn (Liu and Wright, 1992). Hanby *et al.*, from the same laboratory, also suggested that GMD might be differentiated from Brunner's gland duct epithelium, because the gastric trefoil-peptide (TFF1) protein and mRNA was found in both GMD and Brunner's duct epithelium (Hanby et al., 1993). However, Rio *et al.* found no evidence of TFF1 protein or mRNA expression in the Brunner's duct epithelium (Rio et al., 1991). Importantly, because GMD can occur in areas of the intestine that lack Brunner's glands, these glands apparently are not necessary for the development of GMD. Longman e*t al.* suggested that in areas lacking Brunner's glands, GMD may originate from the PAS-positive ulcer-associated cell lineage (UACL), occurring at sites of chronic gastrointestinal ulceration. The UACL in the intestine evolves as a bud from the base of the crypts, which matures to form a coiled acinar structure with a duct leading to the mucosal surface epithelium (Longman et al., 2000). In contrast, Shaoul *et al.* have suggested that goblet cells expressing both intestinal and gastric antigens may represent local precursors of GMD undergoing a transition to gastric-like cells of mixed phenotype at the site of early metaplastic patches. As GMD becomes more widespread, a purer gastric phenotype emerges (Shaoul et al., 2000b). In summary, GMD is characterized by a secretory phenotype that

expresses secretory mucins and TFF-peptides. Therefore, we studied whether and if so, how, these secretory proteins in GMD differ from the normal intestinal secretory protein expression pattern. In addition, we analyzed the mucin and TFF expression in Brunner's glands in normal duodenum and in Brunner's glands where GMD is present at the crypt/villus epithelium, to investigate possible differences in expression pattern.

Materials and Methods

Patients, Tissue, and Pathology

Biopsy specimens of the duodenal bulb of from 95 patients (ranging in age from 0 to 83 years, 26 with gastric *H. pylori*-infection) were collected through endoscopy, as part of a prospective study on the occurrence of *H. pylori* in pediatric and adult patients with upper abdominal complaints. Patients were asked to abstain from anti-acid drugs for least 1 week before the endoscopy. The biopsy specimens were collected in the Academic Medical Center in Amsterdam with the permission of the Medical Ethics Committee. For each patient, 2 biopsy specimens were obtained from the duodenal bulb and immediately fixed in phosphate-buffered saline (PBS)-buffered 4% (wt/vol.) paraformaldehyde solution for 4 hours and then processed into paraffin blocks following standard procedures. *H. pylori*-infection was assessed in biopsy specimens from the antrum taken as part of the same endoscopic procedure, and the bacterium was detected using standard histologic staining and microbiology. The presence of *H. pylori* in the stomach was recorded as either positive or negative.

Immunohistochemistry

The following polyclonal antibodies raised in rabbits were used: anti-MUC5B, anti-MUC6, anti-TFF1, anti-TFF2, and anti-TFF3 (Table 1). The following mouse monoclonal antibodies were also used: anti-sucrase-isomaltase (SI), anti-MUC2, and anti-MUC5AC (Table 1). Tissue sections were deparaffinized through 3 changes of xylene and then rehydrated through a series of decreasing concentrations of ethanol solution to distilled water. Endogenous peroxidase activity was deactivated in 3% (vol./vol.) hydrogen peroxide in PBS for 30 minutes and washed in PBS for 5 minutes. Antigen retrieval was performed by heating the sections for 10 minutes at 100°C in 10 mM citrate buffer, pH 6.0, then letting them cool to room temperature for 20 minutes. The sections were washed 3 times for 5 minutes in PBS and incubated with 1% (wt/vol.) blocking agent (Boehringer, Mannheim, Germany) in PBS for 30 minutes. Primary antibodies were diluted in PBS (Table 1) and were incubated with the tissues for 16 hours at 4°C. Slides were washed 3 times for 5 minutes in PBS, followed by incubation with biotinylated secondary antibodies and avidin-biotin peroxidase complex (Vectastain ABCkit, Vector laboratories, Burlingame, UK) according to the manufacturer's protocol. Staining was performed using 0.5 mg/ml 3,3'-diaminobenzidine/0.03% (vol./vol.) hydrogen peroxide in imidazole (30 mM)/EDTA (1 mM) pH 7.0. Sections were rinsed in water, dehydrated through a series of increasing concentrations of ethanol solutions, and mounted under cover slips.

Control staining was performed leaving each of the primary antibodies out of the procedure, resulting in an absence of staining.

Table 1. Characteristics of antibodies used for immunohistochemical methods.

Name in this study	Name in Reference	Epitope	Dilution	Reference
anti-MUC2	WE9	Peptide domain	1:50	(Tytgat et al., 1995)
anti-MUC5AC	45M1	Peptide domain	1:50	(Bara et al., 1998)
anti-MUC5B	BGBM	Deglycosylated polypeptide	1:3000	(Van Klinken et al., 1998)
anti-MUC6	M6.1	Peptide in VNTR* domain	1:200	(De Bolos et al., 1995)
anti-TFF1	anti-pS2	C-terminal peptide	1:4000	(Rio et al., 1991)
anti-TFF2	anti-hSP	C-terminal peptide	1:2000	(Srivatsa et al., 2002)
anti-TFF3	HM:169	C-terminal peptide	1:3000	(Podolsky et al., 1993)
anti-SI	HBB 2/219/20	Peptide domain	1:1000	(Hauri et al., 1985)

*VNTR, variable number of tandem repeats.

GMD

Metaplastic cells were detected histochemically in each biopsy specimen based on characteristic morphology using hematoxylin and eosin and alcian blue/periodic acid-Schiff (PAS) staining. Expression of the aforementioned proteins was determined within GMDs after positive identification by this histochemical staining. The extent of metaplasia was scored as follows: 0, absent; 1, ≤ 20 metaplastic cells in a continuous stretch on 1 villus; 2, > 20 metaplastic cells in a continuous stretch on 1 villus; 3, metaplastic cells on more that 1 villus.

Crypt-to-villus ratio

The crypt-to-villus ratio was assessed in a double-blind assay using light microscopy at low magnification (100x), yielding scores of 0 for total villus atrophy, 1 for moderate villus atrophy (crypt-to-villus ratio < 1), 2 for mild villus atrophy, and 3 for normal morphology according to established criteria (Mercer et al., 1990). Our findings were checked against the pathologist's analysis of duplicate biopsy specimens from each patient.

Semi-Quantitative Scoring of Immunohistological Staining

The presence of immunohistochemical staining was determined using light microscopy at magnification x200 to 400. Based on the characteristic expression patterns of the secretory proteins under study, we chose to score protein expression in 3 separate structures: (1) normal goblet cells along the crypt-villus axis, (2) Brunner's glands, and (3) metaplastic cells. Goblet cells were present in all biopsy specimens (n = 95). Brunner's glands were present in biopsy specimens of 70 patients, whereas metaplasia was observed in the specimens of 37 patients. The presence of each secretory protein in each of these structures was recorded as follows: 0, staining absent; 1, staining in up to 1/3 of cells; 2, staining in up to 2/3 of cells; 3, staining in >2/3 of the cells. There was 1 notable exception to this classification scheme-MUC5AC appeared to be expressed in sporadic goblet cells and was scored in these cells as follows: 0, absent; 1,

expression in 1 to 5 goblet cells; 2, expression in 6 to 10 goblet cells; 3, expression >10 goblet cells. SI was recorded in the apical membranes of normal villus enterocytes and metaplastic cells. We distinguished 4 semiquantitative classes of staining intensity scored on the staining of the villus brush border: 0, absence of staining; 1, very weak staining in patches of enterocytes; 2, evenly distributed moderate staining; and 3, evenly distributed dark staining (Van Beers et al., 1998).

Statistics

The χ^2 test or Fisher's exact test was used to analyze differences in frequencies. Kendall's tau-ranks sum test or Spearman's correlation test was used to analyze correlations between parameters. Statistical significance was defined at $P < 0.05$.

Results

Pathology and Occurrence of GMD

GMD was detected at the villus tips and by the absence of staining by alcian blue, rendering the metaplastic cells magenta in alcian blue (pH 2.5)/ PAS-staining (Fig. 1, see *Appendix*). The size of these patches of metaplastic cells ranged form very small, constituting only few cells, to very large, covering several entire villi. All of these cells had a characteristic "gastric phenotype", that was clearly and unequivocally distinguishable from both enterocytes and goblet cells.

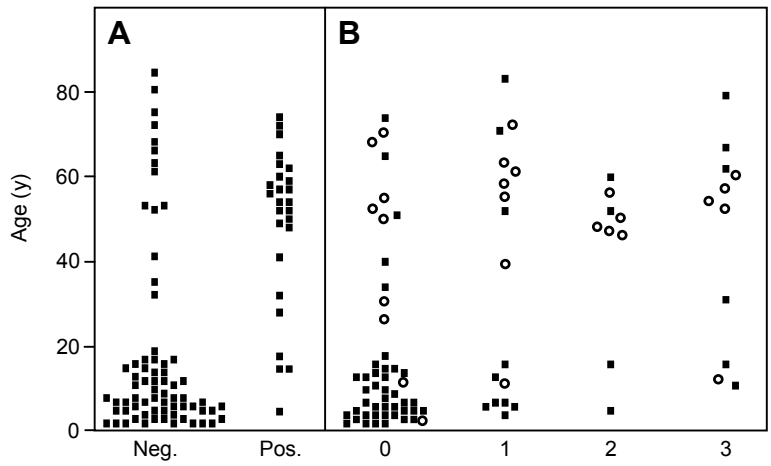

Figure 2. Correlations between age and *H. pylori* status in the stomach with the occurrence of GMD. The age of the individual patients (n = 95) was plotted against the absence (Neg.) or presence (Pos.) of *H. pylori* in the stomach at time of endoscopy (A), and against the extent of GMD (B). Each symbol represents 1 individual. In (B), the squares indicate patients without gastric *H. pylori*-infection, and the circles indicate *H. pylori*-infected patients. The extent of GMD was scored as described in Materials and Methods.

Based on the analysis of antrum biopsy specimens, 26 patients were found to suffer from *H. pylori*-infection at the time of endoscopy. Patient age was positively and closely correlated with infection with *H. pylori* (R=0.420, P <0.0005; Kendall's tau-test) (Fig. 2A), and also the size of the GMD was highly significantly correlated with the age of the individuals (R=0.356, P <0.0005; Kendall's tau-test) (Fig. 2B). The correlation between patient age and GMD size was also considered separately for patients without gastric *H. pylori*-infection and was also found to be positive and statistically significant (R=0.300, P <0.0002; Kendall's tau-test) (Fig. 2B). Independently, GMD size correlated significantly and positively with the presence of *H. pylori* (ρ=0.311, P <0.002; Spearman's test) (Fig. 3A). Conversely, GMD size correlated negatively with the crypt-villus morphology; that is, when villus atrophy was more pronounced, the likelihood of GMD increased (ρ= -0.305, P <0.003; Spearman's test) (Fig. 3B). As noted in a previous study (Van Beers et al., 1998), SI expression proved to be a sensitive indicator for tissue damage. SI expression on the duodenal villi correlated closely with crypt-villus morphology (ρ=0.447, P <0.0005, Spearman's test; data not shown).

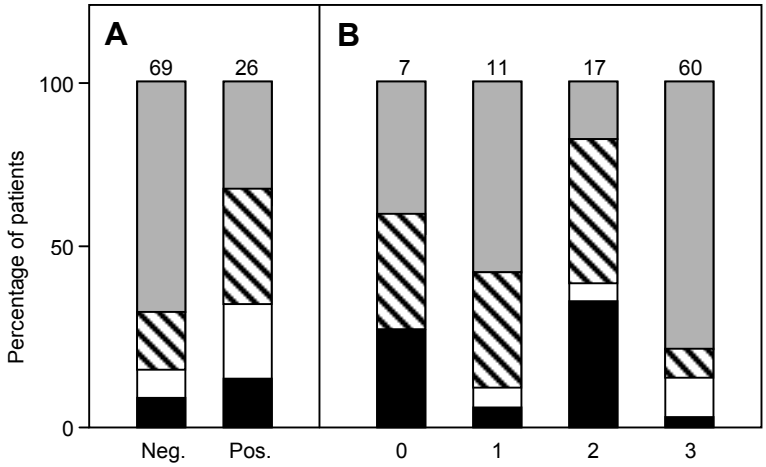

Figure 3. Correlations between GMD and presence of *H. pylori* in the stomach and tissue morphology. The extent of GMD in the patients (n = 95) was plotted against the absence (Neg.) or presence (Pos.) of *H. pylori* in the antrum of the stomach at time of endoscopy (A) and against the morphology of the intestinal tissue (B). The number of patients in each group is indicated above the bars. The extent of metaplasia was scored as 0, absent (gray); 1, ≤ 20 metaplastic cells on 1 villus (cross-hatched); 2, > 20 metaplastic cells on 1 villus (white); or 3, metaplastic cells on more than 1 villus (black). Morphology was scored as described in Materials and Methods.

Protein Expression in GMD

GMD was found in 37 patients, and protein expression in GMD was detected by immunohistochemistry (Fig. 4, see *Appendix*, Table 2). The only marker that was unequivocally expressed in all metaplastic cells, as detected by AB/PAS-staining, was MUC5AC (Fig. 4B, *Appendix*). Expression of MUC5AC ranged from only a few cells at the tips of morphologically normal villi (Fig. 5B, see *Appendix*) to very large patches covering the entire villi in extensive GMD (Fig. 5D, *Appendix*). TFF1 and TFF2 were expressed in most

patches of GMD and also in most of the metaplastic cells. The extent of TFF1 expression in GMD correlated positively and statistically highly significantly with the extent of TFF2 expression in GMD (ρ=0.700, P <0.0005; Spearman's test). Also, MUC6 was frequently found in cells of GMD, but the extent of MUC6 expression in GMD appeared not to correlate significantly with any other parameter, including patient age, *H. pylori*-status, GMD size, or the extent of expression of any other protein in GMD. The other proteins, MUC2, MUC5B, TFF3, and SI, were hardly ever detected in GMD (Fig. 4, *Appendix*, Table 2).

Table 2. Expression of proteins in the small intestine.

	Goblet cells	Metaplasia	Brunner's glands
MUC2	100% (95/95)	8% (3/38)	0% (0/70)
MUC5AC	77% (73/95)	100% (38/38)	1% (1/70)
MUC5B	1% (1/95)	5% (2/38)	97% (62/64)
MUC6	4% (4/95)	65% (24/37)	99% (65/66)
TFF1	93% (88/95)	84% (31/37)	11% (7/65)
TFF2	96% (91/95)	92% (34/37)	99% (66/67)
TFF3	91% (86/95)	16% (6/38)	47% (31/66)
SI*	91% (86/95)*	3% (1/38)	4% (3/70)

NOTE: Indicated are the percentages and numbers of patients showing expression of the protein in a given structure within the tissue of the duodenal bulb (positive patients/ total patients).
*SI expression was not scored in goblet cells, but in the brush border of villus enterocytes.

Protein Expression in Brunner's Glands

Brunner's glands are often present in biopsy specimens of the duodenal bulb. In this study, tissue sections of 70 of the 95 patients showed Brunner's glands, and protein expression in these glands was detected through immunohistochemistry (Fig. 4, *Appendix*). MUC5B, MUC6, and TFF2 were expressed in almost all cells of each Brunner's gland (Fig. 4, *Appendix*, Table 2). MUC2 was not found in any Brunner's gland specimen, and MUC5AC, TFF1 and SI were nearly always absent as well (Fig. 4, *Appendix*, Table 2). Interestingly, TFF3 was expressed in about 50% of the patients, exhibiting an all-or-nothing type of expression-either all cells or no cells were stained for TFF3.

Protein Expression in Goblet Cells

Protein expression of MUC2, MUC5AC, MUC5B, MUC6, TFF1, TFF2, TFF3, and SI was detected in duodenal tissue through immunohistochemistry (Fig. 4, *Appendix*, Table 2). The only unequivocal marker of intestinal goblet cells was MUC2, which was expressed in all identified goblet cells of each patient. Most patients also expressed TFF1, -2, and -3 in their goblet cells (Fig. 4, *Appendix*, Table 2). The extent of TFF expression in goblet cells was very closely correlated. When the extent of expression of each TFF was pairwise related to the extent of expression of each of the other TFFs, all correlations were positive and highly significant (ρ=0.334 to 0.595, P <0.001; Spearman's test) for each pairwise correlation.

MUC5AC was expressed in goblet cells in most patients (Fig. 4, *Appendix*, Table 2), although the expression was often limited to very small numbers of positive goblet cells per section (Fig. 5B, *Appendix*). Interestingly, the number of MUC5AC-positive goblet cells increased when GMD was more extended (Fig. 5D, *Appendix*). MUC5B and MUC6 were almost completely absent from goblet cells (Fig. 4, *Appendix*, Table 2). Likewise, SI was completely absent from goblet cells, but was detected in nearly all patients in the normal brush border of villus enterocytes that surrounded the goblet cells (Fig. 4, *Appendix*, Table 2).

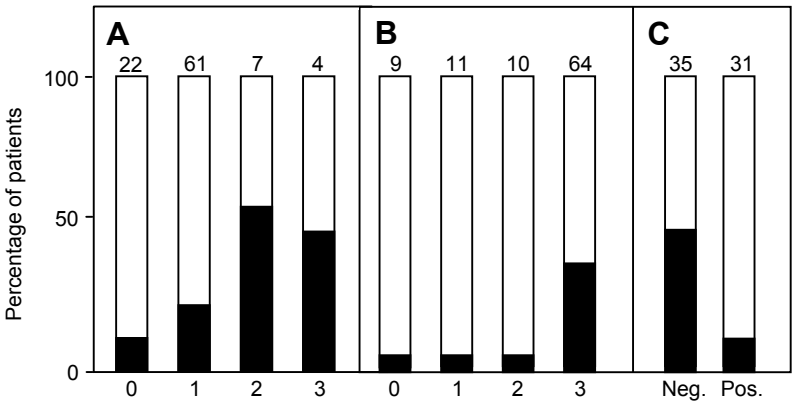

Figure 6. Effect of *H. pylori*-infection in the stomach on the extent of MUC5AC and TFF3 expression in the duodenum. (A) The extent of expression of MUC5AC in goblet cells was scored in all patients (n = 95) as 0, no expression; 1, expression in 1 to 5 goblet cells; 2, expression in 6 to 10 goblet cells; and 3, expression in >10 goblet cells. (B) TFF3 expression in goblet cells was scored in all patients (n = 95) as 0, no expression; 1, expression in up to 1/3 of goblet cells; 2, expression in up to 2/3 of goblet cells; and 3, expression in >2/3 of goblet cells. (C) TFF3 expression in the Brunner's glands was scored (n = 66) as positive (i.e., all cells positive) or negative (i.e., no staining). *H. pylori*-infection was indicated as either present (black) or absent (white). The number of patients in each group is indicated above the bars.

Effects of Age and Pathology on MUC5AC and TFF3 Protein Expression

The extent of expression of all proteins was scored semiquantitatively and correlated with *H. pylori*-infection, GMD size and patient age. These factors clearly affected the extent of expression of only 2 of the proteins in specific tissue structures: MUC5AC expressed in goblet cells and TFF3 expressed in either goblet cells or Brunner's gland.

H. pylori-infection in the stomach was correlated to increased extent of expression of both MUC5AC (ρ=0.240, P <0.02; Spearman's test) and TFF3 (ρ=0.251, P <0.02; Spearman's test) in goblet cells (Fig. 6A and B, respectively). Whereas the extent of TFF3 expression in the Brunner's glands was negatively correlated with the existence of gastric *H. pylori*-infection (ρ= -0.356, P <0.003; Spearman's test) (Fig. 6C). Also, the presence and size of GMD correlated positively with the expression of MUC5AC in goblet cells (ρ=0.443, P <0.005; Spearman's test) (Fig. 7A). The correlation between TFF3 expression in goblet cells and GMD size did reached only near-statistical significance (ρ=0.2, P =0.07; Spearman's test; data not shown); however, TFF3 expression in the Brunner's glands was negatively correlated with GMD (ρ= -0.264, P <0.04; Spearman's test) (Fig. 7B). The expression of both MUC5AC and TFF3 in goblet cells was positively related to patient age (R=0.278, P <0.001 and

R=0.229, *P* <0.005, respectively; Kendall's tau-test) (Fig. 8), whereas the expression of TFF3 in Brunner's glands was decreased with increasing age (R= -0.470, *P* <0.005; Kendall's tau test) (Fig. 8C). When these relations of MUC5AC and TFF3 expression with age were analyzed separately for only the non-*H. pylori*-infected individuals, it appeared that TFF3 expression in goblet cells and in Brunner's glands correlated positively and negatively, respectively, with patient age (R=0.248, *P* <0.01 and R= -0.403, *P* <0.001, respectively; Kendall's tau-test) (Fig. 8B and 8C, respectively).

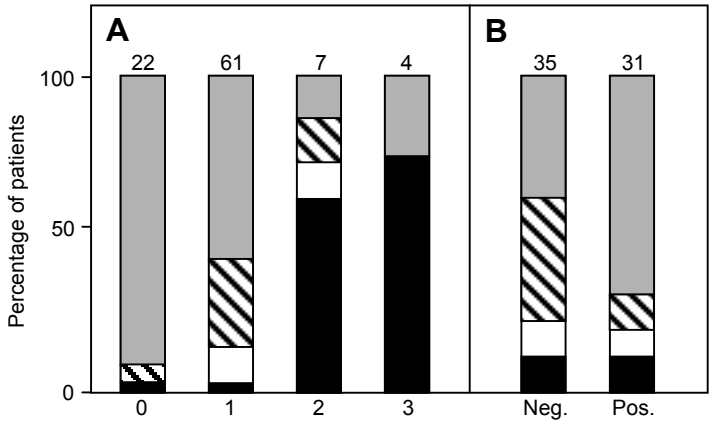

Figure 7. Effect of GMD on the extent of MUC5AC and TFF3 expression in the duodenum. (A) The extent of expression of MUC5AC in goblet cells was scored in all patients (n = 95), as described in the legend of Fig. 6. (B) TFF3 expression in Brunner's glands was scored (n = 66) as positive (i.e., all cells positive) or negative (i.e., no staining). The extent of GMD was scored as described in the legend of Fig. 3. The number of patients within each group is indicated above the bars.

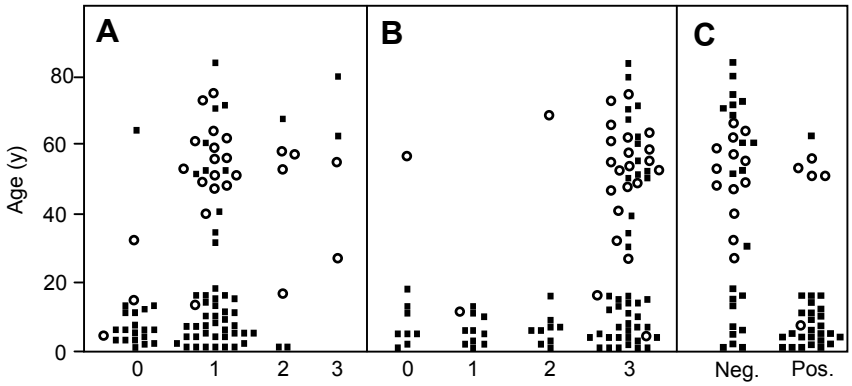

Figure 8. Correlation of age with MUC5AC and TFF3 expression in the duodenum. Expression of MUC5AC (A), and TFF3 (B) in goblet cells was scored in all patients (n = 95), as described in the legend of Fig. 3. (C) TFF3 expression in the Brunner's glands was scored (n = 66) as positive (pos; all cells positive) or negative (neg; no staining). Each symbol represents 1 individual, the squares indicating non-*H. pylori*-infected individuals and the circles indicating *H. pylori*-infected patients.

Discussion

In this study we examined immunohistochemically the protein expression of mucins (MUC2, MUC5AC, MUC5B, and MUC6), TFFs (TFF1, TFF2, and TFF3), and SI of goblet cells, metaplastic cells, and Brunner's glands in the duodenal bulb, in an attempt to better understand the phenotype and origin of GMD. This is the first report to examine each of the secretory mucins and TFFs in GMD and normal duodenum. GMD was characterized by PAS-positive staining of patches of metaplastic cells at villus tips and ranged from only a few cells to very large areas covering the entire villi. Interestingly, PAS-positive goblet cells were also identified in the deeper duodenal crypt region at the junctional areas of Brunner's gland ducts with crypt bases. This was also recently observed by Shaoul *et al.* (Shaoul et al., 2000b), who described the presence of goblet cells with a mixed phenotype, expressing both intestinal and gastric markers.

Among the 95 patients studied, 27% of patients suffered from gastric *H. pylori*-infection. We found positive correlations between the patient age and *H. pylori*-infection, and between age and the extent of GMD. Independently, both age and occurrence of GMD were positively correlated in the non-*H. pylori*-infected individuals, but these correlations were less pronounced. Separately, we demonstrated a positive correlation between the presence of *H. pylori* and the size of GMD. These results are in concordance with earlier observations (Wyatt et al., 1990) and suggest that *H. pylori* may be responsible, directly or indirectly, for extending preexisting GMD. In addition, the extent of GMD was positively correlated with villus atrophy and negatively with SI expression, a sensitive marker for villus damage (Van Beers et al., 1998). This implies that GMD is an important factor in development of villus damage.

It was demonstrated that *H. pylori* can both colonize the gastric antrum and areas of GMD, leading to chronic active inflammation in the duodenum and eventually to erosions and ulcerations (Walker and Crabtree, 1998). This sequence of events may result in villus atrophy. Therefore, GMD and *H. pylori* are considered to be prerequisites for the development of villus damage. The strong association, as found between *H. pylori* gastritis and duodenal ulcer disease and the finding that duodenal ulceration seldom recurs if *H. pylori*-infection is cured (Rauws and Tytgat, 1990), indicates that *H. pylori* plays a critical role in the pathogenesis of duodenal ulcer. Most likely, GMD and antrum epithelium express the same antigens necessary for *H. pylori* colonization. In an earlier study we showed that *H. pylori* in the antrum specifically co-localized with MUC5AC-producing cells, and not with MUC6-producing cells (Van den Brink et al., 2000). As MUC5AC is expressed in GMD, the MUC5AC-producing cells of GMD possibly play a similar role in *H. pylori* colonization, as in the antrum.

To help better understand the phenotype and origin of GMD cells, we compared protein expression in the normal duodenum, GMD, and Brunner's glands. The results are schematically illustrated in Fig. 9. In the normal crypt/villus epithelium, MUC2 was found in all goblet cells, but MUC5AC was found in only a few scattered goblet cells on the villi, co-expressed with MUC2. This mixed expression of intestinal and gastric mucins in goblet cells of the normal duodenum was also observed by Shaoul *et al.* (Shaoul et al., 2000a),

Nonetheless, the function is of these "mixed goblet cells" in the normal villus epithelium remains unclear, as does whether these cells are part of the pathologic processes in GMD formation. To the best of our knowledge, the present study is the first report of MUC5B expression in Brunner's glands. Like MUC5B, MUC6 expression was only found in the Brunner's glands of the normal duodenum (Fig. 9). Others have also described MUC6 expression in Brunner's glands (Ho et al., 1995; Longman et al., 2000). In normal duodenum, all TFFs were expressed in goblet cells in nearly all patients, as was previously found for TFF1 (Hanby et al., 1993; Shaoul et al., 2000a). TFF2 and TFF3 were expressed in Brunner's glands. TFF2 expression was found in 99% of patients; TFF3 expression in about 50%. The demonstration of TFF2 expression in Brunner's glands was in agreement with other recent studies (Elia et al., 1994; Khulusi et al., 1995a; Longman et al., 2000), however TFF3 expression in Brunner's glands has not been described. In summary, the goblet cells in the crypt/villus epithelium of the normal duodenum expressed MUC2, TFF1, TFF2, and TFF3, whereas the Brunner's gland epithelium expressed MUC5B, MUC6, TFF2, and TFF3.

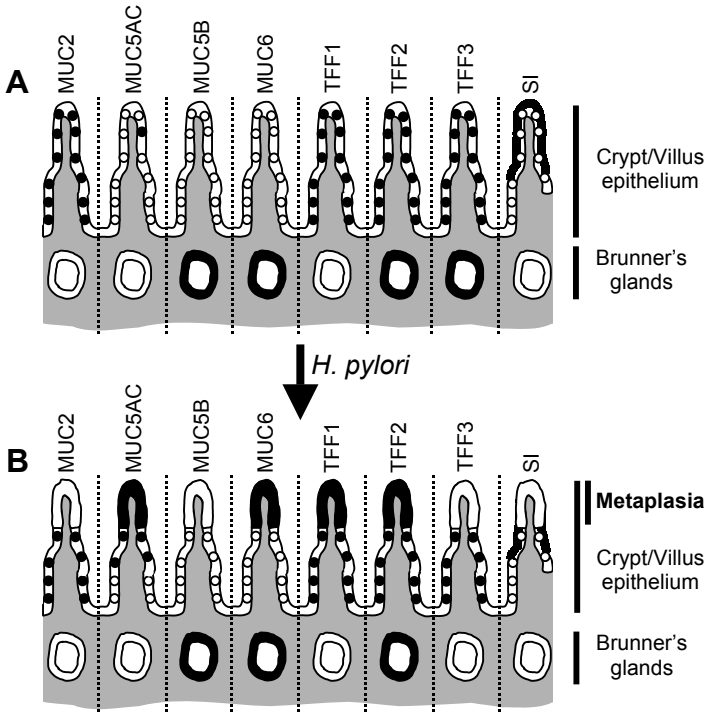

Figure 9. Schematic representation of protein expression in GMD. The expression of each of the proteins studied is indicated in the normal epithelium, particularly in the goblet cells (○), and in the Brunner's glands. Only the expression of SI is indicated as a thick line on the brush border. Expression is marked in black when expression was found in more than 50% of the patients in a particular structure. *H. pylori* is indicated as a probable cause of the transition towards GMD. GMD is indicated only at the villus tips, but may more rarely also replace the entire crypt/villus epithelium. In those cases the expression within goblet cells is lost completely, as goblet cells do not form part of the metaplastic epithelium.

Because we found a positive correlation between the antral presence of *H. pylori* and the extent of GMD, it seemed very likely that *H. pylori* was involved in the process, in which protein expression was changed from normal villus epithelium to metaplastic epithelium. GMD was characterized by the replacement of both goblet cells and enterocytes at the villus tip by metaplastic mucous cells. MUC5AC was ubiquitously expressed in GMD, and MUC5AC expression was also found in seemingly normal goblet cells near the GMD. The number of MUC5AC-positive goblet cells increased with the extent of GMD. Interestingly, MUC5AC expression in goblet cells was rarely found in the junctional area between the crypt bases and Brunner's glands duct. These 2 observations are in agreement with recent findings (Shaoul et al., 2000b) and support the hypothesis that goblet cells expressing both intestinal- and gastric-type antigens may represent local precursors of GMD. In nearly all patients MUC6, TFF1, and TFF2 expression was observed in GMD. Earlier studies also demonstrated TFF1 expression in GMD, but failed to demonstrate TFF2 expression (Hanby et al., 1993; Khulusi et al., 1995a). As GMD appeared, the expression of SI on the brush border of normal villus enterocytes disappeared. Thus, the GMD phenotype is very different from the enterocytes, constituting a complete switch in phenotype from intestinal to a gastric epithelium. As in normal duodenum, the Brunner's glands in tissue containing GMD expressed MUC5B, MUC6, and TFF2. Interestingly, TFF3 expression in Brunner's glands was negatively correlated with both extent of GMD and presence of gastric *H. pylori*. Nonetheless, the mechanisms by which *H. pylori* and GMD may influence the TFF3 gene expression in Brunner's glands, and the consequences of this expression, remain unknown.

We demonstrated that GMD in the duodenal bulb specifically expressed the secretory proteins MUC5AC, MUC6, TFF1, and TFF2. The major source of expression for these proteins is the gastric epithelium, as has been described previously (Van de Bovenkamp et al., 2001; Van den Brink et al., 2000). In gastric antrum, a function of mucins is to protect the gastric surface epithelium against high luminal acid concentrations.(Van Klinken et al., 1995) TFFs in the stomach, expressed in close association with secretory mucins, very likely play a role in mucosal defense and repair (Wong et al., 1999). Therefore, it is not unrealistic to assume that GMD appears as response to the high acid load in the proximal duodenum, which could be the result of gastric *H. pylori*-infection. Generally, GMD could be recognized as a mechanism to protect the underlying epithelium of the duodenum.

The origin of the metaplastic cells remains a subject of debate. Liu and Wright have suggested that GMD is an outgrowth of Brunner's gland ducts into the villi (Liu and Wright, 1992). Our results show that this is unlikely, because MUC5AC and TFF1 expression as found in GMD was not observed in Brunner's glands. In addition, Brunner's glands expressed MUC5B, whereas GMD exhibited virtually no MUC5B expression. Goblet cells co-expressed MUC2 and MUC5AC in 77% of the patient. It is possible that these goblet cells with this dual phenotype represent an early form of metaplasia and shift further from phenotype when moving upward to the villus tip and eventually become cells with a gastric phenotype. Because proliferation at the villi tips seldom occurs, transdifferentiation of goblet cells and enterocytes into cells with a gastric phenotype would explain the appearance of GMD cells.

However, in normal duodenal epithelium, goblet cells and enterocytes are ablated when they reach the villus tip, ending their short lifespan of about 4 days. It is unclear how GMD cells could be maintained on the villus tip, unless we assume that normal epithelial cells continuously transdifferentiate from their normal intestinal phenotypes into the gastric phenotype of GMD. The hypothesis of "mixed" goblet cells, which become cells with a gastric phenotype, fits in the concept of local goblet cell transformation as described by Shaoul *et al.* (Shaoul et al., 2000b). However, we cannot exclude a second scenario in which early reprogramming occurs at the stem cell level, particularly in patients with very extensive GMD in whom the entire crypt/villus epithelium is replaced with GMD. In these cases of extensive GMD, the cells with a gastric phenotype may arise in the crypt and move upward to the villus tip as a cohort of cells (Liu and Wright, 1992).

In conclusion, we demonstrated that GMD expressed the gastric-type protective secretory proteins MUC5AC, MUC6, TFF1, and TFF2. Based on the protein expression, we suggest that goblet cells and normal enterocytes, when moving upward to the villus tips, shift from an intestinal-type gene expression pattern to a gastric-type expression pattern, which is characteristic for GMD. Goblet cells with a mixed intestinal-gastric type of protein expression may constitute early markers of this transdifferentiation into gastric-type epithelium. The positive correlation between the extent of GMD and gastric *H. pylori*-infection suggests that the bacterium plays an inductive role in the development of GMD.

Acknowledgement

This research was financially supported by the Dutch Digestive Diseases Foundation, Irene Foundation, Jan Dekker/Ludgardine Bouman Foundation and Foundation "De Drie Lichten". The authors thank the donors of the antibodies for their kind cooperation: dr G. Offner (anti-BGBM), dr C. de Bolós (anti-M6.1), dr A. Giraud (anti-pS2 and anti-hSP), prof. D.K. Podolsky (HM:169 and WE9), and prof H.P. Hauri (anti-SI). The authors thank dr E.H. van Beers and T.M. Rolf for human biopsy specimens.

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Chapter 7

MUC5AC glycoprotein is the primary receptor for *Helicobacter pylori* in human stomach

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Helicobacter 2003; *in press*

Abstract

Background and Objectives: *Helicobacter pylori* shows a characteristic tropism for the mucus-producing gastric epithelium. In infected patients, *H. pylori* co-localizes *in situ* with the gastric secretory mucin MUC5AC. The carbohydrate blood-group antigen Lewis B (LeB) was deemed responsible for the adherence of *H. pylori* to the gastric surface epithelium. We sought to determine if MUC5AC is the carrier of LeB, and thus if MUC5AC is the underlying gene product, functioning as main receptor for *H. pylori* in stomach. Methods: We studied three types of human tissue producing MUC5AC: Barrett's esophagus (BE), normal gastric tissue, and gastric metaplasia of the duodenum (GMD). Tissue sections were immuno-fluorescently stained for MUC5AC or LeB, and subsequently incubated with one of three strains of Texas red-labeled *H. pylori*, one of which was unable to bind to LeB. We determined the co-localization of MUC5AC or LeB with adherent *H. pylori*. Results: The binding patterns for the two LeB-binding strains to all tissues were similar, whereas the strain unable to bind to LeB did not bind to any of the tissues. In normal gastric tissue, the LeB-binding strains always bound to MUC5AC-and LeB-positive epithelial cells. In four non-secretor patients co-localization of the LeB-binding strains was found to MUC5AC-positive gastric epithelial cells. In BE, the LeB-binding *H. pylori* strains co-localized very specifically to MUC5AC-positive cells. MUC5AC-producing cells in GMD contained LeB. Nonetheless, LeB-binding *H. pylori* did not only co-localize to MUC5AC or LeB present in GMD, but bound also to the LeB-positive brush border of normal duodenal epithelium. Conclusions: Mucin MUC5AC is the most important carrier of the LeB carbohydrate structure in normal gastric tissue and forms the major receptor for *H. pylori*.

Abbreviations: BabA, blood group antigen-binding adhesin; LeB, Lewis B blood group antigen; FITC, Fluorescein isothiocyanate; Gal, galactose; GlcNAc, *N*-acetyl-glucosamine; TR, Texas red.

Introduction

Since the discovery of *Helicobacter pylori* (Marshall, 1983; Warren and Marshall, 1983), chronic infection with this bacterium was identified as the major etiological factor in gastritis, gastric ulcers, gastric atrophy, and gastric carcinoma (Marshall and Warren, 1984; Parsonnet et al., 1991). As a result, in 1994, the international Agency for Research on Cancer (IARC, Lyon, France), classified *H. pylori*-infection as a carcinogenic agent class I.

Within the human body, *H. pylori* resides primarily in the gastric mucus layer. This mucus layer consists mainly of the gel-forming mucin MUC5AC, which is, like all gel-forming mucins, a heavily *O*-glycosylated and large glycoprotein (Strous and Dekker, 1992). The gene encoding MUC5AC has been cloned (Escande et al., 2001; Klomp et al., 1995; Van de Bovenkamp et al., 1998), and is expressed by the surface mucous cells of the gastric glands (Nordman et al., 1995). Besides MUC5AC, the normal antral mucosa of the stomach expresses the secretory mucin MUC6, and these two mucins have distinct expression patterns, being expressed by different subsets of cells within the antral glands. MUC6 is mainly expressed in the deeper antrum gland epithelium, whereas MUC5AC is expressed by the surface epithelium (Van den Brink et al., 2000).

Different blood-group antigens, especially Lewis (Le) antigens, are correlated with the distribution of normal gastric mucin expression. Le blood-group antigens are carbohydrate structures carried on both glycoproteins and glycolipids. They are widely distributed in the body, and found not only on erythrocytes, but also in secretions and particularly on epithelial cells. The antigenic specificity is determined by relatively small differences in the sugar composition and inter-residue linkage of the oligosaccharide moieties (Lloyd, 1987). These antigenic structures are carried by two types of O-glycan backbone structures, named type 1 [containing Gal(β1-3)GlcNAc] and type 2 [containing Gal(β1-4)GlcNAc]. Fucosylation of the type 1 backbone structure leads to the expression of the blood group antigens, Lewis A (LeA), Lewis B (LeB), and H-1, whereas fucosylation of type 2 chain leads to the expression of the blood group antigens, Lewis X (LeX), Lewis Y (LeY), and H-2 (Sakamoto et al., 1989). In the human stomach, the expression of MUC5AC is associated with the type 1 blood group structures, LeA and LeB, and MUC6 expression is tightly associated with type 2 antigens LeX and LeY (De Bolos et al., 1995).

A number of molecules have been implied as receptor for *H. pylori* adhesins (reviewed in Evans, 2000). The LeB structure was shown to mediate the attachment of *H. pylori* to human gastric epithelium, when assayed on tissue sections (Boren et al., 1993). The *H. pylori* adhesin, which interacts with LeB, has been cloned and was designated as blood group antigen-binding adhesin (BabA) and belongs to a gene family with approximately 30 members within the *H. pylori* genome (Ilver et al., 1998; Tomb et al., 1997). Separate studies with transgenic mice expressing the human LeB structure in their gastric epithelial cells indicated that LeB can function as a receptor for *H. pylori* adhesins and mediates attachment of *H. pylori* to gastric pit cells and surface mucous cells (Falk et al., 1995).

We previously demonstrated that *H. pylori* in the antrum of infected patients co-localizes *in situ* with extracellular MUC5AC as well as with the apical domain of MUC5AC-

producing cells of the superficial epithelium (Van den Brink et al., 2000). As the expression of MUC5AC is tightly associated with the presence of the LeB antigen in the human stomach, we suggest that MUC5AC, as the main carrier of LeB, constitutes the primary receptor for *H. pylori* attachment in the human stomach. To test this experimentally, we determined the co-localization of LeB and MUC5AC in different tissues of the upper gastrointestinal tract: normal stomach, esophagus and duodenum. We also examined metaplastic tissue of the esophagus (Barrett's esophagus, BE) and of the duodenum (gastric metaplasia of the duodenum, GMD), which were both demonstrated to express MUC5AC within specific cells (Van de Bovenkamp et al., 2003; Warson et al., 2002).

Adherence of *H. pylori* to MUC5AC or LeB was studied by an *in vitro* binding assay with fluorescently-labeled *H. pylori* on sections of each of these tissues. We used three strains of *H. pylori* with closely defined LeB-binding characteristics: (1) CCUG17875, which avidly binds LeB (Ilver et al., 1998); (2) P466, strongly binding LeB (Boren et al., 1993); and (3) *H. pylori* TIGR26695, which is unable to bind to LeB (Ilver et al., 1998).

With the data described, we will show that the MUC5AC and LeB antigens usually strongly co-localize in the epithelia of the human upper gastrointestinal tract. The two LeB-binding *H. pylori* strains applied to the tissue sections co-localized to both MUC5AC and LeB, whereas the non-LeB-binding strain did not bind. Thus, it is probable that MUC5AC, as the main carrier of LeB in gastric epithelium, is the main receptor for *H. pylori* in the human stomach.

Materials and Methods

Patients and Tissue

Biopsy specimens of normal (i.e., non-*H. pylori*-infected and non-inflamed) antrum (n=20) were collected through endoscopy, as part of routine diagnostic endoscopy for upper gastrointestinal complaints. Biopsy specimens from the duodenal bulb (n=12) were retrieved from archive, and were selected for the presence of gastric metaplasia, as determined previously (Van de Bovenkamp et al., 2003). The biopsy specimens were collected in the Academic Medical Center in Amsterdam with permission of the Medical Ethics Committee. Per patient 2 biopsy specimens were obtained from the antrum and duodenal bulb and immediately fixed in phosphate-buffered saline (PBS)/4% (wt/vol.) paraformaldehyde solution for 4 hours and then processed into paraffin blocks according to standard procedures. Tissue samples of normal esophagus and Barrett's esophagus (BE, n =14) were retrieved from archive of the Gastrointestinal Pathology Unit, Catholic University Leuven, Belgium. Per patient, 4 to 9 endoscopic biopsy specimens were available, fixed in Carnoy's fixative, and paraffin embedded. *H. pylori*-infection was assessed in biopsy specimens from the antrum taken as part of the same endoscopic procedure, and the bacterium was detected using both standard histological staining and microbiology.

H. pylori strains

The following *H. pylori* strains were used (Table 1): (1) strain CCUG17875 from the Culture Collection at the University of Göteborg, which binds strongly to LeB; (2) strain P466, which binds strongly to LeB (Falk et al., 1993); (3) strain TIGR26695 from The Institute for Genomic Research (TIGR) is unable to bind to LeB. The genome of TIGR26695 has been fully sequenced (Tombe et al., 1997).

H. pylori strains were grown at 37°C on *Brucella* agar supplemented with 10% (vol./vol.) bovine blood and 1% (vol./vol.) IsoVitalex (Becton Dickinson, Franklin Lakes, NJ, USA) under microaerophilic conditions (5% O_2/10% CO_2/85% N_2) and 98% humidity. Two days after inoculation the bacteria were harvested from a plate and gently resuspended in 500 µl PBS/0.2% (vol./vol.) Tween 20 (Sigma, St. Louis MO, USA), and subsequently used for fluorescent labeling procedure.

Table 1. Characteristics of *H. pylori* strains used in this study.

H. pylori strain	Type I (CagA/VacA)	BabA	References
CCUG17875	+	+	(Ilver et al., 1998; Xiang et al., 1995)
P466	+	+	(Ilver et al., 1998; Su et al., 1998)
TIGR26695	+	-	(Ilver et al., 1998; Tomb et al., 1997)

NOTE: Each of the three *H. pylori strains* is a Type I strain, and carries the CagA pathogenicity islet as well as the VacA toxin gene (indicated by +). The presence of a functional BabA protein is also indicated (+, present; -, absent), which constitutes the structural gene for the BabA adhesin protein, the *babA1* gene being a non-functional pseudo-gene (Ilver et al., 1998).

Binding of ^{125}I-labeled LeB glycoconjugate *H. pylori* strains

The binding assay was performed as previously described by Ilver and colleagues (Ilver et al., 1998). In short, LeB antigen glycoconjugate was prepared by the conjugation of purified fucosylated oligosaccharide to human serum albumin (IsoSep AB, Tullinge, Sweden). LeB glycoconjugate was labeled with ^{125}I by the chloramine-T method. One ml of *H. pylori* bacteria ($OD_{600,}$ 0.10) was incubated with 400 ng of ^{125}I-labeled LeB glycoconjugate for 30 minutes in PBS containing 0.5% (wt/vol.) bovine serum albumin (BSA)/0.05% (vol./vol.) Tween 20. After centrifugation, the radioactivity bound to the *H. pylori* pellet was measured with a gamma-counter. Binding experiments were reproducible and performed in triplicate.

Texas Red labeling of *H. pylori*

H. pylori suspensions were centrifugated at 3000 x g for 5 minutes. Each pellet of bacteria was resuspended in 500 µl 0.15 M NaCl/0.1 M sodium carbonate, pH 9.0, by gently pipetting. Ten microliters of a 20 mg/ml solution of Texas red (TR; Sigma, St. Louis MO, USA), freshly prepared in acetonitrile, was added to 1 ml of bacterial cells ($OD_{600,}$ 1.0), which was followed by incubation for 10 minutes at room temperature in the dark. The bacteria were recovered by centrifugation at 3000 x g for 5 minutes, resuspended in 1 ml PBS/0.2% (vol./vol.) Tween 20, and pelleted by centrifugation as above. The washing cycle was repeated three times. After the

last washing cycle, the bacteria were resuspended in PBS/1% (wt/vol.) BSA, and were diluted to 1.00 OD_{600}. The intensity of TR labeling of all bacterial strains in each preparation was similar as judged by inspection of comparable numbers of organisms by fluorescence microscopy.

Immunohistochemistry and *H. pylori* binding to tissue sections

Tissue sections were deparaffinized through three changes of xylene and then rehydrated through a series of decreasing concentrations of ethanol solution to distilled water. Antigen retrieval was performed by heating the sections for 10 minutes at 100°C in 10 mM citrate buffer, pH 6.0, and then left to cool to room temperature for 20 minutes. Sections were washed three times for 5 minutes in PBS and incubated with blocking buffer (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% (wt/vol.) gelatin, 0.05% (vol./vol.) Tween20), pH 8.0, for 30 minutes, followed by washing three times for 5 minutes in PBS/0.2% (vol./vol.) Tween 20. Primary monoclonal antibody diluted in PBS was added, either anti-MUC5AC (45M1; Novocastra, Newcasle, UK; diluted 1:50), or anti-Lewis B, (Signet laboratories, Dedham, MA, USA; diluted 1:40), and incubated for 16 h at 4°C. Sections were washed three times for 5 minutes in PBS/0.2% (vol./vol.) Tween 20, followed by incubation for 1 hour in the dark with PBS-diluted (1:50), fluorescein isothiocyanate (FITC)-labeled anti-mouse secondary antibody (DAKO, Glostrup, DK). Again, the sections were washed three times for 5 minutes in PBS/0.2% (vol./vol.) Tween 20. Per section, 40 µl (1.0×10^8 cfu/ml) of TR-labeled *H. pylori* was added and incubated for 60 min in the dark at room temperature. Slides were washed 5 times for 5 minutes in PBS. Finally, sections were covered in Vectashield (Vector laboratories, Burlingame, UK), and were mounted under cover slips. Co-localization of immunofluorescent staining of tissue antigens and bacteria was analyzed by fluorescent microscopy using a TR/FITC double filter block (NIKON, Tokyo, Japan). Control stainings were performed leaving each of the primary antibodies out of the procedure, resulting in absence of FITC-staining. All binding assays on the tissue of each patient were performed at least in duplicate. The results were highly reproducible.

Separately, closely adjacent sections of each tissue sample of each patient were stained for three other major secretory mucins, MUC2 (monoclonal antibody: WE9; a gift of prof. D.K. Podolsky; diluted 1:50 (Tytgat et al., 1995)), MUC5B (polyclonal antibody: BGBM; a gift from dr. G. Offner; diluted 1:3000 (Van Klinken et al., 1998)), and MUC6 (polyclonal antibody: 6.1; a gift from dr. C. de Bolos; diluted 1:200 (De Bolos et al., 1995)). Single immunohistochemical staining was performed using specific antibodies against each of these mucins and by standard horseradish peroxidase/ diamino-benzidine-based staining according to previously reported procedures (Van de Bovenkamp et al., 2003; Warson et al., 2002).

Results

Binding of soluble LeB conjugate to *H. pylori*

To ascertain the expression of functional LeB antigen-binding adhesin, BabA, each of the *H. pylori* strains was examined for binding soluble LeB conjugate. Strains CCUG17875 and P466 were able to bind LeB conjugate, whereas TIGR26695 barely bound soluble LeB conjugate (Table 2). This binding experiment was performed in triplicate and showed high reproducibility, which confirmed expression of functional BabA for strains CCUG17875 and P466.

Table 2. Binding of soluble LeB glycoconjugate to different *H. pylori* strains.

H. pylori strain	Binding (%)	Range (%)
CCUG17875	28.6	27.2-30.0
P466	21.8	20.7-22.9
TIGR26695	0.7	0.6-0.8

NOTE: Binding of *H. pylori* in suspension to soluble ^{125}I-labeled LeB glycoconjugate was measured by gamma counting of bacteria that were pelleted after 1 hour incubation and extensive washing. Binding is expressed as percentage of the total amount of ^{125}I conjugate added to each of the bacterial suspensions. Data are presented as mean percentage of maximal radioactivity and range of three separate experiments.

Binding of *H. pylori* to gastric epithelium

Antrum biopsies were collected from 20 patients. None of these patients suffered from *H. pylori*-infection as determined by standard histochemistry and microbiology, and the tissue showed no signs of acute or chronic inflammation as assessed by standard hematoxylin and eosin staining (data not shown). Abundant expression of the MUC5AC glycoprotein in the epithelial cells of the surface and of the neck of the antrum glands could be identified in each patient. In addition, extracellular remnants of the gastric mucus-layer were observed, and also these were MUC5AC-positive. As reported earlier (Van de Bovenkamp et al., 2001), staining of adjacent tissue sections of each patient with antibodies against the other major secretory mucins, MUC2, MUC5B, and MUC6, showed that the MUC5AC-positive cells did not contain any of these other secretory mucins (not shown).

Sixteen of the 20 patients showed expression of the blood group antigen LeB in the gastric surface epithelium, as determined with immunofluorescent staining. If a patient expressed LeB, the expression co-localized very strongly with MUC5AC-positive cells as well as with extracellular MUC5AC-positive mucus (Fig. 1, see *Appendix*). In all 20 patients the binding of strains CCUG17875 and P466, was observed to the MUC5AC-positive epithelial cells of the surface and the neck region of the glands and also to the MUC5AC-positive extracellular mucus (Figs 1A and B, *Appendix*). Strain TIGR26695, which was unable to bind to soluble LeB conjugate, showed no binding to the epithelium or the mucus in any of the patients (Fig. 1C, *Appendix*). As LeB showed a very strong co-localization to MUC5AC, strains CCUG17875 and P466 bound very specifically to the LeB-containing cells and mucus, while strain TIGR26695 did not bind to LeB-positive structures in the tissue (Figs 1D-F,

Appendix). Interestingly, in 4 patients, in whom we were not able to detect LeB by immunofluorescence, the strains CCUG17875 and P466 bound to MUC5AC-positive cells and mucus, while again no binding was found for strain TIGR26695 (not shown).

When bacteria were added to the tissue sections without prior staining by antibodies directed towards either MUC5AC or LeB, more bacteria were able to bind to MUC5AC-containing cells and mucus (not shown). This effect was only found for the LeB-binding strains, CCUG17875 and P466, while the binding behavior of TIGR26695 towards the tissue sections was not changed by previous staining with these antibodies.

Binding of *H. pylori* to esophagus and BE epithelium

Recently we showed that BE epithelium expressed the gastric-type mucin MUC5AC (Warson et al., 2002). We were interested if *H. pylori* was also able to bind to MUC5AC or LeB in sections of BE tissue and normal esophagus epithelium. The normal stratified epithelium of the esophagus did not express MUC5AC or LeB, and moreover this epithelium showed no adherence of any bacteria upon addition of the TR-labeled *H. pylori* strains to the tissue sections (not shown).

MUC5AC was expressed abundantly in tissue sections of all BE patients (n=14), and was localized in the surface epithelium of BE, and to a variable extent deeper into the glandular structures of the BE epithelium. In the patients in whom LeB expression was detected by immunofluorescence (in 7 of 14 patients), the expression pattern of LeB co-localized very strongly with the MUC5AC expression pattern (Fig. 2, see *Appendix*). In each BE patient binding to the BE epithelium was observed for both the strains CCUG17875 and P466. The binding of these strains of *H. pylori* bacteria perfectly co-localized with MUC5AC-positive cells in the BE epithelium, whereas strain TIGR26695 showed no specific binding to BE epithelium (Figs 2A-C, *Appendix*). As LeB strongly co-localized with MUC5AC in these BE tissues, the same binding pattern was found as for MUC5AC (i.e., strains CCUG17875 and P466 were found to specifically bind to the LeB-positive epithelium, whereas strain TIGR26695 showed no specific binding to LeB-positive epithelium) (Figs 2D-F, *Appendix*).

Interestingly, in the tissue of 7 patients identified with BE and in whom LeB could not be demonstrated by immunofluorescence, binding of strains CCUG17875 and P466 was specifically found to the MUC5AC-positive epithelial cells of BE (Figs 3A-D, see *Appendix*). Also in these LeB-negative tissues, strain TIGR26695 showed no specific binding to the BE epithelium (not shown).

Like in the normal gastric tissues, it appeared that BE tissues, to which no antibodies were bound, bound more bacteria than sections that were effectively stained with antibodies. For example, when BE tissue sections did not bind any anti-LeB antibodies (as in Figs 3B and 3D, *Appendix*), these sections bound more bacteria than after effective binding of the anti-MUC5AC antibodies (compare Figs 3A with 3B, and 3C with 3D, *Appendix*). This effect was found for both the LeB-binding strains, CCUG17875 and P466 (Figs 3A-D, *Appendix*), while the binding characteristics of TIGR26695 were not changed by previous incubation with either of these antibodies (not shown).

Binding of *H. pylori* to small intestinal tissue and GMD

Previously, others and we demonstrated that MUC5AC in the duodenum was restricted to cells of GMD and occasional goblet cells within the normal small intestinal epithelium (Shaoul et al., 2000; Van de Bovenkamp et al., 2003). Immunohistochemical staining for other secretory mucins on tissue sections of the GMD patients in this study revealed that MUC2 was expressed in all goblet cells of the small intestinal epithelium, and that the Brunner's glands, which were present in most of the biopsy specimens, produced MUC5B and MUC6 but no MUC5AC or MUC2 (not shown), as we demonstrated previously for a large number of individuals (Van de Bovenkamp et al., 2003).

Twelve patients were selected from archive in whom gastric metaplasia was detected in duodenal bulb biopsy specimens. MUC5AC expression was unequivocally found in these patients in patches of metaplastic cells, and in a few goblet cells within the villi of the normal epithelium (Fig. 4, see *Appendix*). In contrast to both antrum and BE tissue, *H. pylori* only adhered to duodenal tissue samples in which we were able to demonstrate LeB by immunofluorescence (i.e., in 9 of 12 patients). In these tissue samples LeB was found in the cells of the GMD, the brush border of the normal enterocytes and in a subset of the normal goblet cells. In 9 of 12 patients in whom we detected LeB-positive small intestinal tissue, there was co-localization between LeB and MUC5AC in the epithelial cells of the GMD and in occasional goblet cells of the normal duodenal epithelium (Fig. 4, *Appendix*). In the small intestinal tissue from three patients in which we could detect no LeB, no specific adherence of *H. pylori* could be demonstrated with any of the three strains (not shown). In LeB-positive tissue, strains CCUG17875 and P466 showed specific binding to MUC5AC-positive cells of the GMD epithelium and to the occasional MUC5AC-positive goblet cells (Figs 4A, B, G, and H, *Appendix*). No specific binding of strain TIGR26695 to MUC5AC-positive cells of the GMD or to MUC5AC-positive goblet cells in the normal epithelium was found (Figs 4C and I, *Appendix*).

In GMD, there was a strong co-localization of LeB with MUC5AC, therefore the adherence of the three *H. pylori* strains to the LeB-positive cells of the GMD was very similar as described above for MUC5AC (Figs 4D-F, *Appendix*). However, the most extensive LeB expression was found in the brush border of the intestinal enterocytes, and in the goblet cells of normal duodenum epithelium (Figs 4J-L, *Appendix*). Binding of strains CCUG17875 and P466 was found to the brush border of enterocytes, and to the goblet cells in those tissue samples in which we were able to show LeB, co-localizing specifically with LeB in the brush border and these goblet cells (Figs 4J and K, *Appendix*). Strain TIGR26695 showed no specific adherence to the LeB-positive brush border of the enterocytes or to goblet cells (Fig. 4L, *Appendix*). Upon immunohistochemical staining of the duodenal tissue sections with antibodies against MUC5AC or LeB, the Brunner's glands in the mucosa of the duodenum were not stained for MUC5AC or LeB, respectively. None of the three *H. pylori* strains showed any adherence to the epithelial cells of the Brunner's glands, both in LeB-positive and –negative small intestinal tissue samples, as detected by immunofluorescence (not shown).

Discussion

Helicobacter pylori is known to reside in the gastric mucus-layer, often close to or attached to the mucus-producing epithelium (Shimizu et al., 1996a; Shimizu et al., 1996b; Van den Brink et al., 2000). Therefore, it is of great interest to establish what is so special about this gastric mucus and gastric mucus-producing cells that *H. pylori* is able to colonize this niche. As we demonstrated previously, *H. pylori* in the antrum of infected patients always co-localizes very specifically with MUC5AC and MUC5AC-producing cells (Van den Brink et al., 2000). MUC5AC is a secretory, mucus-forming mucin that is characteristically produced in very large amounts in the gastric mucosa, making it a likely candidate for a *H. pylori* receptor. Another well-characterized gastric receptor for *H. pylori* is the Lewis B blood group structure, which is a carbohydrate structure primarily present on O-linked glycans (Boren et al., 1993). Mucins are heavily *O*-glycosylated structures and were demonstrated to carry, among others, Lewis antigens, including LeB (Nordman et al., 2002). In order to make both ends of this story meet, our hypothesis is that MUC5AC is the primary gene product that carries the LeB carbohydrate structures, which may form the foothold for *H. pylori* in one of the steps necessary to colonize the human stomach. Thereto, we studied the co-localization of *H. pylori* added onto tissue sections with the presence of both MUC5AC and LeB within the tissue. If *H. pylori* would co-localize to both these entities this would support our hypothesis.

First, we have to consider the uniqueness of MUC5AC as the mucus-forming mucin in the human stomach. Gastrointestinal mucus is generally composed of secretory mucins of the MUC-family, of which four members are generally considered to have the ability to form mucus-gels: MUC2, MUC5AC, MUC5B, and MUC6 (Dekker et al., 2002). Each of these mucins shows a very distinct expression pattern along the human gastrointestinal tract (Reid and Harris, 1998; Van Klinken et al., 1997), indicating distinct and specific functions for each of these four secretory mucins. In the normal esophagus, only MUC5B is produced in the sub-mucosal glands, whereas the surface epithelium does not produce gel-forming MUCs (Arul et al., 2000). However, within Barrett's esophagus (BE), which constitutes gastric-type and intestinal-type metaplastic epithelium of the esophagus, all four mucus-forming MUCs are expressed, although MUC2 is restricted to intestinal-type metaplasia in BE (Warson et al., 2002). The epithelium of the gastric antrum, which forms the natural habitat of *H. pylori* in infected patients, produces MUC5AC in the superficial epithelial cells and the cells of the gastric pits (De Bolos et al., 1995; Ho et al., 1995; Van den Brink et al., 2000). MUC5B and MUC6 are produced in the epithelial cells lower in the antral glands (De Bolos et al., 1995; Ho et al., 1995; Longman et al., 2000; Van den Brink et al., 2000), whereas MUC2 is not produced at all in the normal stomach. At the exit of the stomach, the MUC expression pattern again changes dramatically. The small intestinal epithelium contains characteristic goblet cells expressing MUC2 (Chang et al., 1994). The Brunner's glands, which are characteristic for the proximal duodenum, produce both MUC5B and MUC6 (Ho et al., 1995; Longman et al., 2000; Van de Bovenkamp et al., 2001). MUC5AC is only found in rare goblet cells in the normal intestinal villi, however MUC5AC is expressed unequivocally in all cells of gastric metaplasia that are found in the proximal duodenum (Shaoul et al., 2000; Van de Bovenkamp

et al., 2003). Thus, MUC5AC is very characteristic of the normal habitat of *H. pylori*, which is the surface epithelium of the normal stomach, whereas it is normally absent from both esophagus and duodenum.

Second, we have to consider mucins as carriers of carbohydrates, in particular the human Lewis-type blood group antigens. The expression of Lewis antigens is related to the ABO-blood groups, and is dependent on the "secretor" status of the individual. LeB, like Lewis Y, is only expressed in those individuals ("secretors") that produce the α(1,2)-fucosyltransferase necessary to confer the H-structure to the glycans (Kelly et al., 1995; Lopez-Ferrer et al., 2000). In the Western population, about 80% of the individuals are "secretors", and are able to generate LeB antigens on their carbohydrates. Non-secretors who lack the respective α(1,2)-fucosyltransferase have only very low amounts of LeB on their carbohydrates, in the range of 1-5% of the levels of secretors (Sakamoto et al., 1989). LeB is present abundantly in gastric mucus of secretors, as a carbohydrate side chain of MUC5AC (Nordman et al., 2002), but it is also expressed in the normal esophagus (Davidson and Triadafilopoulos, 1992), the duodenum (Davidson and Triadafilopoulos, 1992; Mollicone et al., 1985), and in some areas of metaplasia in the esophagus and intestine (Kobayashi et al., 1993; Murata et al., 1992). Therefore, the distribution of LeB in the upper gastrointestinal tract is more widespread than the distribution of MUC5AC. The affinity of *H. pylori* towards LeB has been demonstrated in an *in situ* adherence assay. In this assay fresh *H. pylori* adhered to fixed sections of human gastric tissue, whereas both soluble LeB and a monoclonal antibody to LeB interfered with this adhesion (Boren et al., 1993). The bacterial adhesin responsible for LeB antigen binding, BabA, was more recently identified (Ilver et al., 1998). Although the exact pathophysiological role remains to be shown for infection of the human stomach, the BabA-LeB interaction may be an important factor in the outcome of infection, since it worsened the severity of gastritis in transgenic mice, expressing human LeB (Guruge et al., 1998). Nevertheless, both the transgenic animals carrying the LeB structure and their LeB-negative controls were eventually colonized by *H. pylori*. Moreover, it was shown very recently that the *H. pylori babA2* gene is strongly associated to *H. pylori* strains that have caused severe disease (Gerhard et al., 1999). This suggests that *H. pylori* strains, which colonize the epithelium without the expression of BabA adhesin, resulted in a less severe gastric disease. Some workers showed that LeB may not play an essential role in the colonization process of *H. pylori in vivo* in patients (Oberhuber et al., 1997), and *in vitro* experiments in a model system using isolated human gastric epithelial cells (Clyne and Drumm, 1997). It seems likely that the adherence of *H. pylori* via its BabA receptor to the host LeB structures effectively facilitates infection, but is not an absolute necessity for eventual colonization.

In normal stomach and in epithelium of BE, MUC5AC and LeB co-localize to the same epithelial cells and to the extracellular mucus. The *H. pylori* bacteria added to the tissue sections of antrum and BE co-localized very well to both MUC5AC as well as LeB. Only the two LeB-binding strains (CCUG17875 and P466) bound to the cells and mucus, whereas the

strain that is not able to bind to LeB (TIGR26695) did not bind to the tissue. This strongly suggests that LeB carried on the MUC5AC molecules functions as a receptor for *H. pylori*.

We have found indications in both antrum and BE epithelium that *H. pylori* was sometimes able to bind to epithelial cells that could be stained for MUC5AC, whereas we were unable to detect LeB through immunofluorescence. In these seemingly LeB-negative tissues, strains CCUG17875 and P466 bound to MUC5AC-positive cells, which may indicate that other structures of MUC5AC may also function as receptors. Yet, this binding could only be demonstrated for the two strains carrying a functional BabA adhesin. The individuals in whom we were not able to detect LeB, most probably constitute non-secretors, who are known to still express 1-5% of the levels of LeB relative to secretors (Sakamoto et al., 1989). *H. pylori* is possibly able to bind to these residual LeB in the non-secretor tissues.

In the duodenum, *H. pylori* is, in infected patients, always found closely associated with gastric metaplastic cells (Gisbert et al., 2000; Khulusi et al., 1996; Noach et al., 1994; Wyatt et al., 1990). Indeed, we were able to demonstrate co-localization of *H. pylori* added to duodenal tissue sections with MUC5AC as well as LeB present in metaplastic cells. However, in LeB-positive individuals the *H. pylori* bacteria of strains P466 and CCUG17875 also bound to the LeB-positive brush border on the normal villus enterocytes. These strains demonstrated, at least under these experimental circumstances *in vitro*, the ability bind to LeB-structures that are not positioned on MUC5AC. Thus, there is a notable discrepancy between the binding of *H. pylori in vitro* and *in vivo* when the binding to the brush border is considered. A likely explanation is that the *H. pylori* bacteria that may become attached to the brush border of the duodenum *in vivo* do not survive on this surface. It was previously noted that the density of infection of the patches of metaplastic cells in the duodenum was very low, when compared to population densities in the stomach (Gisbert et al., 2000; Khulusi et al., 1996; Noach et al., 1994; Wyatt et al., 1990). This indicates that the circumstances for survival of *H. pylori* in the duodenum are far from optimal. Therefore, *H. pylori* might only be able to survive in very close association with the MUC5AC-producing, gastric-type metaplastic epithelial cells, as demonstrated by electron microscopy (Noach et al., 1994).

It was found consistently in each of the separate experiments that the bacteria of the LeB-binding strains, CCUG17875 and P466, bound better to the tissue sections when these sections were not previously stained with antibodies against MUC5AC or LeB. The antibodies, which bound to either MUC5AC or LeB within the tissue sections, apparently hindered the binding of the bacteria in the second step of our co-localization protocol. Although these binding assays are not quantitative, it is very likely that *H. pylori* and the anti-MUC5AC and anti-LeB antibodies compete for binding to the same structures in the tissue sections. Apparently, these respective antibodies are able to block the interaction between the BabA protein on the *H. pylori* strains and LeB or MUC5AC in the tissue sections.

In summary, we demonstrated *H. pylori* binding to mucin MUC5AC in gastric tissue, Barrett's esophagus epithelium and gastric metaplastic cells in the duodenum. Only *H. pylori* strains that were able to bind to LeB adhered to MUC5AC in the tissue sections. We showed

that MUC5AC is probably the most important carrier of LeB structures in these tissues and forms a major receptor for the bacterium.

Acknowledgements

This research was financially supported by the Dutch Digestive Diseases Foundation (Nieuwegein), Irene Children's Hospital Foundation (Arnhem), Jan Dekker/Ludgardine Bouman Foundation (Amsterdam) and Foundation "De Drie Lichten" (Leiden), all in the Netherlands (JHBvdB). TB was supported by the Swedish Medical Research Council (11218) and the Swedish Cancer Society (4101-B00-03XAB). The authors thank prof dr N.L.E.Y. Ectors (Leuven, Belgium), dr E.H. van Beers (Amsterdam, the Netherlands) and T.M. Rolf (Amsterdam, The Netherlands) for obtaining the human biopsy specimens.

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Chapter 8

Summarizing discussion

Summarizing discussion

In this Chapter the overall results of the preceding chapters will be combined and discussed, and this discussion will be focused on MUC5AC as receptor for the human gastric pathogen, *Helicobacter pylori*. For extensive and detailed discussion of the expression patterns of the different secretory proteins in normal and metaplastic tissue, and the possible light that these expression patterns may shed on the pathogenesis of metaplasia and carcinogenesis, the reader is referred to the separate Chapters 3-6.

The epithelial lining of the upper gastrointestinal tract, from the stomach down to the large intestine is covered by a mucus-layer that protects the mucosa against toxic substances and noxes like acid, microorganisms, mechanical and proteolytic erosion. This mucus layer is formed by gel-forming secretory mucins of the MUC-type mucins family, which are large glycoproteins (Dekker et al., 2002). If epithelial damage occurs, mucin expression is increased together with mucin-associated trefoil factor family peptides (TFFs). TFFs are small bioactive molecules, which are involved in the protection of gastrointestinal epithelium, and act in general by promoting restitution, i.e. the moving of cells across lesions in the epithelium without cell proliferation. These two groups of molecules are most often produced in the same epithelial cells, like the mucous cells in the stomach, the goblet cells in the intestinal epithelium, and mucous acini of the Brunner's glands. Collectively these two groups of molecules seem a powerful warden against epithelial damage. The gel forming mucins holds the danger at bay, whereas the TFFs are instrumental in the fast healing of small lesions, may these occur. The members of these two families are however produced in various combinations, that are both tissue and cell type specific (Chapters 3-6). The details of this tissue and cell type specificity are quite clear from the work of others (Hoffmann and Jagla, 2002) and as described in Chapters 3-6. However the specific function(s) of each of the molecules of these families, either MUCs or TFFs, is not described. Therefore the functions of the tissue-specific combinations of MUCs and TFFs have so far no understood function.

The infection by *H. pylori* is most and for all a gastric antrum phenomenon. In general, normal gastric epithelium of the antrum is characterized by the expression of the secretory mucins: MUC5AC, MUC5B, and MUC6, and trefoil factor family peptides: TFF1, TFF2, and TFF3. As demonstrated in chapter 3, the expression of the MUC5AC (the alleged *H. pylori* receptor), was confined to the surface epithelium of the gastric epithelium. Intestinal metaplasia (IM) of the stomach is characterized by the expression of MUC2, TFF1, TFF2, and TFF3, whereas expression of MUC5AC, MUC5B, and MUC6 is diminished in IM, as discussed in Chapter 3. These results show that the expression of MUC2 is a marker for gastric IM (summarized in Table 1), but also that the expression of the alleged *H. pylori* receptor, MUC5AC is diminished in gastric IM. The latter is well corroborated by the fact that *H. pylori* is normally not found in association with IM in the stomach mucosa (Craanen et al., 1992; Testoni et al., 1995), which lends further support to the idea that MUC5AC is a receptor for the bacterium.

Table 1. Summary of protein expression of secretory mucins (MUC) and trefoil factor family (TFF) peptides in normal and metaplastic tissues of the human upper gastrointestinal tract.

	STOMACH ANTRUM		ESOPHAGUS			DUODENUM		
				Barrett's epithelium				
	NE	IM	NE[a]	GM	IM	NE[b]	GM	BG
MUC2	-	+	-	-	+	+	-	-
MUC5AC	+	-	-	+	+	-	+	-
MUC5B	+	-	-	+	+	-	-	+
MUC6	+	-	-	+	+	-	+	+
TFF1	+	+	-	+	+	+	+	-
TFF2	+	+	-	+	+	+	+	+
TFF3	+	+	-	+	+	+	-	+

NOTE: Protein expression is indicated as present if the protein is expressed in at least 50% of the patients. NE, normal epithelium; IM, intestinal metaplasia; GM, gastric metaplasia; BG, Brunner's Glands. +, present; -, absent. [a] Indicated is expression in normal stratified epithelium of the esophagus. [b] Indicated is expression in the goblet cells of the villus/crypt epithelium.

In normal stratified esophagus epithelium neither secretory mucins nor TFF peptides are expressed. MUC5AC is, like any other secretory MUC, not expressed normal esophagus epithelium, but MUC5AC-expressing cells are a very common constituent of the metaplastic epithelium of the esophagus. Despite the abundant expression of MUC5AC, *H. pylori* is only very seldom found in association with the BE epithelium. Nevertheless indications have been found that the bacterium can be found *in situ* at BE epithelium (Ectors et al., 1993). Metaplastic tissue of the esophagus (Barrett's esophagus, BE) is classified in gastric metaplasia (GM) and intestinal metaplasia (IM). The IM of the esophagus has some superficial likeness to the IM of the stomach mucosa. Gastric metaplasia in BE is characterized by the expression of the same secretory mucins and TFF peptides as found in the normal gastric epithelium of the antrum, whereas the intestinal type of BE metaplasia expressed all of the secretory mucins and TFF peptides investigated, including MUC2 (Table 1; Chapter 4 and 5). MUC2 was identified as an unequivocal marker of IM in the BE. Interestingly, when the MUC2 expression was more widespread in patients, the orderly gastric-type mucin and TFF-peptide expression resembling the antrum epithelium was increasingly disturbed. This may indicate that MUC2 expression is sign that the BE epithelium may evolve towards malignancy.

H. pylori was associated with metaplastic cells in the proximal duodenum, which was demonstrated by electron microscopy (Noach et al., 1993; Noach et al., 1994). These cells were long recognized for their gastric epithelial morphology. We were however the first to find that these cells actually have a gastric phenotype, resembling antral epithelial cells. The most important observation of secretory protein expression in the duodenum, in this respect, is the expression of gastric-type mucins (MUC5AC and MUC6) and TFF peptides (TFF1 and TFF2) in GM of the duodenum (Table 1). In particular MUC5AC is uniquely and exclusively expressed in the GM of the duodenum. This may indicate that this ectopic MUC5AC

expression may form the foothold for the *H. pylori* found in association with the cells of the GM in the duodenum. Another aspect of the expression of MUC5AC in the GM cells in the duodenum sheds light on their origin. MUC5AC is further only found in sporadic intestinal goblet cells, but not otherwise in the intestinal epithelium, which led us to hypothesize that these sporadic goblet cells are the precursors of the GM in the duodenum.

Taken together, the analyzed metaplastic tissues of the esophagus and duodenum express the mucin MUC5AC, which is the most abundant component of the gel-forming gastric mucus, as was shown in Chapter 2. The MUC5AC molecule could play a prominent role in the process of *H. pylori* infection, as was shown in Chapter 7. It was demonstrated that the MUC5AC in tissue sections of these metaplastic epithelia was able to bind *H. pylori* (Chapter 7). The possible implications of the ectopic expression of MUC5AC in esophagus and duodenum are discussed later in this chapter.

To determine if the presence of gastric *H. pylori* affects protein expression in stomach, esophagus and duodenum, the normal and metaplastic tissues of those organs were analyzed for mucin and TFF peptide expression as summarized in Table 2. It became clear that gastric *H. pylori*-infection was most strongly associated with changes in gastric epithelium and gastric metaplasia epithelium of the duodenum. As described for the gastric antrum in Chapter 3, the MUC5B- and MUC6-expressing cells were increased, whereas MUC5AC-expressing cells were diminished. In gastric metaplasia of the duodenum all gastric-type markers (i.e. MUC5AC, MUC6, TFF1, and TFF2) were increased (Table 2, and Chapter 6). Interestingly there were no correlations found between gastric *H. pylori* infection and the expression of MUCs and TFFs in the epithelium of the Barrett's esophagus.

Table 2. Summary of correlations between gastric *H. pylori*-infection and the changes in expression of secretory mucins (MUC) and trefoil factor family (TFF) peptides in normal and metaplastic tissues of the human upper gastrointestinal tract. The changes in expression of the MUCs and TFFs involve changes in the number of cells expressing the respective proteins.

	STOMACH ANTRUM		ESOPHAGUS			DUODENUM		
				Barrett's epithelium				
	NE	IM	NE[a]	GM	IM	NE[b]	GM	BG
MUC2	-	0	-	-	0	0	-	-
MUC5AC	↓	-	-	0	0	-	↑	-
MUC5B	↑	-	-	0	0	-	-	0
MUC6	↑	-	-	0	0	-	↑	0
TFF1	↓	0	-	0	0	0	↑	-
TFF2	↓	0	-	↓	0	0	↑	0
TFF3	0	0	-	0	0	0	-	↓

NOTE: Protein expression is shown if at least 50% of the patients are positive for a certain protein. NE, normal epithelium; IM, intestinal metaplasia; GM, gastric metaplasia; BG, Brunner's Glands. 0, no correlation between expression levels and the presence of gastric *H. pylori.* -, Protein is not expressed; ↑, expression increased; ↓, expression is decreased. [a] Indicated is expression in normal stratified epithelium of the esophagus. [b] Indicated is expression in the goblet cells of the villus/crypt epithelium.

Now (1) the different normal and metaplastic tissues of the upper gastrointestinal tract were analyzed for MUC and TFF expression, and (2) the influence of gastric *H. pylori*-infection on the expression patterns of these proteins had been studied, we analyzed if (3) *H. pylori* was able to bind to any of the mucins in these tissue structures (Table 3, and Chapter 7). Strikingly, *H. pylori* adhered to each of these tissues, wherever MUC5AC was expressed. One notable exception was the normal duodenum; here *H. pylori* was found to co-localize with the MUC2/Lewis B-producing goblet cells, as discussed in Chapter 7 (Table 3). A very strong co-localization was found between *H. pylori* and the Lewis B blood group antigen, which is a carbohydrate structure, carried on O-linked carbohydrate structures such as in mucins. In fact Lewis B co-localized very specifically with MUC5AC in these different tissues. Lewis B was shown to be a prominent constituent of gastric MUC5AC in several independent studies (De Bolos et al., 1995; Linden et al., 2002; Nordman et al., 2002). In the light of this triple co-localization in the *in vitro* adhesion studies on tissue section, involving MUC5AC, Lewis B and *H. pylori*, it seems very likely that the Lewis B structures on the MUC5AC molecules form the actual receptor structures for *H. pylori*. This implies that the BabA adhesin of *H. pylori*, since it was identified earlier as being responsible for binding to Lewis B (Boren et al., 1993), is instrumental in the binding of the bacterium to MUC5AC. Our identification as Lewis B on MUC5AC, as an important receptor in the onset of *H. pylori*-infection, was very recently corroborated by others (Linden et al., 2002). As infection of patients becomes persistent and the ensuing inflammation chronic, other blood group antigens like sialyl Lewis X likely become important for *H. pylori* binding. *H. pylori* SabA adhesin, which binds to this sialyl Lewis X, has recently been discovered (Mahdavi et al., 2002).

Table 3. Summary of the adherence of *H. pylori* to secretory mucins in normal and metaplastic tissues of the human upper gastrointestinal tract.

	STOMACH ANTRUM		ESOPHAGUS			DUODENUM		
				Barrett's epithelium				
	NE	IM	NE[a]	GM	IM	NE[b]	GM	BG
MUC2	0	-	0	0	-	+[c]	0	0
MUC5AC	+	0	0	+	+	0	+	0
MUC5B	-	0	0	-	-	0	0	-
MUC6	-	0	0	-	-	0	-	-
Lewis B	+	-	-	+	+	+[c]	+	0
Lewis B/MUC5AC[d]	+	n.a.	n.a.	+[e]	+[e]	n.a.	+	n.a.

NE, normal epithelium; IM, intestinal metaplasia; GM, gastric metaplasia; BG, Brunner's Glands. 0, not present in this epithelium; +, adhesion of *H. pylori*; -, no adhesion of *H. pylori*; n.a., not applicable. [a] Indicated is expression in normal stratified epithelium of the esophagus. [b] Indicated is expression in the goblet cells of the villus/crypt epithelium. [c] Indicated is adherence of *H. pylori* to the Lewis B-producing brush border of the enterocytes, and the MUC2/Lewis B-producing duodenal goblet cells. [d]: +, indicates co-localization of *H. pylori* with both MUC5AC and Lewis B. [e]: In some patients *H. pylori* co-localized to MUC5AC, in absence of detectable levels of Lewis B.

During infection, the antral epithelium becomes heavily infested by the bacterium, which implies that *H. pylori* likely affects the structure and/or synthesis of MUC5AC. The effects of *H. pylori* on the gastric gel-forming mucus layer have been studied with conflicting results.

One study shows that *H. pylori*-infection per se does not decrease the thickness of the mucus barrier. However, in the same study, in individuals with *H. pylori*-associated gastric atrophy, a small, but statistically significant, decrease in mucus thickness was observed (Newton et al., 1998). In contrast, one study has shown as much as 50% decrease in mucus thickness in *H. pylori*-infected individuals (Sarosiek et al., 1991). Analysis of gastric mucins from *H. pylori*-infected and non-infected patients showed no decrease in viscosity of the gastric mucus gel (Markesich et al., 1995). An *in vitro* study on the MUC5AC-producing KATO III human gastric cell-line, showed an *H. pylori*-depended decrease in expression of MUC5AC (Byrd et al., 2000). In addition, the Lewis carbohydrate structures of gastric epithelial mucins are altered during *H. pylori*-infection (Byrd et al., 1997; Mahdavi et al., 2002; Ota et al., 1998). The above studies showed that *H. pylori* does not affect the composition of the mucus gel-layer. That is, MUC5AC is also the most important constituent of the gastric mucus layer during infection by *H. pylori*, and no other secretory mucins are apparently induced by the infection. Conversely, from these studies the MUC5AC expression seems consistently decreased.

On the other hand, several reports indicate that *H. pylori*, as are other bacteria, are able to stimulated mucin secretion. Lipopolysaccharide (LPS) from *H. pylori* has been reported to transiently stimulate mucin secretion in gastric mucosa (Liau et al., 1992). Another example of enhanced MUC5AC transcription and translation was shown in patients suffering from cystic fibrosis (CF) lung disease (Dohrman et al., 1998). In CF patients, who suffered from infection with the Gram-negative bacterium *Pseudomonas aeruginosa*, an up-regulation of MUC5AC mRNA and protein was detected compared with non-infected CF patients. Components of *P. aeruginosa* that mediate this up-regulation were not identified. However, in the infected CF patients the expression of inflammatory mediator tumor necrosis factor-α (TNF-α) was also increased. It has been demonstrated in a HT29-MTX MUC5AC-secreting goblet cell-line (Lesuffleur et al., 1993), that TNF-α stimulates the production and secretion of MUC5AC (Smirnova et al., 2000). If we turn back to *H. pylori*-infected patients, it was shown that TNF-α production is stimulated during infection (Crabtree et al., 1991), which could lead to enhanced MUC5AC production. Thus, it can be possible that *H. pylori* is capable of inducing its own receptor. In a recent study, on *H. pylori*-infected patients, it was shown that TNF-α was expressed by the antral G cells, and that in these same cells the expression of activated transcription factor NF-κB was enhanced (Van den Brink et al., 2000). This transcription factor mediates both the acute and chronic inflammation reactions through the regulation of many pro-inflammatory proteins (Barnes and Karin, 1997). One of these pro-inflammatory proteins is TNF-α. Thus, *H. pylori* very likely stimulates the gastric MUC5AC expression and secretion indirectly via induction of enhanced TNF-α expression, which is stimulated by NF-κB that is also up-regulated by the infection with *H. pylori*. This autocrine stimulation of protein expression (i.e., MUC5AC as receptor for *H. pylori*) due to the inflammation induced by *H. pylori*, has a clear advantage for the bacterium.

Another possible route, by which MUC5AC expression could be increased, is via direct transcriptional activation of the MUC5AC gene. We have cloned the 5'-end of the MUC5AC gene (Chapter 2), and found that the promoter region contained two potential NF-κB binding sites. *H. pylori* induces, directly or indirectly, gastric NF-κB expression, and the activated NF-κB protein can bind to the MUC5AC promoter-region to increase transcription. Once again this seems to be in the advantage of the bacterium, as it induces its own receptor.

H. pylori seems to proliferate almost exclusively on human gastric epithelial cell surfaces and within the overlying mucus, as discussed in Chapter 7. It does not invade the epithelial tissue aggressively. Nevertheless, many *H. pylori* strains can adhere to gastric epithelial cells, into which they can inject at least one bacterial protein, the CagA protein, by bacterial type IV protein secretion (Odenbreit et al., 2000). CagA undergoes tyrosine phosphorylation by a host-cell kinase, and the new phosphoprotein may bind the Src homology region 2 domain (SH2)-containing proteins, which forms a signaling complex that promotes the reorganization of the actin cytoskeleton and, possibly, of some membrane components of the apical membrane (Covacci and Rappuoli, 2000). Hypothetically, *H. pylori* could inject an own receptor via the bacterial type IV protein secretion system into the epithelial cells, and this receptor protein could be modified in the affected host cell and transported to the surface. As a result, *H. pylori* could bind strongly to the gastric surface epithelium without being dependent on protein synthesis of the host cell. An example of this phenomenon is the translocated intimin receptor (Tir) of enteropathogenic *Escherichia coli* (EPEC). This bacterial pathogen inserts its own receptor (Tir) into mammalian cell surfaces, to which it then adheres via the "intimin" adhesin and triggers additional host signaling events and actin nucleation (Kenny et al., 1997).

The reactions of the gastric mucosa on *H. pylori* infection and adhesion are multiple. As described in the introduction (Chapter 1), *H. pylori* induces a gastric inflammatory response, and pro-inflammatory cytokines, and T and B cells are activated. This can also lead to a reaction of the gastric surface epithelium to reduce the number of cells that produce its receptor (i.e. MUC5AC), which results in a decrease of MUC5AC-producing cells as observed in *H. pylori*-infected patients (Chapter 3), or even atrophy of the gastric mucosa. Others showed also a decreased expression of MUC5AC in the gastric surface epithelium in *H. pylori*-infected individuals (Byrd et al., 1997). They showed that in *H. pylori*-infected patients not only MUC5AC protein expression was decreased, but also the expression of *MUC5AC* mRNA was decreased. In addition, they found aberrant expression of MUC6 protein, which is normally expressed in the deeper mucous glands, in the surface epithelium. This example could suggest that the infected gastric surface epithelium protects itself by down regulation of its receptor expression (i.e. MUC5AC), and up regulation of a secretory protein (i.e. MUC6) that can form the mucus gel-layer to protect the underlying epithelium. Thus the host may defend itself by the reduction of the number of MUC5AC-producing cells.

Now that the most important receptors for *H. pylori* in the human gastric stomach are known (carbohydrate structures on MUC5AC), one can think of selecting the respective *H. pylori* adhesins for either therapeutic or prophylactic immunization. *H. pylori*-infection is a key etiological factor in gastric carcinoma, and gastric cancer is ranked as the second most common fatal malignancy after lung cancer (Pisani et al., 1999). Successful eradication of *H. pylori* is thus an important goal. Nowadays, treatment involves antibiotic therapy, and using this therapy the bacterium can be successfully eradicated in most patients. However, antibiotics-resistant strains of *H. pylori* are emerging (Megraud, 1998), and treatment of mass populations, like in developing countries, is only likely to increase the number of *H. pylori* strains resistant to these therapies (Graham, 1998). Therefore, an alternative approach is to develop a vaccine, which would not only clear the bacterium in infected patients (therapeutic vaccination), but also protect against re-infection (prophylactic vaccination). Despite the considerable humeral and cellular responses of the immune system against *H. pylori*, the bacterium can survive in the gastric mucus for a human lifetime. Thus, the bacterium has developed the ability to evade, or to adapt to, the host's immune response. A major subset of lymphocytes involved in immunity against invading pathogens is the $CD4^+$ T-lymphocytes, divided into either Th1 or Th2 phenotypes (Romagnani, 1999). Th1 cells are involved in cell-mediated immunity, whereas Th2 cells are involved in antibody formation, particularly IgA and IgG1, which are the types of antibody most active at mucosal surfaces and most likely to be protective against bacteria trying to colonize mucosal surfaces. The Th1 and Th2 cells have different cytokine profiles when stimulated with a specific antigen. Th1 cells produce interferon (INF)-γ, TNF-α and –β, and interleukin (IL)-2, whereas Th2 cells produce IL-4, 5, 6, 10, and 13 (Ernst and Gold, 2000). Curiously enough, most infected humans have a Th1 response, which does not eradicate *H. pylori*, whereas one would expect a Th2 gastric immune response. Thus, the bacterium forces the gastric immune response in the, for *H. pylori*, least harmful one. Thus for successful vaccination against *H. pylori* it is important to switch from a Th1 to a Th2 immune response. In animal studies there are promising results with regard to therapeutic immunization (reviewed by Kusters, 2001). The first results from human vaccine trails lead to increased antibody titers, but not in the elimination of *H. pylori*-infection (Kreiss et al., 1996; Michetti et al., 1999).

Two *H. pylori* adhesins, BabA and SabA (Ilver et al., 1998; Mahdavi et al., 2002), have been cloned, and were shown to mediate bacterial binding to human blood group antigen structures present on MUC5AC. A recent study showed that BabA was present on proximally 80% of the isolated *H. pylori* strains, and that the presence of BabA in the bacterium was associated with pre-neoplastic gastric lesions (Yu et al., 2002). The *sabA* gene was identified by PCR in six sialyl-Lewis X-binding and six non-sialyl-Lewis X-binding isolates, which suggests that *sabA* is present in the majority of *H. pylori* isolates (Mahdavi et al., 2002). BabA, together with SabA, makes these two adhesins, alone or in combination, candidates for the production of therapeutic vaccines. An effective vaccine against *H. pylori* will be of enormous benefit in the fight against gastric lesions, especially gastric cancer.

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Chapter 9

Samenvatting

Samenvatting

De bovenste organen van het spijsverteringsstelsel omvatten de oesofagus, maag en duodenum. Deze compartimenten dienen voor transport en tijdelijke opslag van ingenomen voedsel, en initiëren de vertering van voedsel. Ziektes van het bovenste spijsverteringsstelsel zijn een belangrijke bron van morbiditeit en mortaliteit. Belangrijke ziekten in dit verband zijn: gastritis, maag en duodenale ulcers, oesofagitis en gastro-oesofagale reflux ziekte. Bovendien wordt in de Westerse wereld een toename waargenomen van maag en oesofagus carcinomen. Sinds 1983 is bekend dat de aanwezigheid van een bacterieel pathogeen in verband kan worden gebracht met verschillende van deze veel voorkomende ziekten. Deze bacterie, *Helicobacter pylori*, komt wereldwijd voor en wordt door de Wereld Gezondheidsorganisatie (WHO) geclassificeerd als een type I carcinogeen. *H. pylori* is de eerste bacterie waarbij een relatie is gelegd tussen de chronische aanwezigheid van een bacterie en de aanwezigheid van adenocarcinomen met name van het maagepitheel. De "natuurlijke" omgeving van *H. pylori* is de maag. Om de maag te koloniseren moet de bacterie overleven in de zure omgeving van de maaginhoud. *H. pylori* heeft hiervoor een aantal aanpassingen ontwikkeld. Als één van de weinige bacteriën bevat *H. pylor*i het enzym urease, waarmee het in staat is om ureum te hydrolyseren in koolstofdioxide en ammonia, waardoor de zure maaginhoud plaatselijk wordt geneutraliseerd. Tevens heeft de bacterie aan één zijde flagellen, waarmee het door de mucus (slijm)-laag kan voortbewegen naar het oppervlakte van het maagepitheel waar een neutrale pH heerst. Om niet te worden uitgewassen met het transport van voedselresten naar het duodenum, zal de bacterie zich moeten hechten in het maagslijm (mucus) of aan de mucus-producerende epitheelcellen die zich aan de oppervlakte van de maag bevinden. Het hoofdbestanddeel van mucus zijn secretoire mucinen van het zogenaamde MUC-type. Deze secretoire mucinen zijn grote glyco-proteïnen die een visco-elastische gel-laag kunnen vormen en die het onderliggende epitheel beschermen. In de mens zijn er tot op heden 18 mucine genen van het MUC-type beschreven, die respectievelijk coderen voor: MUC1, -2, -3A, -3B, -4, -5AC, -5B, -6, -7, -8, -11, -12, -13, -15, -16, -17 en -18. Hiervan zijn MUC2, -5AC, -5B en –6 genetisch geclusterd op chromosoom 11p15.5. Het zijn met name deze laatste 4 genoemde MUC-type mucinen die verantwoordelijk zijn voor de mucuslagen in het maagdarmkanaal en ander holle organen van het lichaam, zoals de longen en de uro-genitale kanalen.

Een zeer belangrijke kandidaat voor de *H. pylori* receptor in de maag is het maagmucine. Mucine vormt de belangrijkste structurele component van de mucuslaag die de maag bekleed. Uit resultaten van *in situ* onderzoek is gebleken dat de bacterie aanwezig is in de mucuslaag, of wordt aangetroffen geassocieerd met de mucine-producerende cellen van het oppervlakte epitheel van de maag. De sequentieanalyse van een maag cDNA-bank toonde aan dat MUC5AC het prominente secretoire maag mucine is, dat verantwoordelijk is voor de opbouw van de mucuslaag in de maag (zie hoofdstuk 2). Tevens werden 3024 baseparen aan nieuwe

MUC5AC-sequentie verkregen aan de 5'-kant van de reeds bekende MUC5AC cDNA sequenties, waarmee de totale sequentie van het humane MUC5AC cDNA bekend werd.

Infectie van de maag door *H. pylori* heeft in vrijwel iedere patiënt ontsteking van de maag mucosa tot gevolg, wat vervolgens gevolgen kan hebben voor de verschillende celtypen in het maagepitheel. In hoofdstuk 3 is gekeken naar de gevolgen van *H. pylori* infectie op de verschillende celtypen in het maagepitheel van het antrum met betrekking tot de expressie van secretoire eiwitten. In antrum biopten van met *H. pylori* geïnfecteerde en niet-geïnfecteerde personen werden verschillende celtypen geanalyseerd. De volgende secretoire eiwitten werden immunohistologisch geanalyseerd: secretoire mucinen (MUC2, MUC5AC, MUC5B en MUC6) en 'trefoil factor family' (TFF) peptiden (TFF1, TFF2 en TFF3). TFF peptiden zijn relatief kleine eiwitten, betrokken bij het herstel van epitheel na schade als gevolg van bijvoorbeeld ontstekingen. De laatste groep van secretoire eiwitten zijn de endocriene eiwitten, gastrine en chromogranine A. Gastrine is een hormoon dat voornamelijk betrokken is bij regulatie van zuursecretie door het maagepitheel. Bovendien stimuleert gastrine de epitheliale proliferatie. Chromogranine A speelt een rol bij de stabiliteit van granula en de processing van hormonen die worden geproduceerd in endocriene cellen. Tevens werd voor beide patiëntengroepen (al dan niet geïnfecteerd door *H. pylori*), de proliferatie in het epitheel geanalyseerd met behulp van het eiwit Ki-67. De bovenstaande markers voor secretoire eiwitten en de marker voor proliferatie in het antrum epitheel werden eveneens gebruikt voor de analyse van intestinale metaplasie in het antrum. Intestinale metaplasie is een pre-malige aandoening, waarvan wordt verondersteld dat het kan ontaarden in een maagcarcinoom. We hebben bepaald of deze bovengenoemde markereiwitten voor wat betreft het antrum epitheel voorkomen in de intestinale metaplasie.

Uit de analyse van de bovenstaande markereiwitten in het antrum epitheel is gebleken dat infectie met *H. pylori* was gecorreleerd met ontsteking in de maagmucosa, en met atrofie en proliferatie van het maagepitheel. Infectie met *H. pylori* was niet gecorreleerd met de aanwezigheid van intestinale metaplasie in het antrum. Intestinale metaplasie werd gekarakteriseerd door MUC2 expressie, dat in het gezonde antrum epitheel afwezig is, en was gecorreleerd met maagklier atrofie. *H. pylori*-infectie in patiënten was gecorreleerd met een afname van het aantal cellen die MUC5AC, TFF1 en TFF2 tot expressie brengen. Deze eiwitten zijn typerend voor de epitheliale cellen gelegen in het oppervlakte epitheel van het antrum. Tegelijkertijd werd in deze patiënten een toename geconstateerd van de aantallen cellen die MUC5B en MUC6 tot expressie brengen. Deze cellen zijn typerend voor de epitheelcellen uit de diepere delen van de maagklier. De verschillende secretoire celtypen, die normaal bij niet-geïnfecteerde individuen uitsluitend in de onderste regionen van de antrum klieren voorkomen, waren bij *H. pylori* geïnfecteerde personen "verschoven" naar de bovenste regionen van het maagklierepitheel. Het gevolg van *H. pylori* infectie is dat het aantal MUC5AC-producerende cellen afneemt, en deregulatie van de verschillende secretoire celtypen.

Een andere afwijking aan het bovenste spijsverteringsstelsel is de zogenaamde Barrett's oesofagus, die wordt gekenmerkt door een hoge expressie van secretoire mucinen in het epitheel. In een Barrett's oesofagus is de normale epitheliale bekleding, het meerlagig niet-verhoornde plaveisel epitheel, vervangen door cylindrisch epitheel met duidelijke secretoire eigenschappen. Barrett's oesofagus is een metaplasie van de oesofagus en wordt onderverdeeld in een maag-type metaplasie en intestinaal-type metaplasie. Het intestinale type metaplasie van Barrett's oesofagus wordt in het algemeen erkend als voorstadium van een oesofagus adenocarcinoom (zie hoofdstuk 4 en 5). In Barrett's epitheel komen secretroire mucinen tot expressie, zoals wordt aangetoond met behulp van histochemische kleuringen, die echter geen uitsluitsel geven over de identiteit van deze secretoire mucinen. Mogelijk zou *H. pylori*, via binding aan MUC5AC, een rol kunnen spelen bij het ontstaan van maligniteit in de oesofagus, net als in de maag het geval is. In hoofdstuk 4 en 5 is de expressie van de vier MUC-type secretoire, gelvormende mucinen (MUC2, -5AC, 5B en -6) en drie TFFs (TFF1, -2 en -3) bepaald in Barrett's oesofagus weefsel van patiënten met of zonder een *H. pylori*-infectie in de maag. De *H. pylori* status was van belang om te kunnen bepalen of er een correlatie bestond tussen de aanwezigheid van *H. pylori* in de maag, en de expressie van de secretoire eiwitten in het epitheel van de Barrett's oesofagus. De resultaten in hoofdstuk 4 laten zien dat MUC5AC tot expressie wordt gebracht in het weefsel van vrijwel alle patiënten met een Barrett's oesofagus, en dat het expressie patroon van MUC5AC vergelijkbaar is met dat van patiënten die tegelijkertijd een Barrett's oesofagus en een *H. pylori* infectie in de maag hebben. Overigens correleerde de expressie van geen enkel secretoir mucine met de aanwezigheid van *H. pylori* in de maag van de patiënten. Het expressie patroon van de MUC-type mucinen en van de TFF-peptiden in het maag type metaplasie lijkt in vele opzichten op het expressiepatroon in het antrum van de maag. De expressie van intestinaal mucine MUC2 in Barrett's epitheel vertoonde een correlatie met progressie naar een adenocarcinoom. Een meer gedetailleerde analyse van het expressiepatroon van de secretoire mucinen en TFF-peptiden in Barrett's epitheel laat zien dat de expressiepatronen van deze eiwitten meer "chaotisch" wordt bij een toename van MUC2 expressie (zie hoofdstuk 5). Het "normale" patroon van Barrett's epitheel heeft celtypen die lijken op de klieren van het antrum epitheel, en wordt daarbij in toenemende mate onherkenbaar. Dit proces heeft een duidelijke toename in de MUC2 expressie en proliferatie in het Barrett's oesofagus epitheel tot gevolg. MUC2 expressie in Barrett's oesofagus vormt daarmee waarschijnlijk een goede marker voor de vroege veranderingen in het fenotype van Barrett's oesophagus die zouden kunnen leiden tot een premaligne aandoening.

Na de maag zal het voedsel voor verdere verwerking in het duodenum terechtkomen. Het duodenum staat in nauw contact met de maag. De enige scheiding tussen deze twee organen is een sluitspier, de pylorus. Negatieve veranderingen van de maaginhoud en -functies kunnen, door dit nauwe contact mogelijk ook negatieve gevolgen hebben voor het duodenumepitheel. Een voorbeeld hiervan is het ontstaan van duodenale ulcers. Studies hebben aangetoond dat er een sterke correlatie bestaat tussen de aanwezigheid van *H. pylori* in de maag en de

aanwezigheid van duodenale ulcers (zie hoofdstuk 1). Als gevolg van een *H. pylori*-infectie in de maag kan de zuursecretie sterk toenemen. De zure maaginhoud kan vervolgens schade aanrichten aan het duodenumepitheel. Zo kan er een maag-type metaplasie optreden in het villus epitheel van het duodenum. Wij hebben deze metaplastische cellen onderzocht op de expressie van MUC-type secretoire mucinen (MUC2, -5A, -5B en -6) en TFF peptiden (TFF1, -2, en 3). Dit metaplastisch epitheel bleek te worden gekenmerkt door de expressie van mucinen en TFF peptiden, vergelijkbaar zoals die worden waargenomen in het antrum van de maag (hoofdstuk 6). MUC5AC expressie werd waargenomen in alle metaplastische cellen en vormt daarmee een zeer goede marker voor dit afwijkende celtype. Uit analyse van de expressiepatronen van MUC-type mucinen en TFF peptiden in maag-type metaplasie van het duodenum konden wij verder nog afleiden dat deze metaplastische cellen waarschijnlijk afkomstig zijn van een "switch" in fenotype van de normale, in de villus voorkomende slijmbeker cellen, naar metaplastische cellen. Uit deze bevindingen bleek dat het onwaarschijnlijk is dat deze metaplastische cellen een afzonderlijke cellijn vormen met eigen stamcellen (hoofdstuk 6). De gevonden positieve correlatie tussen de aanwezigheid van *H. pylori* in de maag en maag-type metaplasie in het duodenum, suggereert een inductieve rol voor *H. pylori* bij de ontwikkeling van maag-type metaplasie in het duodenum (hoofdstuk 6).

Nadat uit ons werk bleek dat het secretoire mucine MUC5AC tot expressie wordt gebracht in maagepitheel cellen en in de metaplastische epitheelcellen van de oesofagus en duodenum (hoofdstuk 3-6), werd de hypothese getest of MUC5AC in deze weefsels in staat was om *H. pylori* te binden in een *in vitro* bindingsassay (hoofdstuk 7). In studies van anderen werd gesuggereerd (zie hoofdstuk 7) dat een bepaalde suiker (koolhydraat) structuur, het Lewis B bloedgroep antigen, kan functioneren als receptor voor *H. pylori*. De structuur van het MUC5AC-molecuul is rijk aan eindstandige suikergroepen, waaronder vele Lewis B bloedgroep antigenen. Onze hypothese werd verder uitgebreid met de veronderstelling dat MUC5AC de drager is van Lewis B bloedgroep structuren, die als receptor voor *H. pylori* kunnen dienen. Fluorescent gelabelde *H. pylori* bacteriën van verschillende stammen werden toegevoegd aan weefselsecties van de maag, oesofagus en duodenum, waarvan bekend was dat ze MUC5AC tot expressie brachten. In deze verschillende weefsels werd een zeer sterke co-lokalisatie tussen MUC5AC en Lewis B structuren waargenomen. De *H. pylori* bacteriën die waren toegevoegd aan weefselsecties van deze verschillende weefseltypen co-lokaliseerden met zowel MUC5AC als met de Lewis B structuren (hoofdstuk 7). Alleen de bacteriestammen die in staat waren om Lewis B te binden, konden aan MUC5AC binden. Echter, de *H. pylori* stam die niet in staat was om Lewis B te binden, kon ook niet binden aan MUC5AC. Dit illustreert dat de Lewis B structuur op MUC5AC waarschijnlijk een belangrijke receptor kan zijn voor *H. pylori* tijdens infectie van de maag.

De mogelijke rol die MUC5AC speelt bij een *H. pylori* infectie en de gevolgen voor de MUC5AC synthese worden bediscussieerd in hoofdstuk 8. Tevens worden hier de mogelijke gevolgen besproken van een *H. pylori* infectie op het maagepitheel, en de reactie hierop van de maag mucosa. Verder wordt aangegeven welke moleculen en structuren op *H. pylori* kunnen worden gebruikt om een effectief vaccin tegen te maken, want steeds vaker blijkt er antibiotica resistentie op te treden van *H. pylori* stammen. Een effectief vaccin tegen *H. pylori* zou een enorm voordeel zijn in de strijd tegen maagaandoeningen, en in het bijzonder maagcarcinomen. Door de belangrijke rol die de bacteriële adhesinen voor MUC5AC spelen bij hechting van *H. pylori* lijken deze adhesinen belangrijke kandidaten te vormen voor een dergelijk vaccin.

Curriculum vitae

Jeroen van de Bovenkamp was born on June 8th, 1968 in Nijmegen (The Netherlands). After graduating from the Scholengemeenschap Nijmegen-West in 1984, he started his laboratory education at the Opleiding Laboratoriumpersoneel Arnhem-Nijmegen (OLAN). During these studies he took part in research at the Laboratory of Pathology and Anatomy of the Sint-Maartens Gasthuis, Venlo. In 1988 he past his exam as medical laboratory technician (MLO) and attended the Higher Laboratory Education (HLO) of the Hogeschool Gelderland in Nijmegen. He finished these studies by participating in a research project of the Department of Biochemistry (head: prof. dr. J.H. Veerkamp) at the Catholic University Nijmegen (KUN) and obtained his Bachelor of Sciences degree (ing.) in June 1992. From 1992 to 1995 he studied biology at the Catholic University Nijmegen, where he did research at the Department of Molecular Plant Physiology (head: prof dr. G.J. Wullems) and at the Department of Molecular Biology (head: prof. dr. R.H.N. Konings†). After obtaining his Master of Sciences degree (drs.) in september 1995, he began working at the Laboratory of Pediatric Gastroenterology & Nutrition at the Academic Medical Center (AMC), Amsterdam (The Netherlands). There he started the research that resulted in this thesis, under supervision of dr. J. Dekker, dr. A.W.C. Einerhand, prof.dr. H.A. Büller and prof. dr. G.J. Strous, who is from the Department of Cell Biology at the Utrecht University. In 1998 the research group moved towards the Laboratory of Pediatrics at the Erasmus MC / Sophia Children's Hospital in Rotterdam, where he has finished his Ph.D. project.

Dankwoord

&

Curriculum vitae

Dankwoord

> "Degenen die erudiete werken schrijven die slechts door een paar geleerden beoordeeld worden (…), vind ik eerder beklagenswaardig dan gelukkig, omdat zij zichzelf voortdurend aan het kwellen zijn. Zij voegen toe, veranderen, halen weg, schrijven weer opnieuw, herhalen, werken stukken om en geven uiteenzettingen. Zij laten hun tekst negen jaar liggen en zijn nooit met zichzelf tevreden. Zij kopen hiermee de lof van slechts weinigen, een nutteloze beloning waarvoor ze een hoge prijs betalen, die bestaat uit nachtwaken, het opofferen van slaap (…), zweetdruppels en zelfkwelling. Voeg hierbij de schade die zij aan hun gezondheid toebrengen en aan hun schoonheid. Ze krijgen oogontsteking of worden zelfs blind, ze zijn arm, jaloers, en hebben nooit plezier, ze worden vroeg oud, gaan vroegtijdig dood en meer van dat soort dingen."
>
> uit: Desiderius Erasmus, *Lof der zotheid* (1511), [140-142]

Zelfs voor de huidige generatie promovendi lijkt deze bijna vijfhonderd jaar oude tekst nauwelijks aan waarde te hebben ingeboet. De suggestie dat het schrijven van een proefschrift echter louter en alleen ellende is deel ik niet. In mijn geval begon het schrijven namelijk pas in de afrondingsfase: het ging om het op schrift zetten van jaren onderzoek. Daarbij hebben veel personen op de één of andere manier een bijdrage geleverd aan dat onderzoek, waardoor ik terugkijk op een heel mooie, fijne en leerzame periode.

Veel dank gaat allereerst uit naar de patiënten die 'materiaal' hebben afgestaan voor wetenschappelijk onderzoek. Zonder hen was het grootste deel van deze studie er überhaupt niet geweest.

Een aantal mensen wil ik met name noemen, waaronder Prof. dr. Hans Büller, mijn eerste promotor. Beste Hans, jij hebt mijn onderzoek altijd met veel enthousiasme gevolgd. Van jouw leerde ik dat het ook belangrijk was om je nationaal en internationaal onder onderzoekers te begeven. Dit leidde tot de jaarlijks terugkerende 'KG&V on tour', die meestal begon (met congresbezoek) in Europa en via Amerika weer terug kwam naar Europa. Prof. dr. Ger Strous, mijn tweede promotor. Beste Ger, het is voor mij een hele eer om jou als promotor te hebben. Opmerkelijk is dat jij 13 jaar geleden de co-promotor van mijn huidige co-promotor was. Jij bent de 'geestelijke vader' van mijn onderzoek dat je, zij het op afstand, altijd kritisch hebt gevolgd.

Dr. Jan Dekker, mijn co-promotor. Beste Jan (JeeDee), ik ben je veel dank verschuldigd. Er zijn maar weinig mensen die intact mucine kunnen isoleren, en jij bent er daar één van. Bedankt dat je mij dit 'kunstje' ook hebt geleerd. Jouw begeleiding speelde zich voornamelijk af tijdens de Rotterdamse (Erasmus MC) periode. Daarbij hebben we dagen/weken lang naar microscoopbeelden gekeken, gediscussieerd en heel veel koffie gedronken. Het laatste jaar is het voornamelijk een e-mail begeleiding geworden tussen jou (vanuit Utrecht) en mij (vanuit Amsterdam). Ik sta nog steeds versteld van de snelheid waarmee je mijn manuscripten hebt gelezen en voorzien van kritisch commentaar. Dr. Sandra Einerhand, ook co-promotor. Beste Sandra, jij hebt me voornamelijk tijdens de Amsterdamse (AMC) periode begeleid. Ik heb veel gehad aan jouw moleculair biologische kennis, en je was ook meestal van de partij tijdens de 'KG&V on tour show'. Naast wetenschappelijke

hoogtepunten, waren er tijdens de tour ook niet-wetenschappelijke hoogtepunten, zoals ‘punting’ op de Cam in Cambridge (GB), op de borrel bij de burgemeester in Toulouse (Fr), de ‘swamp tour’ in New Orleans (USA) en de van Gogh tentoonstelling in Boston (USA). Bedankt voor jouw aandeel in de begeleiding van het onderzoek.

Hierbij wil ik ook alle coauteurs bedanken, met name: Chi-Mei Hau, die als HLO-studente heeft bijgedragen aan de opheldering van de MUC5AC DNA-volgorde; Anita Korteland-Van Male, voor haar hulp bij het praktische werk, waaronder de honderden immunohistochemische kleuringen op patiëntenmateriaal; Christian Warson en prof. dr. Nadine Ectors voor het Barrett’s materiaal. Dr. Jafar Mahdavi and dr. Thomas Bóren are gratefully acknowledged for culturing and labeling the *Helicobacter pylori* strains, and for their critical reading of the manuscript.

De medewerkers van het voormalige lab G2/K2 (AMC periode), bedankt voor de gezelligheid en de vele versnaperingen tijdens de koffie pauzes. Ik hoop dat er de komende jaren nog vele culinaire weekenden zullen volgen. Ook de medewerkers van het lab Kindergeneeskunde (Erasmus MC periode) wil ik bedanken voor de hulp en gezelligheid tijdens de afgelopen jaren. Er was altijd wel een reden om te borrelen en daar moet vooral mee worden doorgegaan. Van ‘De Partij’ (LKG) hoop ik in de toekomst nog veel agendaloze vergaderingen bij te mogen wonen.

Verder dank ik de ex KG&V promovendi ten tijde van de AMC periode: Erik van Beers, Jan-Willem van Klinken en Kristien Tytgat; de KG&V promovendi tijdens de Rotterdamse periode: Jos Boshuizen, Barbara de Koning, Mireille Makkink en Maria van der Sluis; en de tot nu toe enige post-doc John Rossen en enige ex post-doc Marjolein Sutmuller. Melissa Verburg en Ingrid Renes, het is alweer een tijdje geleden dat we met z’n drieën vrijwel gelijktijdig zijn begonnen aan ons promotieonderzoek. De afronding valt voor twee van ons bijna samen. Ik wil jullie erg bedanken voor al die jaren van steun en gezelligheid.

Mijn Paranimfen: John Rossen, Marcel Sluijter en Robert van der Kleij. John en Marcel, heel veel dank voor al jullie hulp in het lab en de gezelligheid. Ook buiten ‘kantooruren’ waren we regelmatig samen om van alles te bespreken en te organiseren. Dank dat jullie me ook nu willen helpen als Paranimf. Robert, wat is het toch heerlijk om na een dag werken in het lab, samen met jou hard te lopen in de Amsterdamse Waterleidingduinen voor weer een training van een halve marathon. Ik hoop dat ik je tijdens het lopen niet te veel heb verveeld met mijn verhalen over *Helicobacter*, mucine, binding en fluorescentie. We hebben er samen al duizenden kilometers op zitten en ik hoop dat er nog heel veel zullen volgen.

Mijn ouders, lieve Pa en Ma, bedankt dat jullie me altijd hebben gesteund bij elke keuze die ik heb gemaakt.

Tenslotte, lieve Arja, als geen ander weet jij wat een promotieonderzoek betekent. Heel veel dank voor al de steun die je me heb gegeven, maar bovenal voor alle liefde en warmte. Veel sterkte met de afronding van jouw promotieonderzoek. In meerdere opzichten zal de komende maanden voor ons een nieuw leven beginnen. Ik kan er bijna niet op wachten!

Jeroen, Amsterdam 2003

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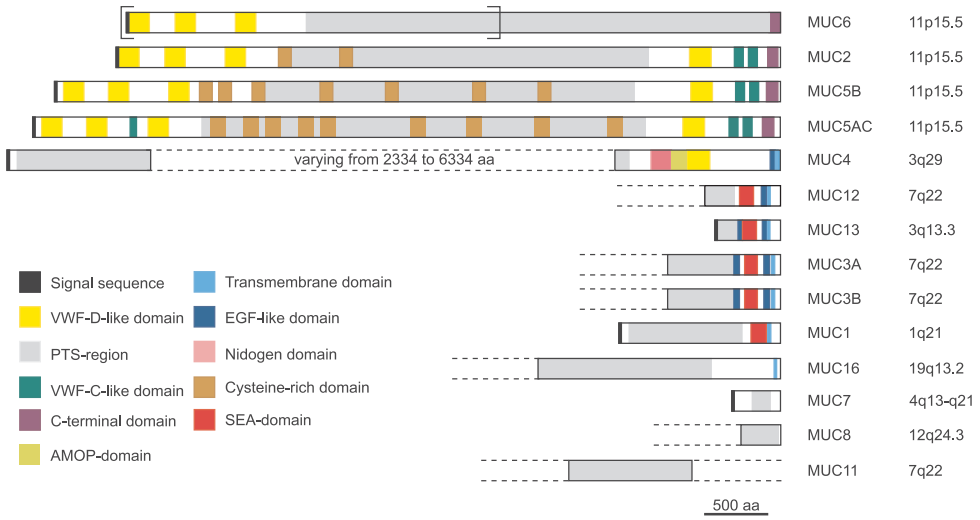
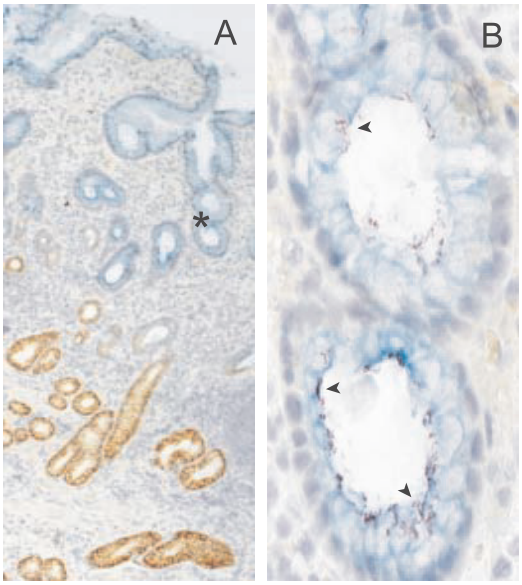
Appendix

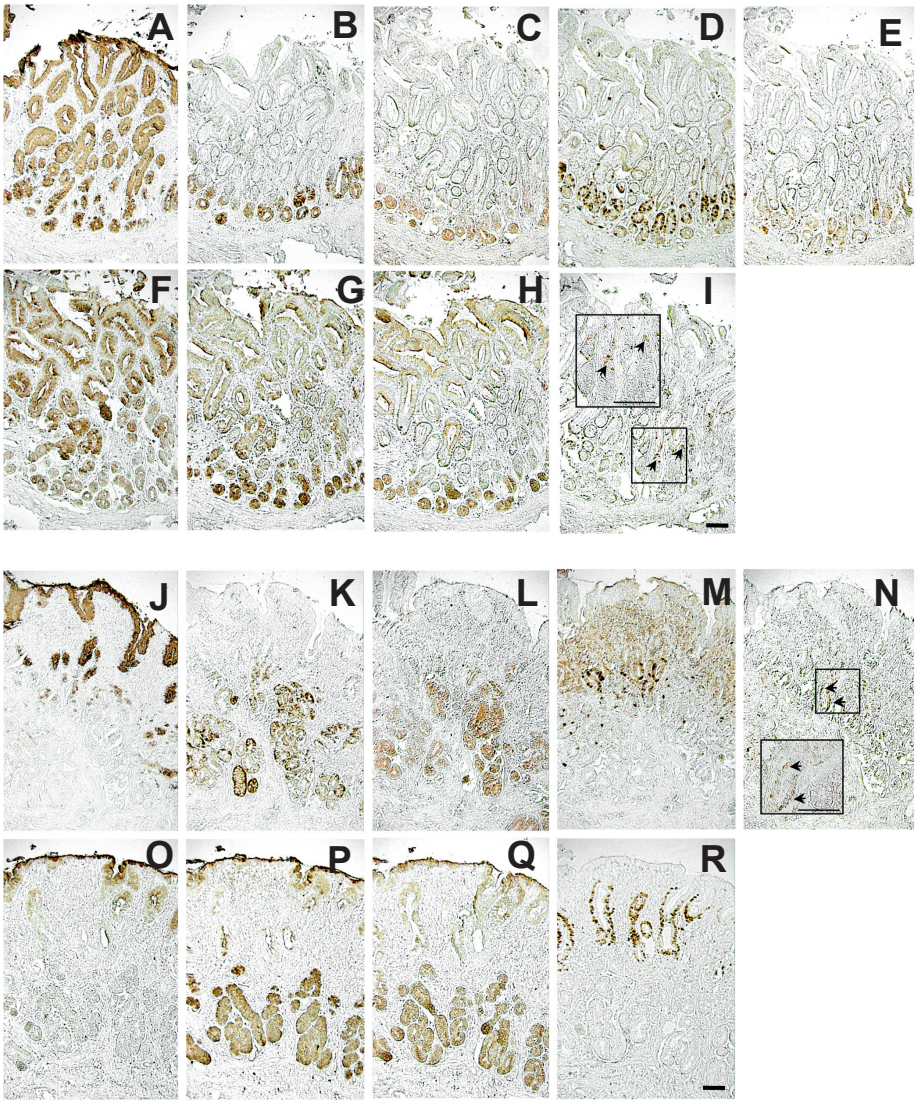
Color Figures



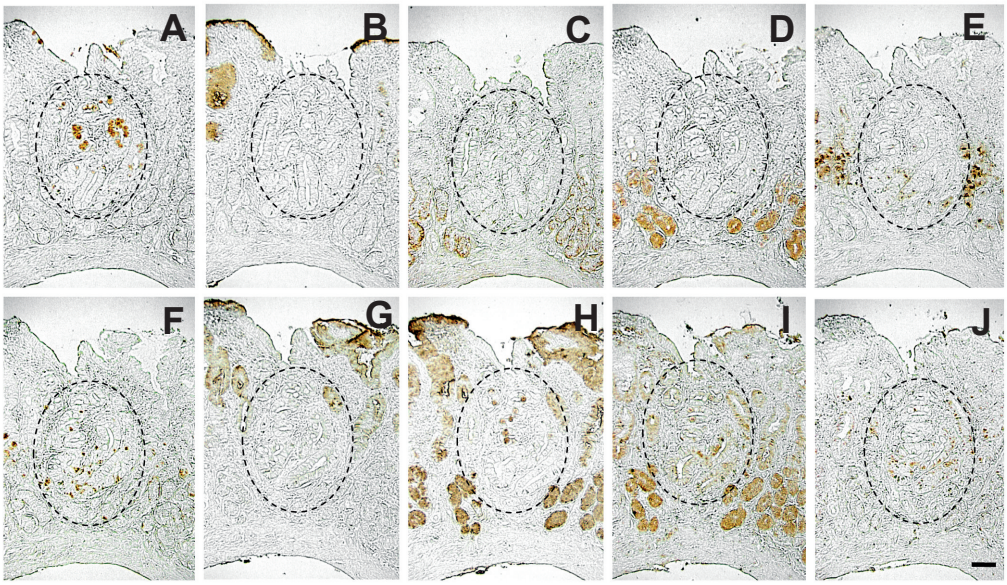
Chapter 1, Figure 3. The relationship between the deduced polypeptide sequences of the mucins. The chromosomal location of each mucin gene is indicated. All mucin sequences were aligned with their C terminus to the right. Each type of peptide domain is depicted in a separate color. Dashed lines indicate unknown sequences. The brackets around the N terminus of MUC6 indicate that this sequences is not publicly available. Adapted from Dekker et al., 2002.



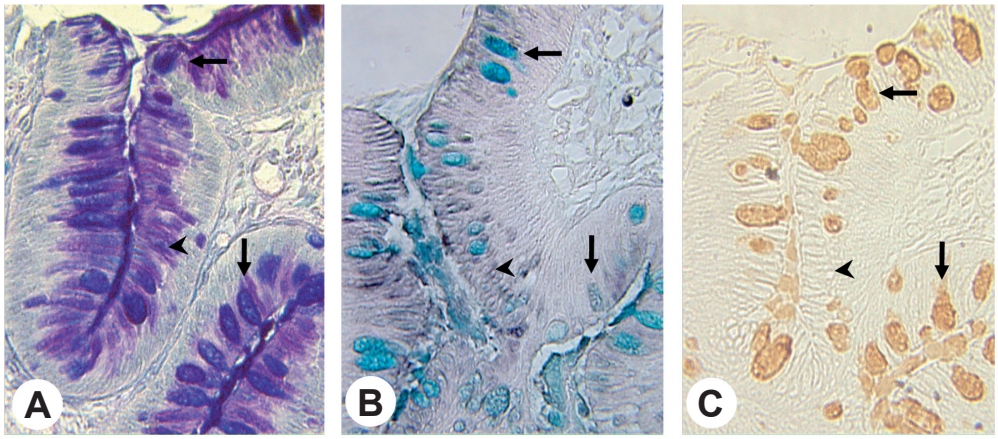
Chapter 1, Figure 4. Immuno-histochemical staining of sections from human antral stomach. Sections were immunohistochemically triple stained using antibodies against MUC5AC (turquoise blue staining), MUC6 (brown staining) and *H. pylori* (reddish purple staining). Sections were counter stained with haematoxylin. (A) Section of *H. pylori* positive patient, showing infection of the mucus layer and epithelium. The star indicates a gland that is shown at higher magnification in (B). Notice in panel A and B that *H. pylori* colocalize with MUC5AC and MUC5AC producing cells, but not with MUC6. Arrows in panel B indicate purple stained, clustered *H. pylori*. Original magnification x 31 (A); x 250 (B). Adapted from Van den Brink et al., 2000b.



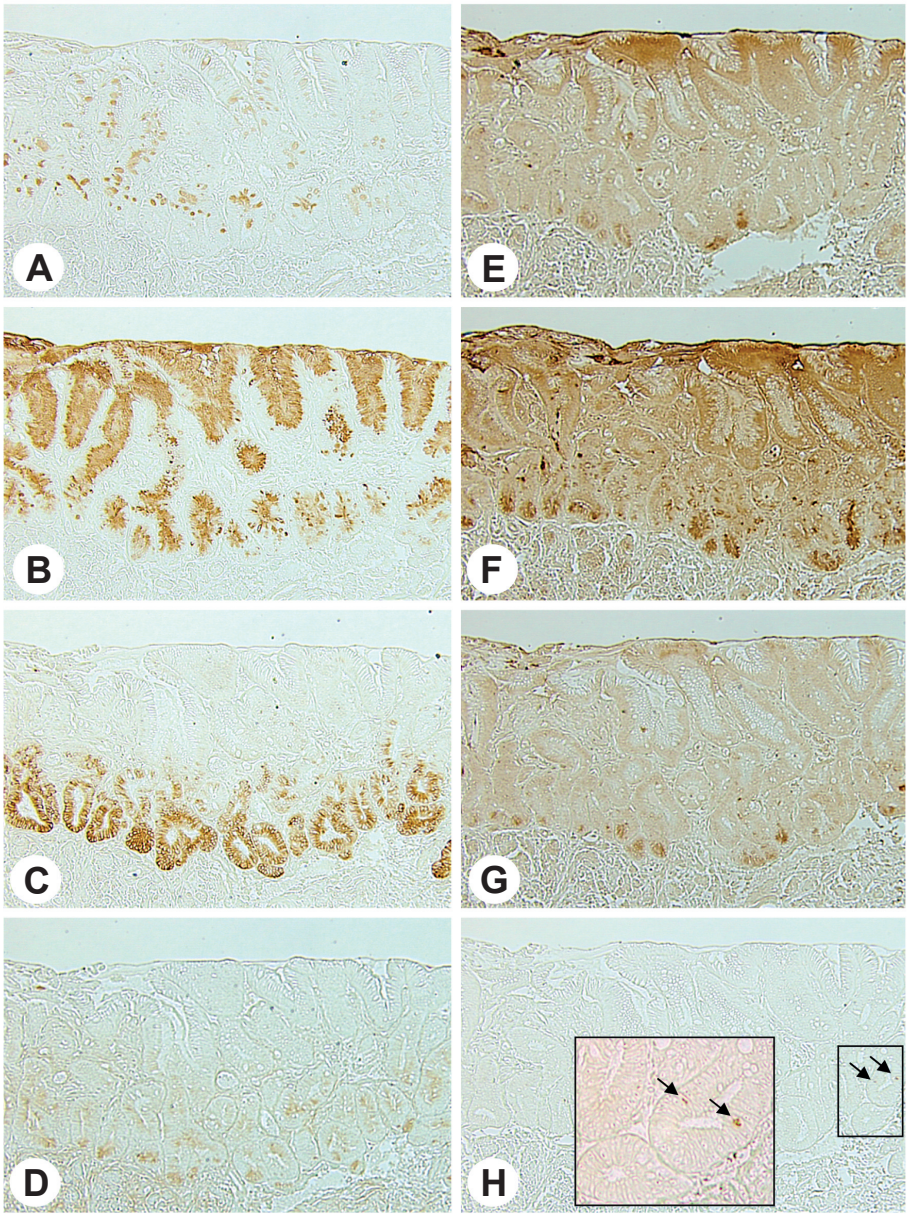
Chapter 3, Figure 4. Gene expression within antrum epithelium as determined by immunohistochemistry. Representative examples are shown of a healthy individual (A-I) and of a *H. pylori*-infected patient (J-R). The expression was assessed by immunohistochemistry of MUC5AC (A and J), MUC6 (B and K), MUC5B (C and L), gastrin (D and M), Chromogranin A, (E and N; arrowheads in Panel N indicate positive cells, higher magnification in insert; Bar = 100 µm), TFF1 (F and O), TFF2 (G and P), TFF3 (H and Q), and Ki-67 (I and R; arrowheads in Panel I indicate positive cells, higher magnification in insert; Bar = 100 µm). Micrographs (A through R) have the same magnification (I and R; Bar =100 µm).



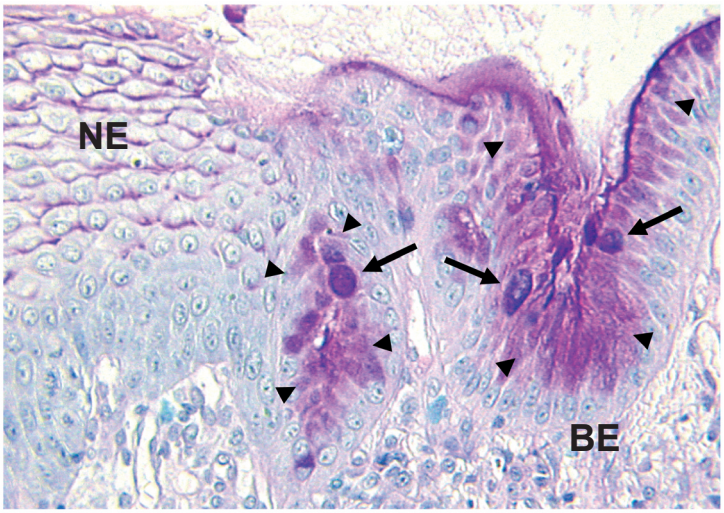
Chapter 3, Figure 8. Expression of secretory proteins and KI-67 within intestinal metaplasia. (A) Expression of MUC2, (B) MUC5AC, (C) MUC6, (D) MUC5B, (E) gastrin, (F) CGA, (G) TFF1, (H) TFF2, (I) TFF3, and (J) Ki-67 was assessed immunohistochemically in a representative biopsy specimen. Dashed lines delineate the position of the intestinal metaplasia (IM). Closely adjacent sections of 1 patient. Micrographs (A through J) have the same magnification (J, Bar = 100 µm).



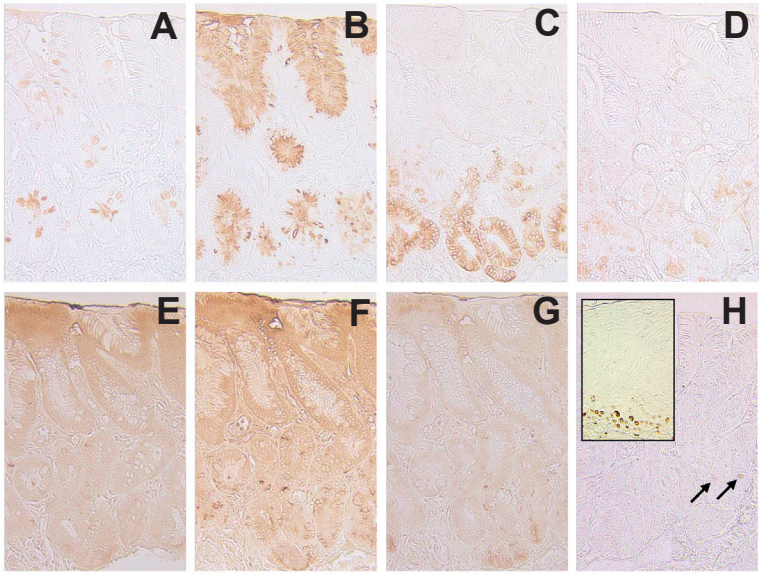
Chapter 4, Figure 1. Example of (A) alcian blue/periodic acid Schiff's reagent, (B) high iron diamine/alcian blue staining, and (C) MUC2 immunohistochemistry of BE epithelium.
Mucins in columnar cells (gastric metaplasia) were stained pink-purple (arrowhead), whereas the goblet cell mucins (arrows, intestinal metaplasia) stained dark purple (A). Sulfomucins were stained in goblet cells (blue/gray, arrows) or in columnar cells (gray, arrowhead) of BE epithelium (B). The goblet cell mucins (intestinal metaplasia) were MUC2-positive (arrows), whereas the columnar cell mucins (gastric metaplasia) were MUC2-negative (arrowhead) (C). Closely adjacent sections of one patient; original magnification x 200.



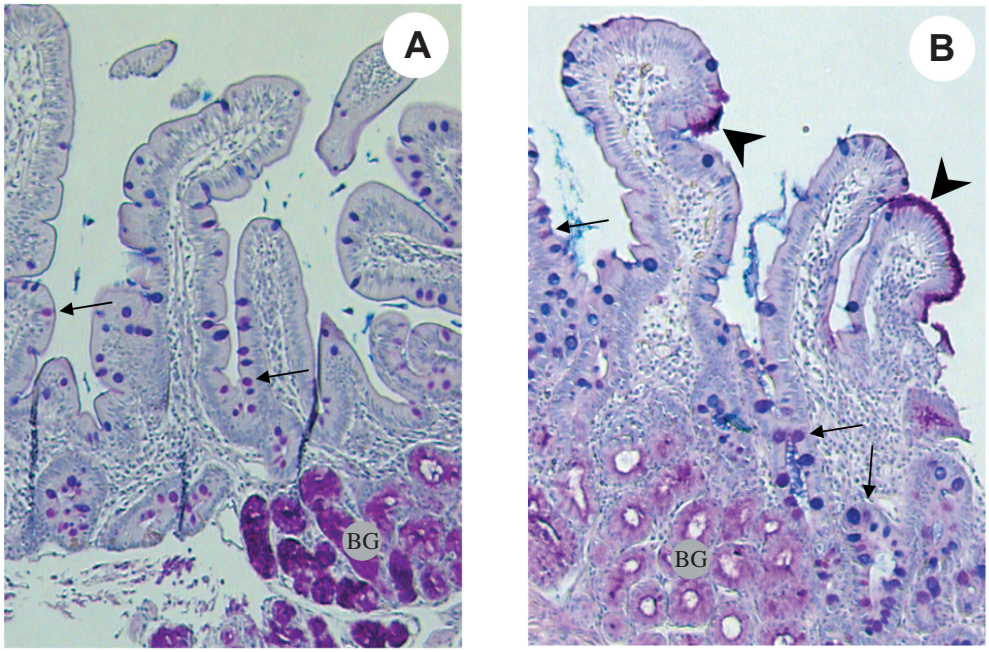
Chapter 4, Figure 2. Mucin, TFF-peptide, and Ki-67 expression in BE epithelium. Examples of staining of BE epithelium with anti-mucin, anti-TFF and anti-Ki-67 antibodies. (A) MUC2 was found in the surface epithelium and associated specifically with goblet cells in IM. (B) MUC5AC was extensively expressed and localized primarily to the surface epithelium. (C) MUC6 localized to the deeper glandular structures. (D) MUC5B was less extensively expressed and localized to the deeper glands. (E) TFF1 expression was primarily found in the surface epithelium. (F) TFF2 was found in the whole continuum of the surface epithelium and the connecting deeper glandular structures. (G) TFF3 was primarily localized in the deeper glandular structures. (H) Ki-67 was found in only a few cells (arrows, higher magnification in insert x 120) and not specifically localized to a particular region. Closely adjacent sections of 1 patient; original magnification x 30.



Chapter 5, Figure 1. Histochemical staining of Barrett's esophagus epithelium. Example of Barrett's esophagus (BE) epithelium with intestinal metaplasia as characterized by the presence of goblet cells, showing also normal stratified epithelium (NE) of the esophagus. Alcian blue/periodic acid Schiff's staining; mucins in columnar cells of the Barrett's esophagus epithelium were stained magenta (arrowheads), whereas the goblet cell mucins (arrows) stained purple.

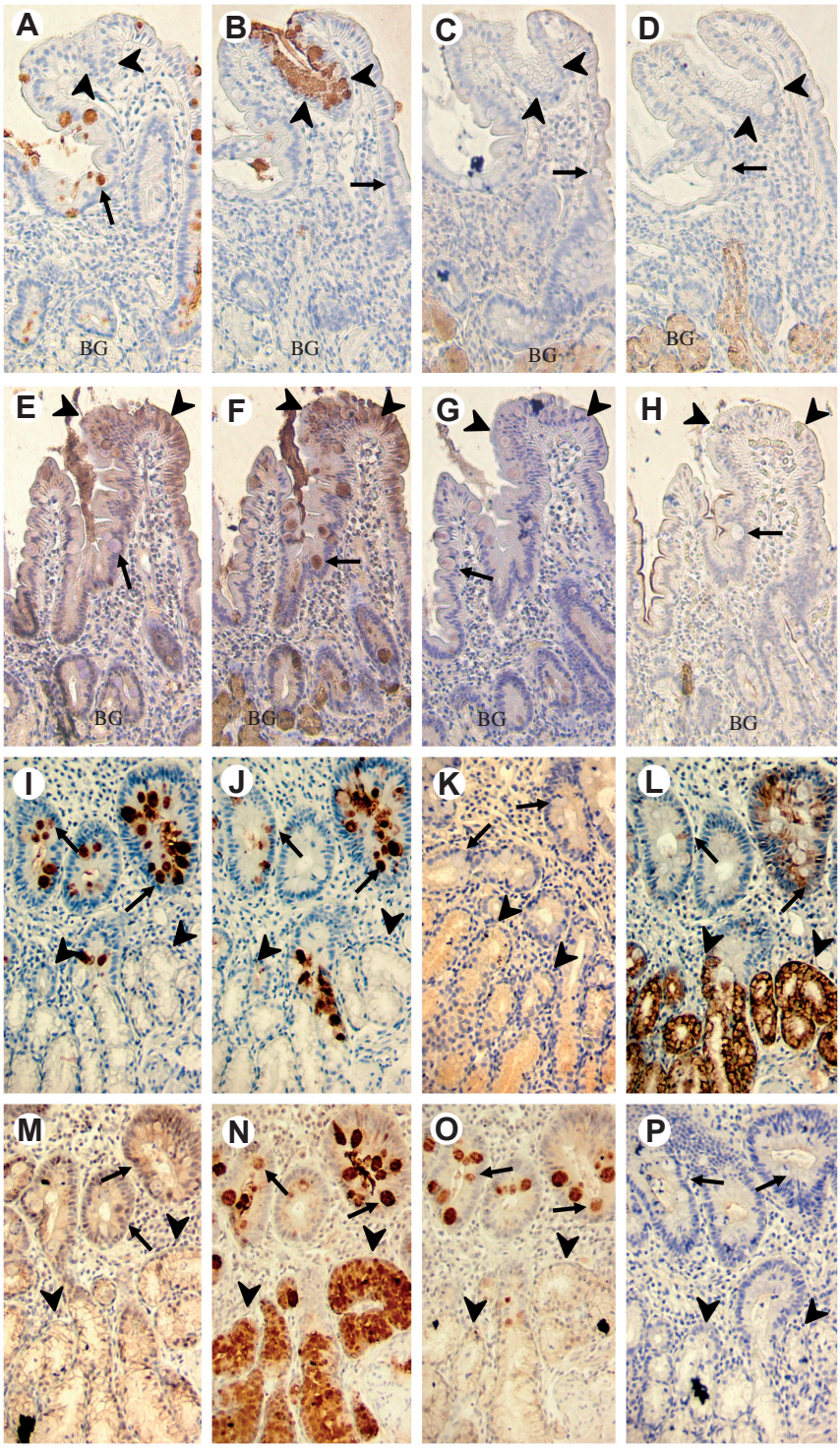


Chapter 5, Figure 2. Immunohistochemistry of secretory proteins and Ki-67 within Barrett's esophagus epithelium. Example of Barrett's esophagus (BE) epithelium with intestinal metaplasia as characterized by the presence of goblet cells. (A) MUC2 expression was uniquely associated with goblet cells in intestinal metaplasia. (B) MUC5AC was mainly localized to the surface epithelium and to some extent to the deeper glandular structures. (C) MUC6 and (D) MUC5B localized to the deeper glandular structures. (E) TFF1 expression was primarily found in the surface epithelium. (F) TFF2 was found in the whole continuum of the surface epithelium and the connecting deeper glandular structures. (G) TFF3 could be found in both surface cells as well in glandular structures. (H) Ki-67 was found in a few cells (arrowheads), not specifically localized to a particular region in the BE epithelium. Inset in (H) shows Ki-67 expression in the normal stratified epithelium of the esophagus, which displayed a discrete layer of proliferating cells.



Chapter 6, Figure 1. Gastric metaplasia in GMD. Example of (A) alcian blue/periodic acid-Schiff reagent staining of a normal intestinal crypt/villus epithelium and (B) villus epithelium showing GMD. (A) Multiple goblet cells that contain acidic mucins were stained purple, whereas a small number of goblet cells with neutral mucins were stained magenta (arrows). (B) Patches of metaplastic cells at the villus tips were stained magenta (arrowheads). Goblet cells, which contain acid mucins, were stained purple, whereas goblet cells with neutral mucins were stained magenta (arrows). Brunner's glands (BG) contain magenta stained, neutral mucins. Nuclei were stained by hematoxylin. Original magnification x20.

Chapter 6, Figure 4 (right page). Protein expression in normal villus epithelium, GMD, and Brunner's glands. Immunohistochemical staining of: (A and I) MUC2, (B and J) MUC5AC, (C and K) MUC5B, (D and L) MUC6, (E and M) TFF1, (F and N) TFF2, (G and O) TFF3, and (H and P) SI. Panels A through H show normal duodenal villus epithelium with goblet cells (arrows) and GMD (arrowheads). (BG indicates Brunner's glands). Panels I through P show normal duodenal crypt epithelium and Brunner's glands (arrowheads). The arrows indicate sporadic goblet cells, which expressed both intestinal- and gastric-type secretory proteins. Nuclei were counterstained with hematoxylin. Original magnification x40 (A through H) and x80 (I through P).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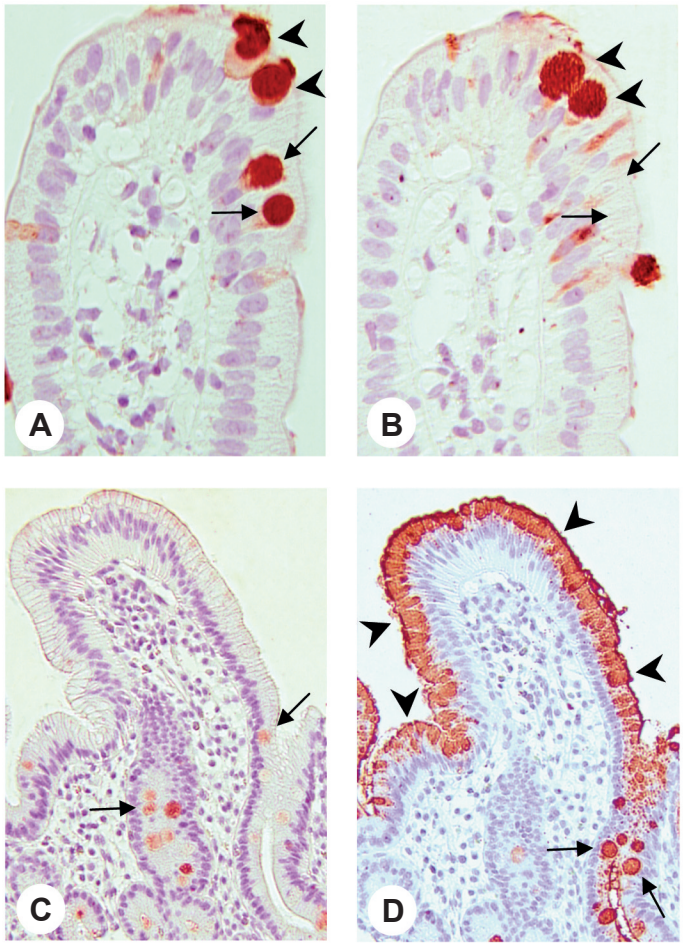
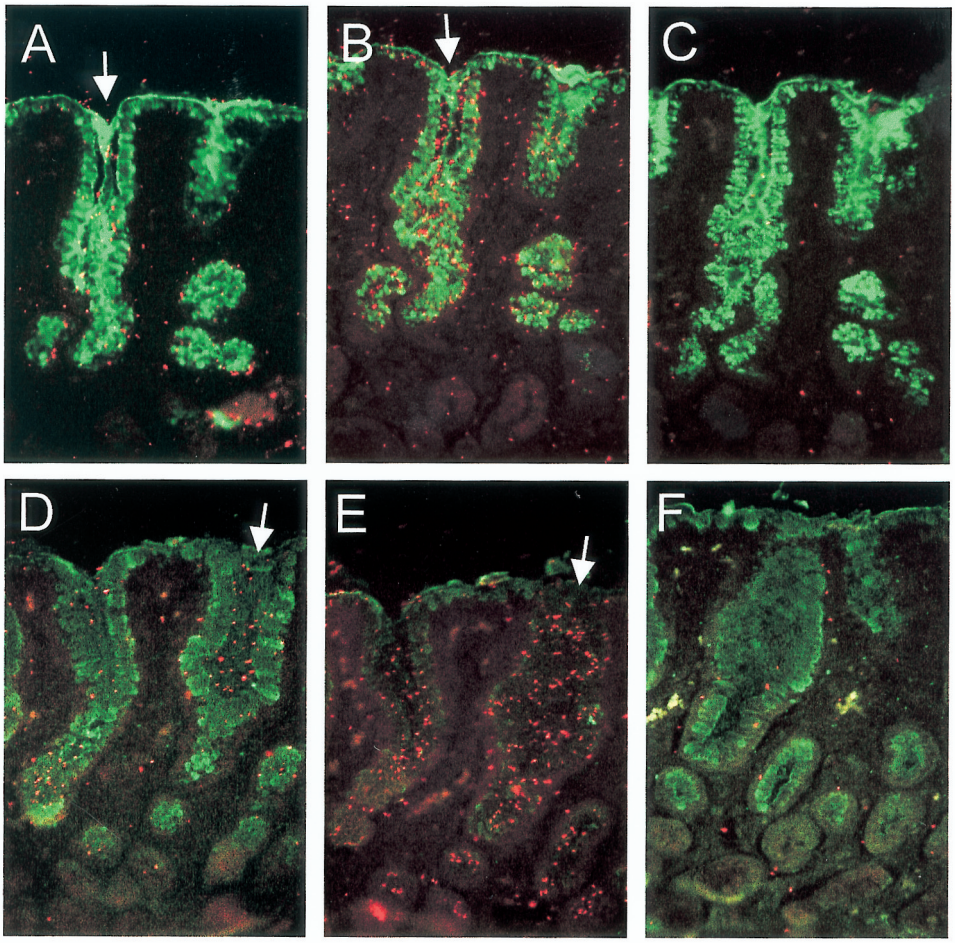
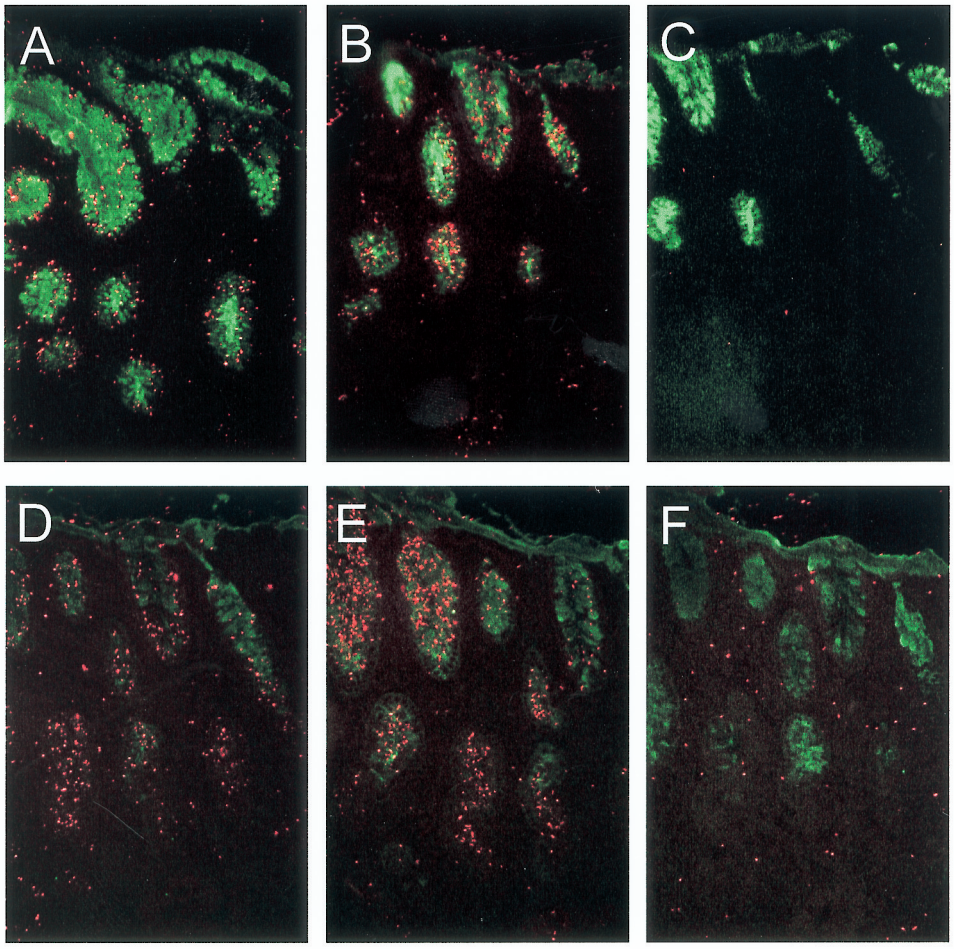


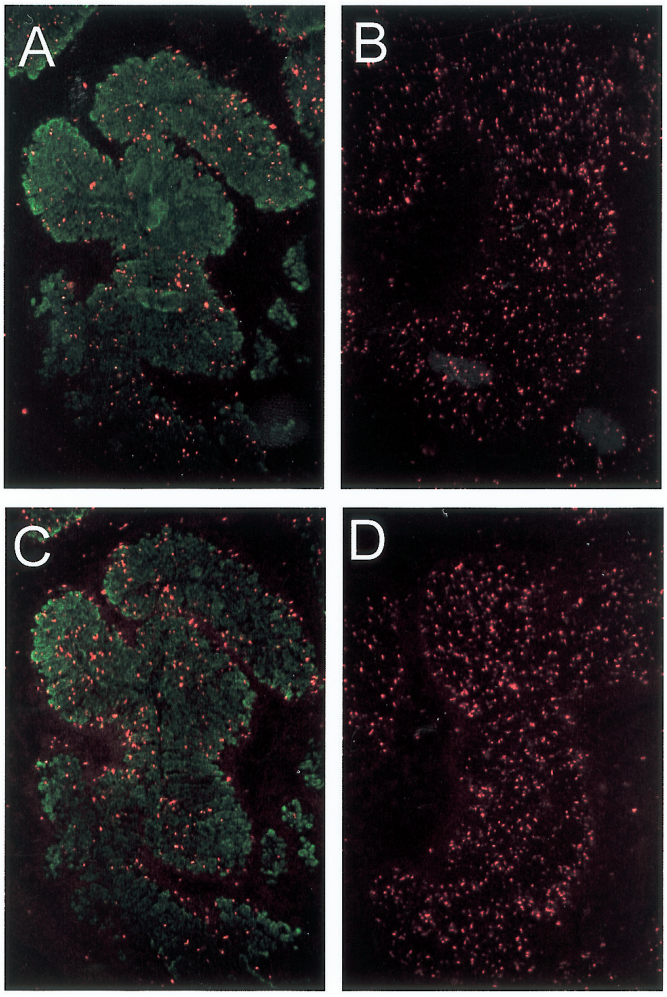
Chapter 6, Figure 5. MUC2 and MUC5AC expression in GMD. Photomicrographs showing examples of mild (A and B) and marked (C and D) GMD. Sections were immunohistochemically stained for (A and C) MUC2 and (B and D) MUC5AC. In mild GMD, a few goblet cells at the villus tip were both MUC2 and MUC5AC positive (arrowheads), whereas other goblet cells were only MUC2 positive (arrows). In marked GMD, only sporadically MUC2-positive goblet cells were found on the villus (C), whereas MUC2-positive goblet cells were normally found in the crypt epithelium (arrows). MUC5AC expression in marked GMD covered the entire villus epithelium (D; arrowheads), also numerous goblet cells expressing MUC5AC were found (D; arrows). Nuclei were counterstained by hematoxylin. Original magnification x120 (A and B) and x50 (C and D).



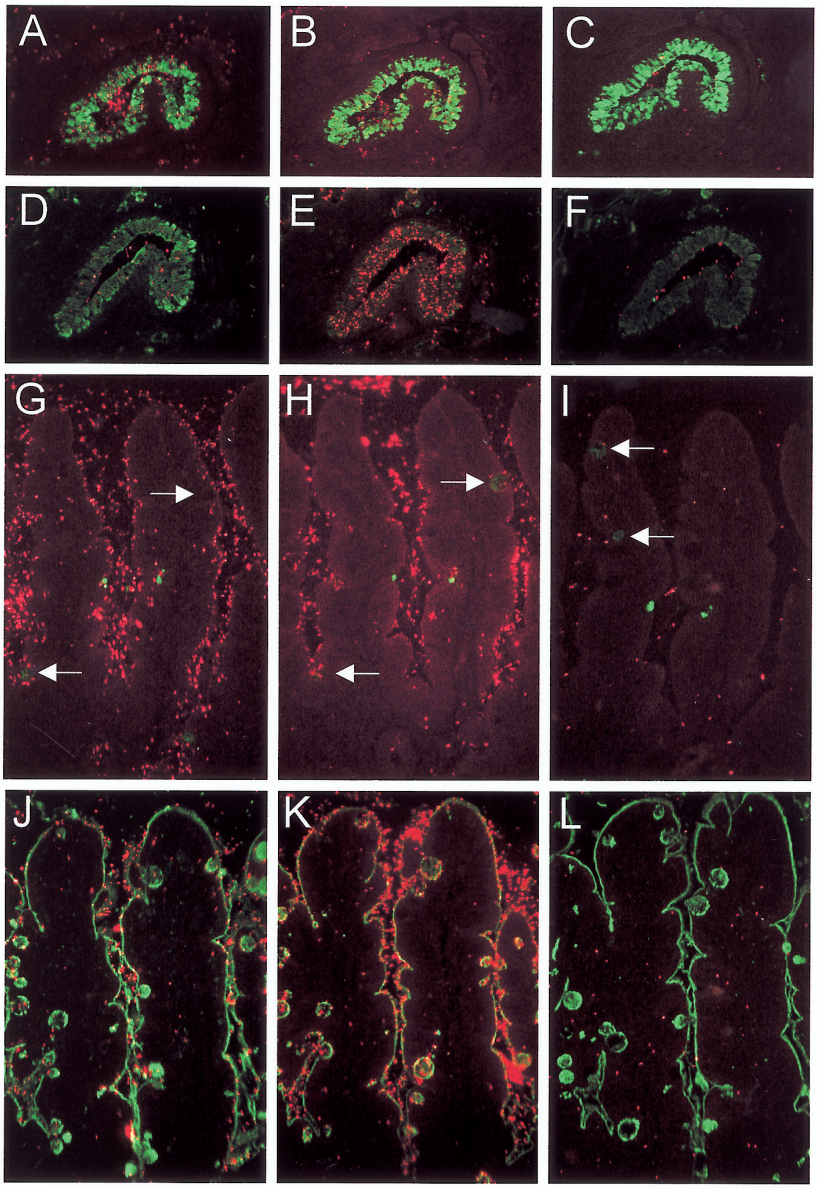
Chapter 7, Figure 1. Binding of *H. pylori* to gastric epithelium. Example of double immuno-fluorescence on human gastric antrum epithelium, with closely adjacent sections of a LeB-positive patient. Co-localization was studied of TR-labeled *H. pylori* (red) that were added to sections that were immunohistochemically stained for either MUC5AC (green; A-C) or LeB (green; D-F). MUC5AC-positive cells co-localized very strongly with LeB-positive cells in the surface epithelium. *H. pylori* strains CCUG17875 (A) and P466 (B) bound specifically to MUC5AC-positive cells and to extracellular MUC5AC-positive mucus (arrows; A and B, respectively) and also to LeB-positive cells and LeB-positive extracellular mucus (arrows; D and E, respectively). Strain TIGR26695 showed no specific binding to any particular structure in the antrum tissue (C and F). Original magnification x40.



Chapter 7, Figure 2. Binding of *H. pylori* to Barrett's esophagus (BE) epithelium Panels A through F showing examples of double immuno-fluorescence on LeB-positive human metaplastic esophagus epithelium. Co-localization was studied of TR-labeled *H. pylori* (red) that were added to sections that were immunohistochemically stained for either MUC5AC (green; A-C) or LeB (green, D-F). MUC5AC-positive cells co-localized very strongly with LeB-positive cells in the BE epithelium. *H. pylori* strains CCUG17875 and P466 bound specifically to MUC5AC-positive cells (A and B, respectively) and also to LeB-positive cells (D and E, respectively). Strain TIGR26695 (C and F) showed no specific binding to any particular structure in this LeB-positive BE tissue. Original magnification x40.



Chapter 7, Figure 3. Binding of *H. pylori* to LeB-negative Barrett's esophagus (BE) epithelium
Panels A through D showing examples of adherence of *H. pylori* to sections of a patient with LeB-negative BE epithelium. These sections were immunohistochemically stained for either MUC5AC (green; A and C) or LeB (green; B and D), followed by incubation with TR-labeled *H. pylori* (red). (A and B) Sections were incubated with CCUG17875. (C and D) Sections were incubated with P466. Strain TIGR26695 showed no specific binding to any structure in this LeB-negative BE tissue (not shown). Original magnification x40.



Chapter 7, Figure 4. Binding of *H. pylori* to normal and gastric metaplastic epithelium (GMD) of the duodenum. Example of double immuno-fluorescence on GMD epithelium (A through F) and normal villus epithelium of the duodenum (G through L). Co-localization was studied of TR-labeled *H. pylori* (red) that were added to sections that were immunohistochemically stained for either MUC5AC (green; A-C and G-I) or LeB (green; D-F and J-L). *H. pylori* strains added to the sections were: CCUG17875 (A, D, G, and J), P466 (B, E, H, and K), and TIGR26695 (C, F, I, and L). The arrows indicate goblet cells in the normal duodenal villi, which were MUC5AC positive. Original magnification x40.